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PSEUDOMONAS AERUGINOSA, BIOLOGY, GENETICS, AND HOST-PATHOGEN INTERACTIONS

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Dara W. Frank

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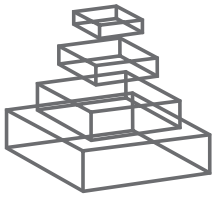

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PSEUDOMONAS AERUGINOSA, BIOLOGY, GENETICS, AND HOST-PATHOGEN INTERACTIONS

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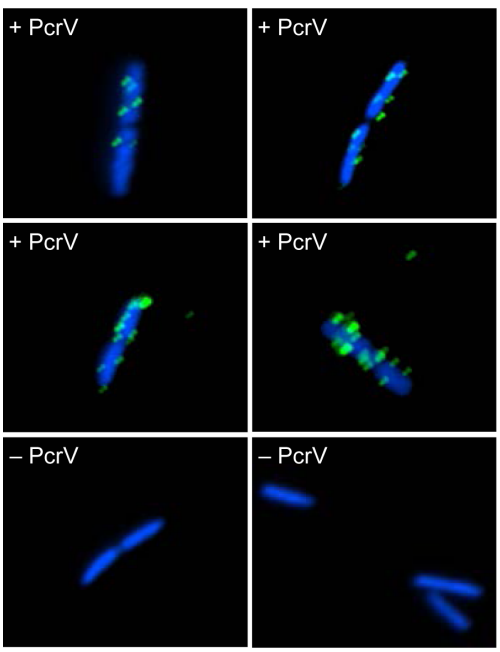

Over the years, Pseudomonas aeruginosa has served as a paradigm or model for many aspects of prokaryotic genetics, metabolism, biology and pathogenesis. This bacterium has a genome that approaches the complexity and size of lower eukaryotes, which has been annotated by a consortium of scientists to provide unprecedented detail and accuracy. The large and plastic genome, coupled with a vast array of regulatory systems, contribute to the versatility of the bacterium for adaptation to almost any environment. During evolution in the soil, competition with other prokaryotes and the acquisition of defensive mechanisms to fend off eukaryotic predators have allowed the maintenance of many antibiotic resistance markers, degradative enzymes and secretion systems, which impact upon human infection. Eleven articles, nine of which are comprehensive reviews, are presented to highlight these topics and provide an up-to-date resource.

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Research topic on *Pseudomonas aeruginosa*, biology, genetics, and host–pathogen interactions

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Over the years, *Pseudomonas aeruginosa* has served as a paradigm for the study of gene expression, metabolism, and pathogenesis. The large genome, which approaches the complexity and size of lower eukaryotes, and an abundance of regulators, facilitates adaptation to almost any environment. During evolution, competition with other prokaryotes and the acquisition of defensive mechanisms to fend off eukaryotic predators have allowed the maintenance of antibiotic-resistance markers, degradative enzymes, and secretion systems, which impact upon human infection. Eleven articles, nine of which are comprehensive reviews, are presented to highlight these topics and provide an up-to-date resource.

GENOMES, PROTEOMES, BIOLOGY

A perspective on the evolution of genomic diversity is provided in the initial article by Klockgether et al. (2011). The core genome of *P. aeruginosa* is highly conserved and collinearly arranged as a single chromosome. The mosaic structure of the genome is interrupted by the accessory genome being made up of regions of plasticity, integrated islands, transposons, phages, or IS-elements. Comparisons of strains from the same clonal complex, yet unrelated geographic origin, show a low substitution rate. Versatility is related to a combination of a large genome, accessory elements, and a variety of regulatory systems.

Rao et al. (2011) use global proteomic approaches to study the differences between a mucoid and a non-mucoid isolate derived from the same patient. Data from mass spectrometry analyses utilizing isobaric tags and 2D-electrophoresis are correlated with microarray results to provide a snapshot of expression profiles. These techniques indicate that the type 6 secretion (T6S) and the mucoid phenotype are inversely correlated. Important technical considerations make this report a valuable resource for refinements of bacterial proteomics.

METABOLISM, MAINTENANCE OF EXTRACELLULAR MATRICES

Targeting bacteria-specific metabolic processes has the promise of eradicating not only actively growing cells but also static or slow-growing cells. Arai (2011) provides a review of the terminal oxidases, denitrification enzymes, their roles in aerobic/anaerobic respiration, and control by inter-related regulatory systems. The control of respiratory function is coordinated by RoxSR, which is predicted to sense electron flow through the terminal oxidases or the redox status of the ubiquinone pool. Overall, control/coordination occurs through terminal oxidases, nitric oxide, and the redox status of the respiratory chain.

The metabolic versatility of *P. aeruginosa* is highlighted by its ability to synthesize extracellular matrices in response to environmental conditions. Franklin et al. (2011) provide a comprehensive review of the biosynthetic pathways for three secreted polysaccharides; alginate, Psl, and Pel. The alginate and Pel pathways share characteristics of a lipid-carrier-independent mechanism similar to the biosynthetic complexes of bacterial cellulose and Pga systems. A lipid-carrier-dependent mechanism is consistent with the Psl pathway, comparable to the *E. coli* capsular synthesis pathways. A framework for additional experimentation is described and questions regarding the biological implications of regulating three pathways are posed.

The plasticity of *P. aeruginosa* in extracellular polysaccharide production is reflected in the ability to produce O-specific antigens that make up the lipopolysaccharides (LPS). Lam et al. (2011) describe the generation of LPS diversity through mechanisms involving horizontal gene transfer, chromosomal insertions, lysogenic conversion, genomic plasticity in the face of *in vivo* selection, and quorum sensing. The biological implications of the complete LPS, O-specific antigens, core oligosaccharide, and lipid A domains are addressed relevant to flagellar and twitching motility, biofilm formation, adherence, recognition by TLR4, CTFR, host lectin, complement components, and the expression of membrane-associated systems including T3SS and phage receptors. The authors also review the use of LPS components in vaccine formulations.

ANTIBIOTIC RESISTANCE

Multi-drug resistant *P. aeruginosa* are of clinical concern and Poole (2011) cautions that pan-resistant isolates, treatable only with colistin, are on the rise. The antibiotic-resistance mechanisms addressed include the acquisition of β-lactamases, particularly the extended-spectrum enzymes, carbapenemases, aminoglycoside-modifying enzymes, and 16S rRNA methylases. Mutational changes causing the upregulation of multi-drug efflux pumps, derepression of *ampC*, modification of antimicrobial targets, and changes in the outer membrane permeability barrier are described with the propensity of *P. aeruginosa* to exist *in vivo* and in the environment as slow-growing organisms imbedded in extracellular matrices. Aspects of hypermutation are discussed relative to cystic fibrosis and a lung environment rich in reactive-oxygen species.

In an effort to identify anti-microbial peptides with properties consistent with therapeutic use, Dean et al. (2011) measure the susceptibility of *P. aeruginosa* to several variations of anti-microbial peptides belonging to the cathelicidin family. This research article describes a series of peptides with properties that not only demonstrate toxic effects to the bacteria but also affect bacterial gene expression, twitching motility, and biofilm formation and maintenance.

PROTEIN SECRETION

Filloux (2011) elegantly deconstructs all five of the secretion systems that operate in *P. aeruginosa*. He summarizes the salient features, known protein structures, secreted proteins, or injected proteins in the case of T3SS and T6SS. The common themes include energy requirements, conduit construction, pore formation in the outer membrane, and protein targeting. The perspective from overviewing all systems leads to insights into universal functions and how these systems may have diversified.

Diaz et al. (2011) focus on the T3SS and review the gene regulatory circuitry involved in expression of this secretory/intoxication system. Intrinsic regulation is described with emphasis on the partner-switching mechanisms involving ExsA, ExsC, ExsD, and ExsE. Also, the various environmental signals and stimuli are compiled. The authors provide valuable insights and caveats to interpreting microarray data, concerning strains that overexpress ExsA, host–cell contact, or low-calcium stimuli used to induce T3SS and the requirement for an intact injectisome relative to T3SS regulation.

The transcriptional control of T3SS is complemented by a review of needle-tip proteins that coordinate the assemblage of a translocon into the host–cell membrane. Structural and functional properties of three needle-tip-protein families are compared with emphasis on biophysical and mechanical aspects of regulation including the propagation/amplification of signals to the T3S nanomachine, the control of energy expenditure, protein secretion, and gene regulation within the bacterium as well as feedback regulation by the action of effectors on host–cell physiology. Sato and Frank (2011) point out how the injection process and its understanding provide a wealth of vaccine and therapeutic targets for *P. aeruginosa*.

EPITHELIAL BARRIERS, CELL POLARITY

Being an opportunistic pathogen, *P. aeruginosa* may be unable to overcome intact epithelial barriers or innate immunity. The interaction of bacteria with intact or wounded epithelium is addressed in the final review article by Engel and Eran (2011). Binding of the bacteria through their pili to host glycans and heparin-sulfate proteoglycans near cell–cell junctions triggers a remodeling of apical surfaces by the recruitment of PI3K resulting in the formation of protrusions. These basolateral-membrane-like protrusions enhance colonization and invasion by this bacterium, suggesting that *P. aeruginosa* subverts epithelial cell regulatory processes that maintain apical–basolateral polarity and prevents wound healing processes.

REFERENCES

Arai, H. (2011). Regulation and function of versatile aerobic and anaerobic respiratory metabolism in *Pseudomonas aeruginosa*. *Front. Microbiol.* 2:103. doi: 10.3389/fmicb.2011.00103

Dean, S. N., Bishop, B. M., and van Hoek, M. L. (2011). Susceptibility of *Pseudomonas aeruginosa* biofilm to alpha-helical peptides: D-enantiomer of LL-37. *Front. Microbiol.* 2:128. doi: 10.3389/fmicb.2011.00128

Diaz, M. R., King, J. M., and Yahr, T. L. (2011). Intrinsic and extrinsic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Front. Microbiol.* 2:89. doi: 10.3389/fmicb.2011.00089

Engel, J., and Eran, Y. (2011). Subversion of mucosal barrier polarity by *Pseudomonas aeruginosa*. *Front. Microbiol.* 2:114.doi: 10.3389/fmicb.2011.00114

Filloux, A. (2011). Protein secretion systems in *Pseudomonas aeruginosa*: an essay on diversity, evolution, and function. *Front. Microbiol.* 2:155.doi: 10.3389/fmicb.2011.00155

Franklin, M. J., Nivens, D. E., Weadge, J. T., and Howell, P. L. (2011). Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Front. Microbiol.* 2:167. doi: 10.3389/fmicb.2011.00167

Klockgether, J., Cramer, N., Wiehlmann, L., Davenport, C. F., and andTümmler, B. (2011). *Pseudomonas aeruginosa* genomic structure and diversity. *Front. Microbiol.* 2:150. doi: 10.3389/fmicb.2011.00150

Lam, J. S., Taylor, V. L., Islam, S. T., Hao, Y., and Kocíncová, D. (2011). Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. *Front. Microbiol.* 2:118. doi: 10.3389/fmicb.2011.00118

Poole, K. (2011). *Pseudomonas aeruginosa*: resistance to the max. *Front. Microbiol.* 2:65. doi: 10.3389/fmicb.2011.00065

Rao, J., Damron, F. H., Basler, M., DiGiandomenico, A., Sherman, N. E., Fox, J. W., Mekalanos, J. J., and Goldberg, J. B. (2011). Comparisons of two proteomic analyses of non-mucoid and mucoid *Pseudomonas aeruginosa* clinical isolates from a cystic fibrosis patient. *Front. Microbiol.* 2:162.doi: 10.3389/fmicb.2011.00162

Sato, H., and Frank, D. W. (2011). Multi-functional characteristics of the *Pseudomonas aeruginosa* type III needle-tip protein, PcrV; comparison to orthologs in other Gram-negative bacteria. *Front. Microbiol.* 2:142. doi: 10.3389/fmicb.2011.00142

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Pseudomonas aeruginosa genomic structure and diversity

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The *Pseudomonas aeruginosa* genome (G + C content 65–67%, size 5.5–7 Mbp) is made up of a single circular chromosome and a variable number of plasmids. Sequencing of complete genomes or blocks of the accessory genome has revealed that the genome encodes a large repertoire of transporters, transcriptional regulators, and two-component regulatory systems which reflects its metabolic diversity to utilize a broad range of nutrients. The conserved core component of the genome is largely collinear among *P. aeruginosa* strains and exhibits an interclonal sequence diversity of 0.5–0.7%. Only a few loci of the core genome are subject to diversifying selection. Genome diversity is mainly caused by accessory DNA elements located in 79 regions of genome plasticity that are scattered around the genome and show an anomalous usage of mono- to tetradecanucleotides. Genomic islands of the pKLC102/PAGI-2 family that integrate into $tRNA^{Lys}$ or $tRNA^{Gly}$ genes represent hotspots of inter- and intraclonal genomic diversity. The individual islands differ in their repertoire of metabolic genes that make a large contribution to the pangenome. In order to unravel intraclonal diversity of *P. aeruginosa*, the genomes of two members of the PA14 clonal complex from diverse habitats and geographic origin were compared. The genome sequences differed by less than 0.01% from each other. One hundred ninety-eight of the 231 single nucleotide substitutions (SNPs) were non-randomly distributed in the genome. Non-synonymous SNPs were mainly found in an integrated Pf1-like phage and in genes involved in transcriptional regulation, membrane and extracellular constituents, transport, and secretion. In summary, *P. aeruginosa* is endowed with a highly conserved core genome of low sequence diversity and a highly variable accessory genome that communicates with other pseudomonads and genera via horizontal gene transfer.

Keywords: *Pseudomonas aeruginosa*, genome, genomic island, core genome, accessory genome, clonal complex, oligonucleotide signature

INTRODUCTION

The genetic repertoire of *Pseudomonas aeruginosa* reflects the lifestyle of this ubiquitous bacterial species. *P. aeruginosa* strains are found in various environmental habitats as well as in animal and human hosts, where they can act as opportunistic pathogens. The colonization of this broad spectrum of habitats goes along with the ability to exploit many different nutrition sources and a high potential for adaptation to new (or changing) environmental conditions (Ramos, 2004).

The metabolic versatility is provided by genes encoding not only the enzymes participating in metabolic pathways, but also by a very high number of transcriptional regulators and two-component regulatory systems. More than 500 regulatory genes were identified in the genome of strain PAO1 (Stover et al., 2000). The genomes of *P. aeruginosa* strains are larger than those of most sequenced bacteria. Within the species, the genome size varies between 5.5 and 7 Mbp (Schmidt et al., 1996; Lee et al., 2006).

The divergence in genome size is caused by the so-called accessory genome. The major part of the genome, the core genome, is found in all *P. aeruginosa* strains with the respective DNA generally collinearly arranged (Römling et al., 1995). The core genome, with few exceptions of loci subject to diversifying selection, is highly conserved among clonal complexes and shows sequence diversities of 0.5–0.7% (Spencer et al., 2003; Lee et al., 2006; Cramer et al., 2011). The accessory genome consists of extrachromosomal elements like plasmids and of blocks of DNA inserted into the chromosome at various loci. The elements of the accessory genome can be present in subgroups of the *P. aeruginosa* population but may also occur only in single strains (Klockgether et al., 2007; Wiehlmann et al., 2007). The individual composition of the accessory genome accounts for most intra- and interclonal genome diversity in *P. aeruginosa*. The elements of the accessory genome were apparently acquired by horizontal gene transfer from different sources including other species or genera. Upon integration into the host chromosome they appear as "foreign" blocks in the core genome. Therefore, a *P. aeruginosa* chromosome is often described as a mosaic structure of conserved core genome frequently interrupted by the inserted parts of the accessory genome.

The individual mosaics also show remarkable plasticity. Ongoing acquisition of new foreign DNA as well as larger or smaller deletion events, mutations of single nucleotides and even chromosomal inversions (Römling et al., 1997; Ernst et al., 2003; Kresse et al., 2003; Smith et al., 2006; Klockgether et al., 2010; Cramer et al., 2011) – all of them potentially affecting parts of the core and/or the accessory genome – continuously modify the genome,

modulate the *P. aeruginosa* strain's phenotype and differentiate it from others.

Genome diversity of *P. aeruginosa* was initially analyzed by low-resolution physical mapping techniques (Schmidt et al., 1996; Römling et al., 1997). Thanks to progress in DNA sequencing technologies *P. aeruginosa* genomes can nowadays be compared by the base (Kung et al., 2010; Silby et al., 2011).

GENOME SEQUENCES

Pseudomonas aeruginosa is ubiquitous in aquatic habitats and colonizes animate surfaces of humans, animals and plants. Complete genome sequences, however, are so far only available for *P. aeruginosa* isolates from human infections (**Table 1**).

The first complete genome sequencing was performed for strain PAO1 (Stover et al., 2000), derived from an Australian wound isolate from the 1950s. The PAO1 strain has been and is still the major reference for genetic and functional studies on *P. aeruginosa*. The PAO1 genome consists of a 6.264-Mbp circular chromosome encoding 5,570 predicted protein coding sequences. Sequence and annotation are deposited at the National Center for Biotechnology Information (NCBI) genome database (Refseq. no. NC_002516) and in the *Pseudomonas* Genome Database (Winsor et al., 2009), which also documents ongoing annotation updates. Thanks to the recently developed deep cDNA sequencing more and more non-coding RNAs are currently being identified in bacterial genomes, and thus we can expect a large number of non-coding genes to be added to the annotation of *P. aeruginosa* genomes as has been executed for *Helicobacter pylori* and *Pseudomonas putida* (Sharma et al., 2010; Frank et al., 2011).

The second *P. aeruginosa* genome sequence was published for the ExoU-positive strain PA14 (NC_008463, Lee et al., 2006), a clinical isolate displaying higher virulence than PAO1. Fifty-four PAO1 regions of at least one open reading frames (ORFs) are absent in the PA14 genome, and 58 PA14 regions are absent in PAO1 including the PA14 pathogenicity islands PAPI-1 and PAPI-2 (He et al., 2004).

LESB58, a so-called "Liverpool epidemic strain," was found to be highly transmissible among CF-patients and displayed the potential to cause severe infections even in non-CF human hosts (Cheng et al., 1996; McCallum et al., 2002). The LESB58 genome (NC_011770) contains previously unknown accessory genome elements (Winstanley et al., 2009).

PA7 is a clinical isolate from Argentina with a notably unusual antimicrobial resistance pattern. Strain PA7 (NC_009656) shares only 93.5% nucleotide identity in the core genome with the other sequenced strains confirming the previous assignment of strain PA7 as a taxonomic outlier within the species *P. aeruginosa* (Roy et al., 2010).

Almost complete genome sequences are also available for strains 2192 (NZ_AAKW00000000), C3719 (NZ_AAKV00000000), PACS2 (NZ_AAQW00000000; Mathee et al., 2008), and 39016 (AEEX00000000; Stewart et al., 2011). Eight additional *P. aeruginosa* genome sequences are listed at NCBI as "In Progress" (last checked on February 23rd, 2011) and numerous *P. aeruginosa* projects are deposited in the European Nucleotide Archive (ENA) hosted by EMBL-EBI[1]. With decreasing costs and increasing speed of sequencing we can expect an avalanche of novel *P. aeruginosa* genome sequence data. Published examples are the comparative sequencing of PAO1 sublines of divergent metabolic and virulence phenotypes (Klockgether et al., 2010), the identification of *de novo* mutations conferring antimicrobial resistance (Moya et al., 2009), the analysis of genomic gradients of sequence diversity in a pool of clinical isolates (Dötsch et al., 2010), and the intraclonal microevolution in the cystic fibrosis lung (Cramer et al., 2011).

THE ACCESSORY GENOME

The accessory genome consists of DNA elements from within the range of a few hundred bases to more than 200 kbp. The minimum size of an accessory element was defined as a block of at least four contiguous ORFs that are not conserved in all *P. aeruginosa* (Mathee et al., 2008). Thirty-eight to 53 accessory elements were identified in the completely sequenced *P. aeruginosa* genomes (**Table 2**). The PAO1 genome only contains inserts of 14 kbp or smaller (Mathee et al., 2008), whereas the LESB58 genome harbors five genomic islands and five inserted prophages of 14–111 kbp in size (Winstanley et al., 2009). **Table 3** lists the subset of genomic islands that were analyzed in detail *in silico* and/or in wet lab experiments.

Within the chromosomally integrated islands, very often phages, transposons, or IS-elements are found indicating that the majority of the accessory genome originates from mobile DNA elements which have been acquired and kept by the host strain. Many elements were irreversibly fixed by secondary mutation or deletions, but a few others have retained their mobility and can still leave the chromosomal insertion site and be transferred elsewhere, as shown for the elements PAPI-1 (Qiu et al., 2006) and pKLC102 (Klockgether et al., 2007). For a detailed description of the different types of accessory elements [integrative and conjugative elements (ICEs), prophages, transposons, etc.], the reader is referred to the recently published review by Kung et al. (2010).

The acquisition of the elements of the accessory genome from other taxa is not only evident from the gene contents with its overrepresentation of mobile DNA elements, but also from global

[1]http://www.ebi.ac.uk/ena/

Table 1 | Features of sequenced *P. aeruginosa* strains.

Strain	PAO1	PA14	PA7	LESB58	PACS2	2192	C3719	39016
Source	Wound	Clinical	Clinical	CF-patient	Clinical	CF-patient	CF-patient	Keratitis
Genome size (Mbp)	6.264	6.538	6.588	6.602	6.492	6.905	6.222	6.667
GC-content (%)	66.6	66.3	66.5	66.5	66	66.2	66.5	66
No. of protein coding ORFs	5570	5892	6286	5925	5676	6191	5578	6401

Table 2 | Regions of genome plasticity (RGP) in seven sequenced *P. aeruginosa* genomes.

RGP		Flanking loci		Strain						
	Insertion Site	In PAO1[1]	In PA14[1]	PAO1	PA14	LESB58	PA7	2192	C3719	PACS2
RGP1		0201/0208	02530/02550	*			i		*	*
RGP2	$tRNA^{Arg}$	0256/0264	03160/03420	*	i	*	i	*	*	
RGP3		0611/0629	07960/08160	*	*	i[2]	i		+	+
RGP4		0641/0648	08300/08330	*	i	i[2]	i	*	i	i
RGP5	$tRNA^{Gly}$	0714/0730	55100/54830	i	i		i	i		i
RGP6	tmRNA	0819/0827	53680/53560	i	i	i	i	i	i	i
RGP7	$tRNA^{Lys}$	0976/0988	51670/51510	i	i		i			
RGP8	$tRNA^{Ser}$	1013/1014	51240/51220				i		i	
RGP9[3]		1087/1092	50340/50290	*	*	*	i	i	*	i
RGP10		1191/1192	49040/48870		i	i	i			
RGP11		1222/1225	48520/48440	*	i	*	i	*	*	*
RGP12		1243/1244	48160/48150				i	i		
RGP13		1367/1373	46630/46490	i	i	i	i	i		
RGP14		1375/1376	46470/46540		i				i	
RGP15		1377/1394	46440/46390	i	i		i[4]	i	i	i
RGP16		1530/1531	44650/44640		i[5]				i	i
RGP17	$tRNA^{His}$	1796/1797	41350/41280				i	i		
RGP19		1964/1965	39130/39110					i		
RGP20		2024/2070	38340/37730	i	*	*	i	i		i
RGP21		2099/2107	37360/37350	*				*		
RGP22		2181/2187	36370/36360	*		*	i		*	*
RGP23		2217/2235	36050/35690	i	i	i	i	i	i	i
RGP24		2422/2423	33370/33290		i		i	i	*	*
RGP25		2455/2464	32860/32770	i	i	i	i	i	i	i
RGP26	$tRNA^{Leu}$	2570/2571	31290/30840		i		i			
RGP27	$tRNA^{Gly}$	2583/2584	30700/30670		i	i	i			i
RGP28	$tRNA^{Pro}$	2727/2737	28895/28730	i	i	i	i	i	i	i
RGP29	$tRNA^{Gly}$	2817/2820	27710/27590	+	*	*	+	i	*	*
RGP30		2950/2951	25900/25880					i		
RGP31[6]		3141/3160	23470/23360	i	i	i	i	*	i	*
RGP32		3222/3223	22560/22490		i					
RGP33		3239/3240	22290/22075		i					
RGP34		3496/3515	18870/18860	i						
RGP35		3536/3537	18620/18610					i		
RGP36		3768/3769	15670/15340		i	i				i
RGP37		3865/3870	13990/13850	*	i			*		
RGP38		4162/4163	10130/10040		*		*			
RGP39		4190/4196	09700/09690	*		*		*	*	*
RGP41	$tRNA^{Lys}$	4541/4542	58900/60190		i			i	i	i
RGP42	$tRNA^{Met}$	4673/4674	61820/61840	i[7]			i	i		i
RGP43		2770/2773	28280/28220	*[8]	i	*[8]	i	*[8]	*[8]	*[8]
RGP44		4100/4108	10850/10820	*	i	*	*	*	*	*
RGP46		0041/0042	00510/00530		i	i	i	i	i	
RGP47		1149/1153	49530/49500	i	i	*		*	i	*
RGP48		1238/1242	48240/48170	*	*			*		*
RGP50		1655/1656	43110/43050		i					
RGP52		1934/1940	39500/39460	i	i	i	i	i	i	i
RGP53		2332/2337	34450/34440	*		*		*	*	*
RGP56		2793/2795	28000/27980	*	*	+	i	+	*	+[9]
RGP58	$tRNA^{Arg}$	3366/3368	20560/20490	*	i	i	*	*	*	i
RGP60[10]	$tRNA^{Thr}$	4524/4526	58700/58750	i	*	+	i	+	*	i

(Continued)

Table 2 | Continued

RGP		Flanking loci		Strain						
	Insertion Site	In PAO1[1]	In PA14[1]	PAO1	PA14	LESB58	PA7	2192	C3719	PACS2
RGP62	$tRNA^{Phe}$	5149/5150	68000/68040							i
RGP63		0069[11]	00810[11]				i			
RGP64		0278[11]	03620[11]				i			
RGP65		0377/0378	04940/04950				i			
RGP66	$tRNA^{Met}$	0574/0575	07450/07500		i		i			
RGP67		3858/3859	14080/14100				i			
RGP68[12]		3840/3844	14290/14340	*	i	*	i	*	*	*
RGP69		3714/3715	16340/16350				i			
RGP70	$tRNA^{Pro}$	3031/3032	24860/24880				i			
RGP71		2650/2651	29820/29830			*	i		*	*
RGP72	$tRNA^{Cys}$	2581/2582	30710/30730				i			
RGP73[13]		2397/2403	33600/33690	*	*	i	+	i	+	+
RGP74		2201/2202	36230/36250				i			
RGP75		1579/1580	44070/44080				i			
RGP76		1425/1428	45980/46010	*	*	*	i	*	*	*
RGP77		1397/1398	46330/46350				*			*
RGP78		4466/4467	57980/57990				i			
RGP79		5290/5291	69840/69850				i			
RGP80		5454/5460	72000/72060	*	*	*	i	*	*	*
RGP81		4138/4139	10420/10380			i				
RGP82		3663/3664	16980/16970			i				
RGP83		3463/3464	19330/19320	i[14]		i		i[14]	*[14]	*[14]
RGP84	$tRNA^{Ser}$	2603/2604	30430/30410			i	[15]			
RGP85		2593/2594	30550/30560			i	[15]			
RGP86		0831/0832	53510/53520			i				
RGP87	$tRNA^{Thr}$	5160/5161	68140/68170					i		i
RGP88		3961[16]	12630[16]							
RGP89		3834/3836	14440/14390	*	i	*	+	i	*	+

*Differentiation of accessory elements in the RGPs: i, strain-specific accessory element; *or +, identical accessory elements in two or more strains. RGPs 1–62 were defined by Mathee et al. (2008) and RGPs 63–80 by Roy et al. (2010). The novel RGPs 81–89 were extracted from the sequences of genomic islands in strain LESB58 (RGP 81–86; Winstanley et al., 2009) and strain PSE9 (RGP 87–89; Battle et al., 2009).*

[1] Insertions are designated by the numbers of the flanking loci in the PAO1 and PA14 genomes (e.g., 0201 is PA0201, 02530 is PA14_02530).

[2] Insertion LESPP-1 between PA0612 and PA0648 homologs comprises RGP3 and RGP4.

[3] Region containing flagellin glycosylation genes (replacement island).

[4] Partial duplication of sequence of the core genome (between RGP27 and RGP28).

[5] No annotated ORF in this insertion.

[6] Region contains O-antigen gene cluster (replacement island).

[7] No insertion in PAO1 reference sequence but in variants PAO1-DSM and MPAO1 (Klockgether et al., 2010).

[8] Identical sequence with discordant ORF annotation for the different strains.

[9] Identical sequence with discordant annotation for PACS2 versus LESB58 and 2192.

[10] Region contains pilA gene (replacement island).

[11] Homologous ORF in PA7 disrupted by the insertion.

[12] Insertion contains exoS gene in PAO1, LESB58, 2192, C3719, and PACS2.

[13] Region contains pyoverdine synthesis gene cluster (replacement island).

[14] < 1 kb insertion in PAO1, 2192, C3719, and PACS2 with no predicted ORF.

[15] Insertion in PA7 comprises RGP84 and RGP85.

[16] Homologous ORF in strain PSE9 disrupted by PAGI-7.

parameters like the oligonucleotide signature. The segments of the core genome share the same oligonucleotide usage, whereas the constituents of the accessory genome exhibit a divergent G + C content and oligonucleotide usage (Reva and Tümmler, 2004, 2005). In the genome atlas of *P. aeruginosa* LESB58 (**Figure 1**), the regions with an anomalous tetranucleotide composition and an underrepresentation of common octa- to tetradecanucleotides coincide with the segments of the accessory genome. **Figure 2**

Table 3 | Genomic islands in *P. aeruginosa* strains described in literature.

Genomic island	Host strain	Size (kb)	RGP locus	Reference
PAPI-1	PA14	108	41	He et al. (2004)
PAPI-2	PA14	10.8	7	He et al. (2004)
LES-prophage 1	LESB58	14.8	3 and 4	Winstanley et al. (2009)
LES-prophage 2	LESB58	42.1	81	Winstanley et al. (2009)
LES-prophage 3	LESB58	42.8	82	Winstanley et al. (2009)
LES-prophage 4	LESB58	26.8	83	Winstanley et al. (2009)
LES-prophage 5	LESB58	39.9	84	Winstanley et al. (2009)
LES-prophage 6	LESB58	7.6	10	Winstanley et al. (2009)
LESGI-1	LESB58	46.4	28	Winstanley et al. (2009)
LESGI-2	LESB58	31.7	85	Winstanley et al. (2009)
LESGI-3	LESB58	110.6	27	Winstanley et al. (2009)
LESGI-4	LESB58	39.4	23	Winstanley et al. (2009)
LESGI-5	LESB58	29.4	86	Winstanley et al. (2009)
pKLC102	C, SG17M	103.5	41	Klockgether et al. (2004)
PAGI-1	X24509	48.9	23	Liang et al. (2001)
PAGI-2	C	105	29	Larbig et al. (2002)
PAGI-3	SG17M	103.3	29	Larbig et al. (2002)
PAGI-4	C	23.4	7	Klockgether et al. (2004)
PAGI-5	PSE9	99.3	7	Battle et al. (2008)
PAGI-6	PSE9	44.4	87	Battle et al. (2009)
PAGI-7	PSE9	22.5	88	Battle et al. (2009)
PAGI-8	PSE9	16.2	62	Battle et al. (2009)
PAGI-9	PSE9	6.6	89	Battle et al. (2009)
PAGI-10	PSE9	2.2	25	Battle et al. (2009)
PAGI-11	PSE9	2	52	Battle et al. (2009)
ExoU-A	6077	81.2	7	Kulasekara et al. (2006)
ExoU-B	19660	29.8	7	Kulasekara et al. (2006)
ExoU-C	X13273	3.7	7	Kulasekara et al. (2006)

shows the genome distribution of the most abundant 8- to 14mers in *P. aeruginosa* LESB58 (Davenport et al., 2009). Regions that lack these strain- or taxon-specific words represent those parts of the accessory genome that is most foreign from the core.

REGIONS OF GENOME PLASTICITY

Elements of the accessory genome are located in all sections of the *P. aeruginosa* chromosome, not concentrated in some regions. Nevertheless, the uptake of accessory DNA apparently did not occur completely at random but at specific genomic loci that are prone to integration of special mobile elements.

A comprehensive comparison of the genomes of strains PAO1, PA14, 2192, C3719, and PACS2 (Mathee et al., 2008) led to the definition of so-called "regions of genome plasticity" (RGPs). Mathee and co-workers searched for segments of DNA not conserved in all five genomes and designated any region containing a block of four or more contiguous ORFs that is missing in at least one of the genomes as an RGP. For each of these RGPs they defined the DNA contained in the accessory blocks and the ORFs annotated within. Also the RGP flanking ORFs conserved in all five strains were listed, referred to as "anchors," which describe the genomic site used for the integration of the foreign DNA.

The approach by Mathee et al. (2008) appears reasonable to describe accessory and core genome of *P. aeruginosa* strains, although small insertions are ignored and deletions affecting the core genome in some, but not all, compared strains will misassign the respective segment to the accessory genome. A secondary check of the oligonucleotide usage will correct these false positives.

Mathee et al. (2008) initially defined 52 RGPs (no. 1–62 in **Table 2**). With the advent of the PA7 genome sequence, a further 18 elements were identified (RGPs 63–80; Roy et al., 2010).

Table 2 moreover lists the novel RGPs 81–89 that comprise yet unknown RGPs from strains LESB58 (Winstanley et al., 2009) and PSE9 (Battle et al., 2009).

On average each sequenced *P. aeruginosa* strain carries about 40 RGPs with insertions. The outlier was strain PA7 with 53 occupied RGPs. tRNA genes serve as integration sites for 20 RGPs. The 3′ end of tRNA genes and the subsequent nucleotides are known to serve as integration sites for ICEs and phage-like elements (Dobrindt et al., 2004). In the majority of RGPs, however, other target sequences had been utilized for the insertion corresponding with the diverse type and origin of the elements of the accessory genome of *P. aeruginosa* (Kung et al., 2010). Most target sequences are located in intergenic regions, but in three RGPs a single ORF was disrupted (RGPs 63, 64, and 88; **Table 2**). Interestingly, insertions in each of these three RGPs were only detected for a single strain so far, while in all other tested genomes the non-fragmented anchor-ORF was present.

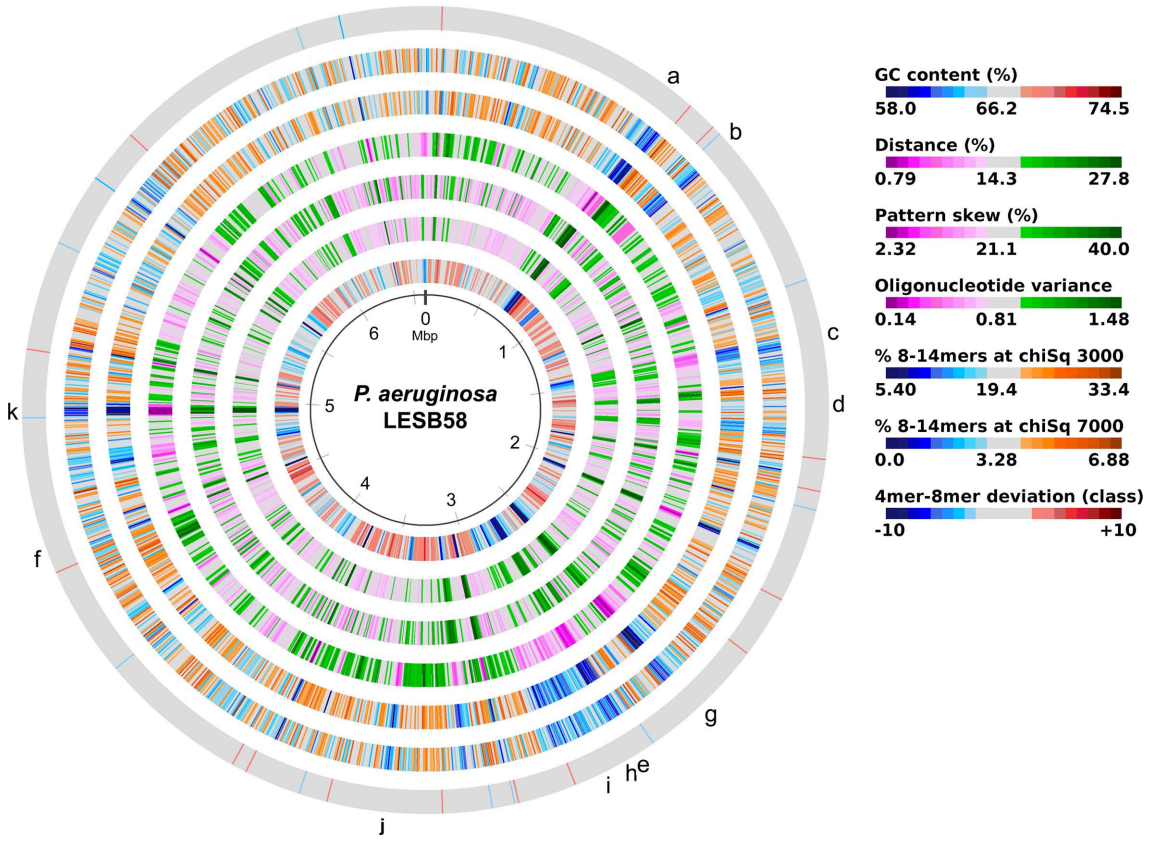

FIGURE 1 | Genome atlas representations of G + C content, tetranucleotide parameters and overrepresented 8- to 14mers in *P. aeruginosa* LESB58 (Davenport et al., 2009). Increasing divergence from average (up to an extreme value at ±3 SD) is indicated by progressively darker colors. G + C content and the three tetranucleotide parameters are plotted on the innermost four rings. Distance (second innermost circle) is the distance between global and local sliding window tetranucleotide patterns, pattern skew (third innermost circle) is the distance between tetranucleotide rankings on direct and reverse strands, and oligonucleotide variance (fourth innermost circle) is the numerical variance of oligomers, where a lower value indicates tetramer usage is more highly restricted (for example in repeat regions). Rings 5 (χ^2 threshold 3000) and 6 (χ^2 threshold 7000) display the number of bases occupied by overrepresented 8- to 14mers in a certain region, with overlaps only counted once, as a percentage. The outermost ring shows the difference (in classes) between a tetranucleotide parameter, oligonucleotide variance, and the 8- to 14mers in ring 5. Figures were created with JCircleGraph. Letters at the outermost ring indicate the regions of the six identified prophages (a–f) and five genomic islands (g–k; Winstanley et al., 2009).

Three regions show an unusual local genome structure. Strains LESB58 and PA7 each carry hybrids of two adjacent RGPs. Moreover, in strain LESB58 a 137-kbp segment of the core genome 3′ to RGB15 was transposed upstream by 83 genes (84.3 kbp; **Figure 3**). No repeats flanking the segment or mobility-related genes such as transposase- or integrase-coding genes were identified so that the underlying mechanism of the transposition remains elusive.

THE pKLC102/PAGI-2 ICE FAMILY

Among the genomic islands of the *P. aeruginosa* accessory genome, members of the pKLC102/PAGI-2 family are highly prevalent. They represent a special group of ICEs that can be described as semi-conserved elements, as they generally consist of individual DNA blocks and sets of genes common to all members (Klockgether et al., 2008; Kung et al., 2010). pKLC102/PAGI-2 family islands have been detected in various bacterial species and genera, mainly in β- and γ-proteobacteria. The fact that a set of genes is conserved among all family members indicates a common origin from an ancient ancestor (Mohd-Zain et al., 2004). This conserved gene set accounts for structural and mobility-related features and conjugal transfer. Individual genes within the islands can encode a broad spectrum of different functions, among them catabolic pathways as well as virulence effectors. Existence of free episomal forms and/or transfer to other strains, even across species barriers, have been monitored for several pKLC102/PAGI-2-like islands, thus confirming their role for (ongoing) evolution of bacterial genomes and, due to the different "cargo" provided by these elements to the host strains, for the genome diversification within bacterial species and emergence of subgroup- or strain-specific phenotypes. For a detailed summary of the role of the common "backbone" genes for integration, mobilization and transfer of pKLC102/PAGI-2-like elements, the reader is referred to the recent review by Kung et al. (2010).

The role of pKLC102/PAGI-2-like islands within the *P. aeruginosa* accessory genome, and thus their contribution to genome

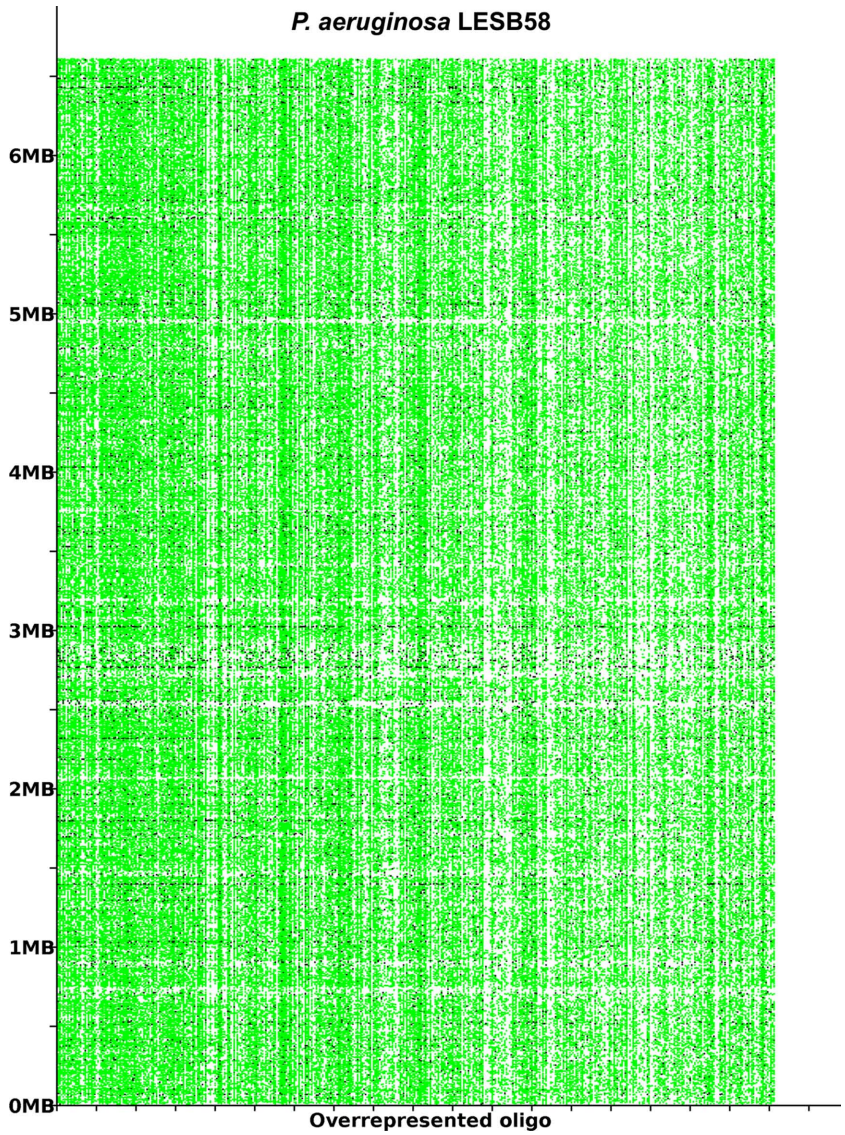

FIGURE 2 | The most overrepresented 8- 14 bp oligomers in *P. aeruginosa* LESB58 sorted by decreasing χ^2 values. The genome position of each oligo is plotted on the *y*-axis. A black dot is printed where an oligonucleotide occurs in non-coding regions and a green dot where an oligonucleotide occurs in coding regions. The figure was created with the program OligoViz (Davenport et al., 2009). The majority of the overrepresented 8–14 bp oligomers is located in coding sequences distributed all over the genome; only in the few cases of white vertical lines the respective oligonucleotide clusters in a few genome positions. Horizontal white lines indicate regions with an atypical oligonucleotide usage that lack these strain- or taxon-specific words and represent those parts of the accessory genome that are most foreign from the core.

diversity, is illustrated by the abundance of many different islands of this family within the population. Hybridization results have indicated the presence of such islands in a majority of strains isolated from different habitats (Klockgether et al., 2007; Wiehlmann et al., 2007). Similarly, searching the available *P. aeruginosa* genome sequences for the typically conserved genes revealed their presence in all strains but PAO1.

Six of the islands listed in **Table 3** are members of that family: pKLC102, PAPI-1, PAGI-5, PAGI-2, PAGI-3, and LESGI-3. All of them are between 99 and 110 kbp in size. Clusters of typically conserved backbone genes were also detected in smaller islands like PAGI-4 or ExoU-A. As significant parts of the backbone, however, were missing, it was hypothesized that PAGI-4 and ExoU-A represent remaining fragments of formerly complete PAGI-2/pKLC102-like islands that underwent recombination and deletion events resulting in the loss of smaller (ExoU-A) or bigger parts (PAGI-4) of the original elements (Klockgether et al., 2004; Kulasekara et al., 2006).

The mentioned *P. aeruginosa* islands split up into two subtypes: PAGI-2-like islands (PAGI-2, PAGI-3, and LESGI-3) contain a phage P4-related integrase gene and are inserted at $tRNA^{Gly}$ genes in RGPs 27 or 29. The well described *clc* element providing

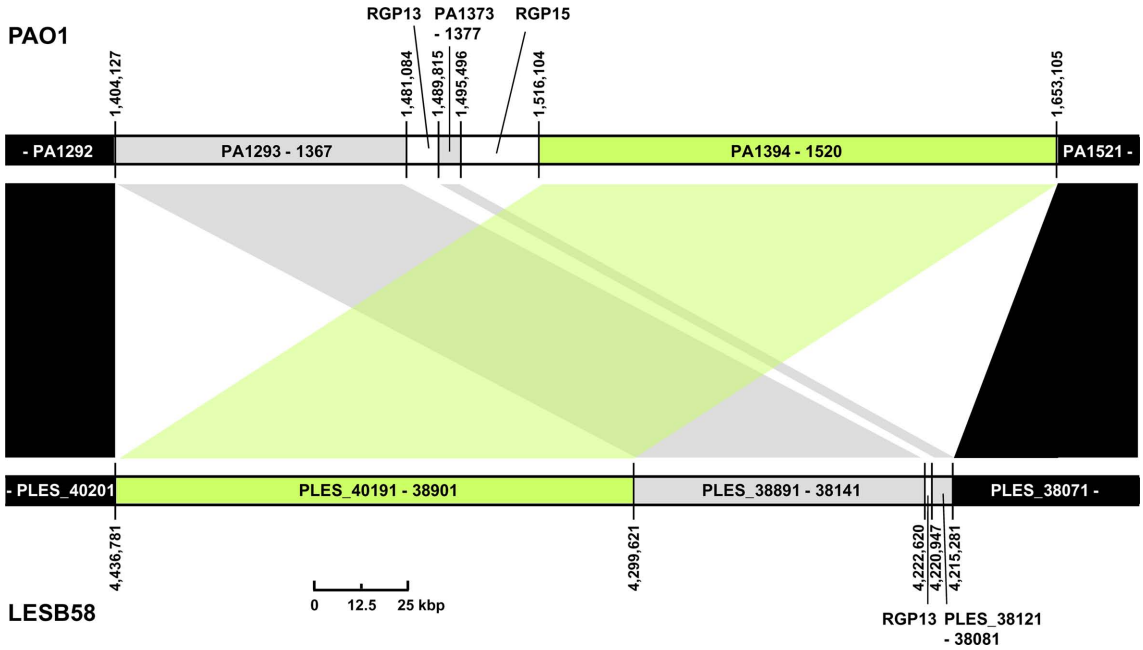

FIGURE 3 | Transposition of core genome DNA in LESB58. The genomic region with different core genome architecture is shown for strains PAO1 and LESB58. One hundred thirty-seven kbp of DNA (green) are located upstream of other core genome DNA blocks (gray) in LESB58 while occurring downstream of them in PAO1 (and other genomes). Surrounding core genome DNA arranged collinearly in both strains is shown in black, strain-specific insertions are represented by white areas. Genome coordinates of the borders of the core genome DNA blocks and numbers of the ORFs within are given for both strains. Accessory DNA blocks are described by the RGP number (see **Table 2**).

features for metabolizing chlorinated aromatic compounds could be assigned to that subtype as well. Present in other *Pseudomonas* species as well as in *Ralstonia* and *Burkholderia* strains, transfer of *clc* to *P. aeruginosa* PAO1 by conjugation was shown *in vitro* (Gaillard et al., 2008). Upon transfer, genomic integration occurred at the usual $tRNA^{Gly}$ genes in RGP27 or RGP29.

The pKLC102-subtype islands (pKLC102, PAPI-1, PAGI-5) are endowed with a XerC/XerD-like integrase gene, and the two copies of a $tRNA^{Lys}$ gene in RGP7 and RGP41 can be used as insertion sites. Transfer of pKLC102-like elements from one RGP to the other has been demonstrated (Kiewitz et al., 2000; Qiu et al., 2006). The "fragmentary" pKLC102-like islands PAGI-4 and ExoU-A are also located in RGP7. The $tRNA^{Lys}$ gene in RP7 is also the insertion site for islands carrying the virulence-associated *exoU* gene and its cognate chaperone *spcU* gene, ExoU-B, ExoU-C, and PAPI-2. Although DNA typical for pKLC102-like islands is scarce in these *exoU*-positive islands, the common insertion site and a few motifs within their sequence indicate a descent from a pKLC102-like element as hypothesized for ExoU-A (Kulasekara et al., 2006).

Kung et al. (2010) described the two subtypes as two families of *P. aeruginosa* ICEs. Due to the conserved function and synteny of the backbone genes, however, we prefer to consider them as members of one family with common ancestry (Klockgether et al., 2007, 2008). The pKLC102/PAGI-2-like islands share 35 conserved orthologs with a variable degree of amino acid identity between 35 and 100%.

Divergent evolution from the ancestor might have caused the early formation of the two pKLC102- and PAGI-2 subtypes that exhibit higher average identity values among the conserved backbone genes and each carry a subfamily-specific set of genes (**Figure 4**). Eleven genes were specific for the PAGI-2-subtype and 39 genes specific for the pKLC102- subtype including a cluster of conjugative type IV sex pilin genes (Klockgether et al., 2004; Carter et al., 2010). Thus, pKLC102-/PAGI-2-family islands appear as mosaic pieces in *P. aeruginosa* genomes while they are small mosaics themselves, composed of conserved backbone, subtype-specific, and individual cargo genes.

Due to their size, islands of this family can represent a major portion of the accessory genome. Strains with one or two large pKLC102/PAGI-2-family elements are common, but higher numbers per genome are possible. *P. aeruginosa* strain C harbors PAGI-2 and pKLC102, but two more sets of backbone ORFs have been identified in the chromosome indicating four related elements in total, with an overall DNA sequence length of more than 360 kbp (own unpublished data). Of the seven genomes presented in **Table 2**, six contain large pKLC102/PAGI-2-family islands. Strains PA14, C3719, and PA7 each harbor one pKLC102-like island in RGP41 or, in case of PA7, in RGP7. LESB58 also contains one island, but of the PAGI-2 subtype (LESGI-3 in RGP27). Two islands each are located in the 2192- and the PACS2 genomes. Both strains also harbor a pKLC102-like insertion in RGP41 and a PAGI-2-related island, which is in RGP29 for strain 2192 and in RGP27 in PACS2.

The island in 2192 inserted at RGP29 is a nearly identical copy of PAGI-2 itself but is interestingly accompanied by another island of comparable size, the so-called Dit-island which is distinct from the

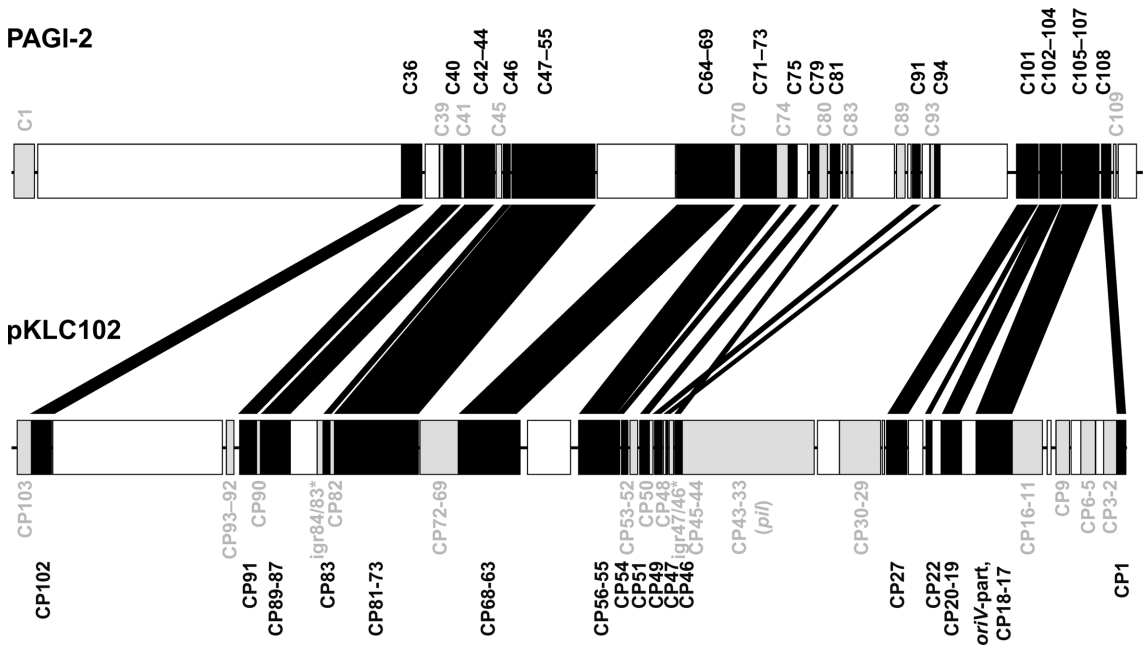

FIGURE 4 | Conserved genes in pKLC102-/PAGI-2-like genomic islands. PAGI-2 (Larbig et al., 2002) and pKLC102 (Klockgether et al., 2004) were chosen as representatives for the respective subtypes among the pKLC102-/PAGI-2 family. The annotated ORFs are labeled according to their conservation. ORFs appearing in all *P. aeruginosa* islands of this family ("backbone genes") are shown in black. ORFs conserved within one of the subtypes are colored in gray. White blocks represent ORFs specific for the single islands ("individual cargo"). Intergenic regions (igr) marked with an asterisk indicate loci with no ORF annotated for pKLC102 but for the highly homologous sequences in other islands from this subtype. Please note that ORF C105 of PAGI-2 is homologous to DNA in pKLC102 described as a part of the replication origin *oriV* of this element. The other part of *oriV* containing 16 57 bp repeats (Klockgether et al., 2004) is not conserved among the island family, not even in other islands from the pKLC102 subtype.

pKLC102/PAGI-2 family (Mathee et al., 2008). Thus an extremely large insertion of about 220 kbp is present in RGP29, which probably resulted from successive acquisition of two elements using the same chromosomal integration site. The RGP41-insertion in strain PA7 also provides hints for a combination of genome islands. Next to the pKLC102-like island with all typically conserved genes a DNA block with a second copy of some of the backbone genes is located, resembling a fragment of a second pKLC102-like element linked to the first one (Klockgether et al., 2008; Roy et al., 2010).

REPLACEMENT ISLANDS

Table 2 also lists the loci in the core genome that are under diversifying selection, the so-called replacement islands: RGP9 (flagellin glycosylation genes), RGP31 (O-antigen biosynthesis genes), RGP60 (pilin gene), and RGP73 (pyoverdine gene cluster). The RGPs only encompass those genes that fulfill the definition of less than 70% nucleotide sequence identity between homologs and thus do not necessarily comprise the complete functional units (Mathee et al., 2008).

The types of each replacement island were identified by comparative sequencing of the respective gene clusters in *P. aeruginosa* strain collections. The 20 known O-antigen serotypes, for example, were assigned to 11 groups according to the criterion of more than 98% sequence identity in the major O-antigen biosynthesis gene cluster (Raymond et al., 2002).

RGP60, containing the *pilA* gene that encodes the major subunit for type IV attachment pili, was classified into groups I–V (Kus et al., 2004). This "major pilin" region adjacent to a $tRNA^{Thr}$ gene contains, besides *pilA* for all groups but group II, several *tfp* genes that are involved in type IV pilus assembly and modification. More *tfp* genes are located downstream in the "minor pilin" region. Each of the five major pilin regions is associated with a specific set of minor pilins, and unrelated strains with the same major pilin type have identical minor pilin genes (Giltner et al., 2011). The absolute linkage disequilibrium between major and minor pilin groups provides evidence that both regions were derived from one large island. Consistent with this interpretation more pilin assembly genes are located between the major and minor pilin groups. These genes, however, were not subject of diversifying selection. Moreover a tRNA gene cluster is located between the major and the minor pilin region that serves as a hotspot for integration of large pKLC102-like islands (RGP41). Thus, the genome distance between major and minor pilin gene clusters varies between 136 kbp in strain PA14 and only 29 kbp in PAO1.

The pyoverdine gene clusters I, II, and III encode the three pyoverdine types and their specific receptor. Intratype divergence driven by recombination, positive selection, and horizontal gene transfer have enhanced the diversity of this genomic region (Smith et al., 2005).

The two flagellins a and b differ in their primary amino acid sequence and their glycosylation from each other (Spangenberg et al., 1996). b-type flagellins are conserved in sequence and glycosylation (Verma et al., 2006). In contrast, six *fliC* single nucleotide substitutions (SNPs) haplotypes (Spangenberg et al., 1996) and

differential glycosylation patterns lead to a large diversity of a-type flagellins (Arora et al., 2004). The variability of the a-type glycosylation gene cluster (RGP9) is high, even within the subtypes A1 and A2 that were defined by phylogenetic relatedness of amino acid sequences.

THE *P. AERUGINOSA* PANGENOME

The pangenome represents the complete gene pool of a bacterial species. Thus the description of a pangenome depends on the amount of sequence data available. For species with an extended accessory genome like *P. aeruginosa*, the addition of each new genome sequence will enlarge the overall pool of genes. The size of the core genome that is present in all strains will decrease concurrently.

To define the core genome and pangenome, the genomes are sequentially screened for orthologs by searching for reciprocal best BLAST hits. Genes that lack an ortholog in the already investigated gene pool are added to the pangenome.

We used the tool "Comparative Genome Search" provided by the *Pseudomonas* Genome Database[2] to define the number of orthologs representing reciprocal best blast hits in the four fully sequenced genomes of PAO1, PA14, LESB58, and PA7 (BLASTP comparisons, *E*-value cutoff: 1×10^{-4}). The tool also allows the determination of individual genes per genome, so the number of genes contributing to the pangenome could be counted with paralogs excluded. The results are shown in **Figure 5**. Please note that the PAO1 gene pool is lower than the overall number of ORFs in this genome (5520 compared to 5570) due to this exclusion of paralogs. As expected the core genome decreases and the pangenome increases each by a few hundred genes with the addition of a new genome. Although the analysis of just four genomes is insufficient for the extrapolation of the gene pool of core genome and pangenome of *P. aeruginosa*, we can assume that the pangenome does not approach a saturation value. Each novel genome sequence will contribute a yet unknown gene set to the pangenome. The large genomic islands of the pKLC102/PAGI-2 family contribute a broad variety of cargo to the species. Each strain possesses an individual set of islands that is acquired by horizontal gene transfer preferentially from beta- and gamma-proteobacteria (Klockgether et al., 2008). In other words, *P. aeruginosa* has wide, but not unrestricted access to the gene pool of prokaryotes.

INTRACLONAL GENOME DIVERSITY

The comparison of published genome sequences of clonally unrelated strains uncovered an interclonal sequence diversity of the *P. aeruginosa* core genome of 0.5–0.7% (Spencer et al., 2003; Cramer et al., 2011). The intraclonal diversity of members of the same clonal complex, however, is yet unknown. Of the strains with completely sequenced genomes, only strain PA14 belongs to a common clonal complex in the *P. aeruginosa* population (Wiehlmann et al., 2007). Hence we decided to sequence another strain of the PA14 clonal complex by Illumina sequencing-by-synthesis technology [study accession number ERP000390 at the Nucleotide Read Archive (ENA) of the EBI]. This strain RN3 was isolated from the first *P. aeruginosa*-positive airway specimen of an individual with cystic fibrosis who was living in North–West Germany. Strain PA14 is a clinical isolate from California. Thus the two strains are of unrelated geographic origin.

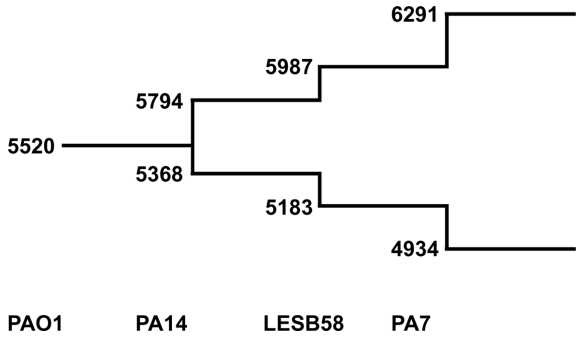

FIGURE 5 | The *P. aeruginosa* pangenome. The extent of the *P. aeruginosa* core- and pan-genome is shown as a stepwise development going along with the availability of complete genome sequences. The numbers at the lower branch give the amount of genes identified as best reciprocal blast hits in the indicated genomes (core genome). Numbers of the upper branch describe amount of genes making up the pangenome. For each genome the number of genes are added that are neither ortho- nor paralogs of genes from the existing pool.

The strain PA14 and strain RN3 genomes match in genome size and differ in 231 SNPs from each other (**Table 4**) which corresponds to a sequence diversity of 3.5×10^{-5}. Transitions ($n = 148$) occurred significantly more frequently than the expected ratio of transitions to transversions of 55: 176 of a random distribution ($\chi^2 = 206.3$; $P < 0.001$). The number of SNPs in inter- and intragenic regions roughly corresponded with their proportions in the genome. Within the coding regions synonymous SNPs were significantly overrepresented ($\chi^2 = 23.2$; $P < 0.001$) indicating that *de novo* amino acid substitutions had been subject to purifying selection.

Of the 231 SNPs, only 33 SNPs followed the statistics of a random distribution in the genome (**Figure 6**). In other words, 198 SNPs were non-randomly distributed in the genome implying that the affected loci had been subject to diversifying selection.

The major hotspot is the phage Pf1-like gene cluster (PA14_48890–PA14_49000) with 87 SNPs, i.e., 38% of all SNPs. Thus phage Pf1 seems to be the most rapidly evolving part of the PA14 genome consistent with the view that phages span a high degree of genetic diversity and are prone to frequent horizontal transfer (Hatfull, 2008).

Non-synonymous SNPs were mainly found in the functional categories of transcriptional regulators, membranes, cellular appendages, transport, and secretion (**Table 4**). Hotspots of sequence diversity in single genes between the PA14 and RN3 genomes are *ftsZ, armB (mexH)*, and *cynS* with six, five, and four SNPs, respectively. FtsZ is the major tubulin-like cytoskeletal protein in the bacterial cytokinesis machine (Erickson et al., 2010) and hence we noted with surprise that the FtsZ proteins of strains PA14 and RN3 differ at five positions in their amino acid sequence. The substitutions P-L, M-L, G-D, T-N, and P-T are located within a stretch of 35 amino acids of the 394 aa

[2]http://www.pseudomonas.com/geneSearch.jsp

Table 4 | Single nucleotide substitutions in RN3 sequence (compared to PA14 reference).

Intragenic position	nt	Locus_tag	aa	Gene name	Encoded product
72440	T – C	PA14_00740	K – E		Putative lipoprotein
96307	A – C	PA14_00970	syn.		Hypothetical protein
273734	C – T	PA14_03110	D – N		Hypothetical protein
477483	G – A	PA14_05410	syn.	*chpC*	putative chemotaxis protein
480880	T – C	PA14_05450	syn.		16S ribosomal RNA methyltransferase RsmE
480915	T – C	PA14_05450	K – E		16S ribosomal RNA methyltransferase RsmE
522777	G – T	PA14_05890	E – stop[1]		putative stomatin-like protein
741080	G – A	PA14_08660			$tRNA^{Gly}$
747764	G – A	PA14_08760	G – D	*rpoB*	DNA-directed RNA polymerase subunit beta
791890	A – G	PA14_09280	N – D	*pchF*	Pyochelin synthetase
888038[2]	C – T	PA14_10290	P – L	*acoR*	Transcriptional regulator AcoR
888039[2]	T – G	PA14_10290	P – L	*acoR*	Transcriptional regulator AcoR
927917	T – C	PA14_10770	I – T		Putative sensor/response regulator hybrid
982940	A – G	PA14_11290	syn.		Putative permease
1071133	G – C	PA14_12430[3]		*ladS*	Homolog to lost adherence sensor LadS
1082958	A – G	PA14_12630	syn.		Putative ATP-dependent helicase
1356548	G – C	PA14_15920	R – G	*yhjE*	Major facilitator transporter
1441164	T – C	PA14_16820	syn.		Putative efflux transmembrane protein
1468998	C – T	PA14_17130	syn.	*dxr*	1-deoxy-d-xylulose 5-phosphate reductoisomerase
1551564	G – A	PA14_18080	A – V		TetR family transcriptional regulator
1558205	A – G	PA14_18150	syn.	*acsL*	Putative acetyl-CoA synthetase
1612742	A –G	PA14_18740	syn.	*argG*	Argininosuccinate synthase
1640196	G – T	PA14_18985	P – H		Hypothetical protein
1640394	A – G	PA14_18985	F – S		Hypothetical protein
1880872	C – G	PA14_21690	A – G	*lhr1*	Putative ATP-dependent DNA helicase
1960256	C – A	PA14_22520	R – L		Hypothetical protein
2027678	C – G	PA14_23360	P – R	*wzz*	O-antigen chain length regulator
2149425	T – C	PA14_24600	syn.		Putative carboxypeptidase
2156146	C – A	PA14_24665	Q – K		Hypothetical protein
2209674	A – G	PA14_25250	K – E	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase
2318606	A – G	PA14_26600	syn.		RNA polymerase sigma factor
2407435	C – G	PA14_27755	syn.	*yliJ*	Glutathione S-transferase
2407463	A – G	PA14_27755	K – E	*yliJ*	Glutathione S-transferase
2510099	A – G	PA14_29030	T – A		Putative FMN oxidoreductase
2545609	T – C	PA14_29390	syn.		Hypothetical protein
2545663	T – C	PA14_29390	syn.		Hypothetical protein
2553747	T – C	PA14_29440	D – G		LysR family transcriptional regulator
2651339	T – C	PA14_30600	F – L		Putative permease
2651357	A – G	PA14_30600	N – D		Putative permease
2762006	A – G	PA14_31750	K – E		Putative acyltransferase
2787777	C – G	PA14_32015[3]		*czcA*	Homolog to RND efflux transporter CzcA
2787784	T – G	PA14_32015[3]		*czcA*	Homolog to RND efflux transporter CzcA
2807266	G – C	PA14_32300	V – L		Putative kinase
2885933	G – C	PA14_32985	syn.	*gcvH2*	Glycine cleavage system protein H
2955357	A – G	PA14_33600	syn.		Hypothetical protein
2955433	A – G	PA14_33600	syn.		Hypothetical protein
2955468	A – G	PA14_33600	syn.		Hypothetical protein
2985345	A – G	PA14_33650	K – E	*pvdD*	Pyoverdine synthetase D
3198441	T – G	PA14_35940	syn.		Acyl-CoA synthetase
3373667	G – C	PA14_37830	syn.	*iscS*	Putative pyridoxal-phosphate dependent enzyme
3374601	A – G	PA14_37830	F – S	*iscS*	Putative pyridoxal-phosphate dependent enzyme

(Continued)

Table 4 | Continued

Intragenic position	nt	Locus_tag	aa	Gene name	Encoded product
3387854	A – C	PA14_37965	Y – S	*cynS*	Cyanate hydratase
3387881	A – C	PA14_37965	M – L	*cynS*	Cyanate hydratase
3387884	T – C	PA14_37965	F – L	*cynS*	Cyanate hydratase
3387941	A – C	PA14_37965	M – L	*cynS*	Cyanate hydratase
3390498	A – C	PA14_38000	Stop – S[4]		Hypothetical protein
3423281	A – G	PA14_38410	syn.	*amrB/mexH*	Multidrug efflux protein
3423414	C – G	PA14_38410	Q – E	*amrB/mexH*	Multidrug efflux protein
3424176	A – G	PA14_38410	T – A	*amrB/mexH*	Multidrug efflux protein
3424199	A – G	PA14_38410	syn.	*amrB/mexH*	Multidrug efflux protein
3425614	A – T	PA14_38410	H – S	*amrB/mexH*	Multidrug efflux protein
3442543	G – A	PA14_38580	G – D		Hypothetical protein
3443292	C – G	PA14_38580	P – A		Hypothetical protein
3541978	G – A	PA14_39750	syn.		Putative amino acid permease
3543662	A – G	PA14_39770	T – A		Putative regulatory protein
3558172	A – G	PA14_39910	F – L	*phzE2*	Phenazine biosynthesis protein PhzE
3559401	T – C	PA14_39925	K – E	phzD2	Phenazine biosynthesis protein PhzD
3566716	A – G	PA14_40020	Q – R		Hypothetical protein
3566730	A – G	PA14_40020	K – E		Hypothetical protein
3566749	A – G	PA14_40020	Q – R		Hypothetical protein
3566751	A – G	PA14_40020	N – D		Hypothetical protein
3566769	A – G	PA14_40020	K – E		Hypothetical protein
3566788	A – G	PA14_40020	Q – R		Hypothetical protein
3670384	A – G	PA14_41150	syn.		Putative permease of ABC transporter
3711749	T – C	PA14_41563	syn.	*cobA*	Uroporphyrin-III C-methyltransferase
3711791	G – C	PA14_41563	V – L	*cobA*	Uroporphyrin-III C-methyltransferase
3764383	A – G	PA14_42220	I – M		Membrane sensor domain-containing protein
3769180	C – G	PA14_42250	syn.	*pscL*	Type III secretion system protein
3879553	A – G	PA14_43570	F – L		Hypothetical protein
3906764	G – C	PA14_43870	R – G		Hypothetical protein
3933352	C – G	PA14_44190	syn.		Putative sugar MFS transporter
4346242	C – G	PA14_48890	syn.		Hypothetical protein
4346254	G – A	PA14_48890	syn.		Hypothetical protein
4346325	A – G	PA14_48890	syn.		Hypothetical protein
4346329	G – A	PA14_48890	syn.		Hypothetical protein
4346413	G – A	PA14_48890	syn.		Hypothetical protein
4346434	G – A	PA14_48890	syn.		Hypothetical protein
4346485	A – G	PA14_48890	syn.		Hypothetical protein
4346497	G – A	PA14_48890	syn.		Hypothetical protein
4346500	G – A	PA14_48890	syn.		Hypothetical protein
4346665	C – T	PA14_48890	syn.		Hypothetical protein
4346713	C – T	PA14_48890	syn.		Hypothetical protein
4346731	T – C	PA14_48890	syn.		Hypothetical protein
4346763	A – G	PA14_48890	syn.		Hypothetical protein
4346845	A – G	PA14_48890	syn.		Hypothetical protein
4346890	A – C	PA14_48890	syn.		Hypothetical protein
4346926	G – A	PA14_48890	syn.		Hypothetical protein
4346938	C – T	PA14_48890	syn.		Hypothetical protein
4347034	C – T	PA14_48890	syn.		Hypothetical protein
4347190	A – G	PA14_48890	syn.		Hypothetical protein
4347211	G – A	PA14_48890	syn.		Hypothetical protein
4347241	G – A	PA14_48890	syn.		Hypothetical protein

(Continued)

Table 4 | Continued

Intragenic position	nt	Locus_tag	aa	Gene name	Encoded product
4347256	C – T	PA14_48890	syn.		Hypothetical protein
4347283	T – C	PA14_48890	syn.		Hypothetical protein
4347289	G – C	PA14_48890	syn.		Hypothetical protein
4347310	T – C	PA14_48890	syn.		Hypothetical protein
4347322	T – C	PA14_48890	syn.		Hypothetical protein
4347346	C – G	PA14_48890	syn.		Hypothetical protein
4347358	G – A	PA14_48890	syn.		Hypothetical protein
4347376	C – G	PA14_48890	syn.		Hypothetical protein
4347642	G – A	PA14_48900	A – V		Hypothetical protein
4347673	T – A	PA14_48900	T – S		Hypothetical protein
4347701	C – A	PA14_48900	syn.		Hypothetical protein
4347825	G – T	PA14_48910	P – T		Hypothetical protein
4348119	C – T	PA14_48910	A – T		Hypothetical protein
4348192	A – G	PA14_48910	syn.		Hypothetical protein
4348221	G – A	PA14_48910	P – S		Hypothetical protein
4348224	T – C	PA14_48910	T – A		Hypothetical protein
4348308	G – A	PA14_48910	syn.		Hypothetical protein
4348378	G – T	PA14_48910	syn.		Hypothetical protein
4348501	A – G	PA14_48910	syn.		Hypothetical protein
4348684	A – G	PA14_48910	syn.		Hypothetical protein
4348966	T – G	PA14_48910	syn.		Hypothetical protein
4349128	A – C	PA14_48920	syn.		Bacteriophage protein
4350200	A – T	PA14_48930	syn.		Putative coat protein A of bacteriophage Pf1
4350213	G – C	PA14_48930	A – G		Putative coat protein A of bacteriophage Pf1
4350484	T – C	PA14_48930	N – D		Putative coat protein A of bacteriophage Pf1
4350502	T – C	PA14_48930	T – A		Putative coat protein A of bacteriophage Pf1
4350656	G – A	PA14_48930	syn.		Putative coat protein A of bacteriophage Pf1
4350884	A – G	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4350911	G – C	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4350917	A – G	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4350941	A – G	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4350959	T – C	PA14_48940	syn.	*coaB*	Coat protein B of bacteriophage Pf1
4351186	C – T	PA14_48950	A – T		Hypothetical protein
4351199	G – A	PA14_48950	syn.		Hypothetical protein
4351316	A – G	PA14_48950	syn.		Hypothetical protein
4351503	G – A	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351563	T – C	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351617	A – G	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351641	C – G	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351722	A – G	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4351857	G – A	PA14_48970	syn.		Helix destabilizing protein of bacteriophage Pf1
4352075	A – G	PA14_48980	syn.		Hypothetical protein
4352106	T – C	PA14_48980	D – G		Hypothetical protein
4352113	C – A	PA14_48980	D – Y		Hypothetical protein
4352144	G – C	PA14_48980	S – R		Hypothetical protein
4352234	G – A	PA14_48980	syn.		Hypothetical protein
4352294	G – C	PA14_48980	syn.		Hypothetical protein
4352384	C – T	PA14_48980	syn.		Hypothetical protein
4352465	G – T	PA14_48990	syn.		Hypothetical protein
4352471	G – A	PA14_48990	syn.		Hypothetical protein
4352545	C – A	PA14_48990	A – S		Hypothetical protein

(Continued)

Table 4 | Continued

Intragenic position	nt	Locus_tag	aa	Gene name	Encoded product
4352560	G – A	PA14_48990	P – S		Hypothetical protein
4352594	C – G	PA14_48990	syn.		Hypothetical protein
4352607	C – T	PA14_48990	R – Q		Hypothetical protein
4352676	G – C	PA14_48990	A – G		Hypothetical protein
4352700	T – C	PA14_48990	H – R		Hypothetical protein
4352821	T – C	PA14_49000	I – V		Hypothetical protein
4352865[2]	A – C	PA14_49000	I – G		Hypothetical protein
4352866[2]	T – C	PA14_49000	I – G		Hypothetical protein
4352921	C – A	PA14_49000	M – I		Hypothetical protein
4450619	T – G	PA14_50060	L – R		Hypothetical protein
4565005	C – G	PA14_51360	G – A	*phnA*	Hnthranilate synthase component I
4565040	A – G	PA14_51360	syn.	*phnA*	Hnthranilate synthase component I
4565093	C – G	PA14_51360	G – R	*phnA*	Hnthranilate synthase component I
4707658	G – C	PA14_53110	syn.		Hxidoreductase
4707787	G – C	PA14_53110	syn.		Oxidoreductase
4760743	A – G	PA14_53670	L – P		Hypothetical protein
4901696	A – G	PA14_55180	M – V	*migA*	Glycosyl transferase
4912690	C – T	PA14_55330	D – N		Hypothetical protein
4947683	A – G	PA14_55600	H – R		Hypothetical protein
4997786	T – C	PA14_55980	K – E	*yjgR*	Hypothetical protein
5041775	A – T	PA14_56550	syn.		Hypothetical protein
5103224[2]	A – C	PA14_57275	P – L	*ftsZ*	Cell division protein FtsZ
5103225[2]	G – A	PA14_57275	P – L	*ftsZ*	Cell division protein FtsZ
5103259	T – G	PA14_57275	M – L	*ftsZ*	Cell division protein FtsZ
5103291	C – T	PA14_57275	G – D	*ftsZ*	Cell division protein FtsZ
5103303	G – T	PA14_57275	T – N	*ftsZ*	Cell division protein FtsZ
5103322	G – T	PA14_57275	P – T	*ftsZ*	Cell division protein FtsZ
5236534	A – G	PA14_58760	syn.	*pilC*	Type 4 fimbrial biogenesis protein pilC
5404627	C – T	PA14_60630	L – F		Hypothetical protein
5464577	C – T	PA14_61200	G – D		Hypothetical protein
5530315	A – G	PA14_62000	F – L	*hitA*	Ferric iron-binding periplasmic protein HitA
5722900	A – C	PA14_64230	D – A	*retS/rtsM*	RetS, regulator of exopolysaccharide and type III Secretion
5757525	G – C	PA14_64620	Q – E		Putative oxidoreductase
5757527	G – C	PA14_64620	P – R		Putative oxidoreductase
5809365	T – C	PA14_65190	K – E	*yjfH*	TrmH family RNA methyltransferase, group 3
5866730	T – G	PA14_65860	syn.		Putative two-component sensor
5905079	A – G	PA14_66270	syn.	*glnE*	Glutamine-synthetase adenylyltransferase
5968025	C – G	PA14_66820	P – A	*phaC1*	Poly(3-hydroxyalkanoic acid) synthase 1
6070122	T – C	PA14_68020[3]			Homolog to hypothetical protein PA5149
6076066	G – C	PA14_68100	syn.		Hypothetical protein
6412470	G – C	PA14_71930	R – G	*wbpX*	Glycosyltransferase WbpX
6441338	T – C	PA14_72300	L – P		Hypothetical protein

Intergenic position	nt	Intergenic region
151966	A – G	igrPA14_01660-01670
187759	G – T	igrPA14_02050-02060
208430	T – G	igrPA14_02310-02330
208433	G – C	igrPA14_02310-02330
888497	A – C	igrPA14_10290-10300
966217	T – C	igrPA14_11110-11120
1144646	C – G	igrPA14_13320-13330

(Continued)

Table 4 | Continued

Intergenic position	nt	Intergenic region
1375947	A – G	igrPA14_16150-16160
1725505	A – G	igrPA14_20020-20030
1748240	T – C	igrPA14_20290-20300
1923008	C – T	igrPA14_22080-22090
2354149	A – G	igrPA14_27090-27100
2362330	A – C	igrPA14_27180-27190
2362363	G – C	igrPA14_27180-27190
2589402	C – T	igrPA14_29890-29900
2840442	T – C	igrPA14_32700-32710
2840444	T – C	igrPA14_32700-32710
3281477	G – C	igrPA14_36810-36820
3356495	G – C	igrPA14_37680-37690
3515863	T – C	igrPA14_39480-39500
3662614	T – C	igrPA14_41070-41080
4347602	G – C	igrPA14_48890-48900
4351470	T – G	igrPA14_48960-48970
4352019	T – G	igrPA14_48970-48980
4352023	C – G	igrPA14_48970-48980
4352432	G – A	igrPA14_48980-48990
4352433	G – A	igrPA14_48980-48990
4407236	A – G	igrPA14_49540-49560
4659805	A – G	igrPA14_52530-52540
4708161	A – G	igrPA14_53110-53120
5198405	T – C	igrPA14_58360-58375
5200474	A – C	igrPA14_58380-58390
5565118	A – G	igrPA14_62380-62390
5648548	A – G	igrPA14_63280-63290
5648573	A – G	igrPA14_63280-63290
5792010	A – G	igrPA14_64980-64990

[1] *Protein length 180 aa instead of 264.*

[2] *Two SNPs in one codon.*

[3] *Annotated as probably inactive protein fragment/putative frameshift gene.*

[4] *Next stop 18 codons downstream.*

protein and are all not neutral (**Table 4**). MexH is a component of the MexGHI-OpmD efflux pump that is required for biofilm formation (Southey-Pillig et al., 2005), facilitates cell-to-cell communication and promotes virulence and growth in *P. aeruginosa* (Aendekerk et al., 2005). MexH of strains PA14 and RN3 differ by three amino acid substitutions (Q-E, T-A, and H-S) in three distant domains of the protein from each other. *CynS* encodes a cyanase (EC 4.2.1.104) that catalyzes the decomposition of cyanate into CO_2 and ammonium (Luque-Almagro et al., 2008). The intraclonal diversity of cyanase between RN3 and PA14 of four amino acid substitutions is similar in number and localization to that of the completely sequenced *P. aeruginosa* strains, i.e., 5–11 amino acid substitutions clustering in the N-terminal region of CynS.

Key genes were also affected by non-synonymous SNPs that may modulate the function of the gene products. The DNA-directed RNA polymerase RpoB of strain RN3 carries a substitution of a glycine by an aspartate, and the global regulator RetS of the sessile and planktonic lifestyle of *P. aeruginosa*, which is involved in the transition from acute to chronic infections (Goodman et al., 2004), harbors a substitution of an aspartate by an alanine.

Of the 34 observed amino acid substitution types, nine are classified by the Dayhoff (1978) matrix as uncommon and associated with an impact on protein function. In contrast, only 12 of the 20 most common neutral amino acid changes were seen. In summary, SNPs non-randomly targeted elements of the cell surface and uncommon non-neutral substitutions (e.g., K-E) were overrepresented in the affected proteins. These facts suggest that in the investigated case the intraclonal diversity did not evolve by random drift, but was driven by selective forces.

Strain RN3 was isolated from the first *P. aeruginosa*-positive specimen taken from an individual with cystic fibrosis. Thus the portion of adaptive mutations that typically emerge during chronic colonization of cystic fibrosis airways (Smith et al., 2006) should be low. Nevertheless some sequence differences between RN3 and PA14 could provide RN3 with

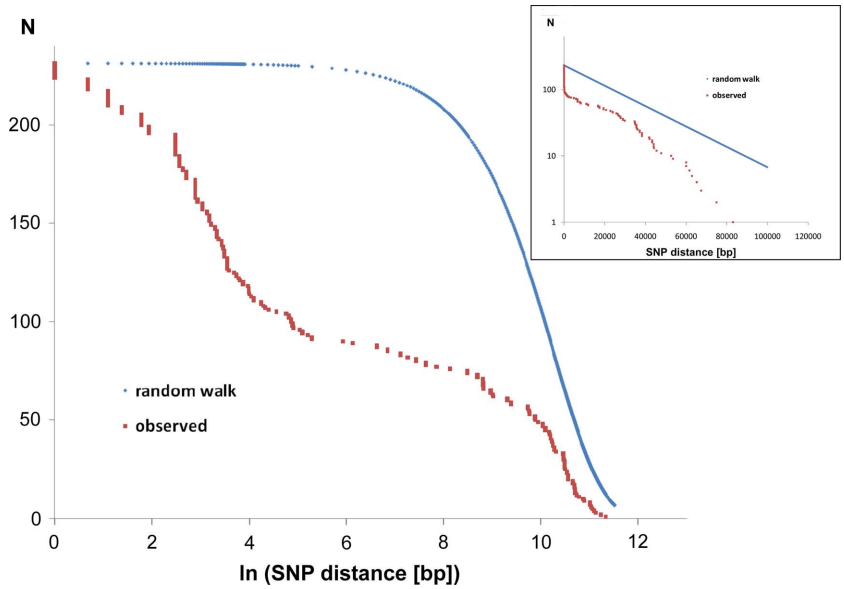

FIGURE 6 | Intraclonal SNP diversity of the *P. aeruginosa* PA14 clone: distribution of nearest SNP neighbors in the RN3 genome. Mapping of the RN3 genome onto the PA14 genome uncovered 231 SNPs. The figure depicts the genomic distribution of the distance between two adjacent SNPs (nearest neighbors). The red graphs show the observed distribution that is compared with a random genomic distribution of the same number of 231 SNPs (blue graphs, one-dimensional random walk statistics). The two semilogarithmic plots visualize the deviation from a random distribution at either a global scale (insert) or with focus on the hotspots of sequence diversity (large figure).

selective advantage to adapt and persist in cystic fibrosis airways. Obvious candidates are loci encoding efflux pumps (*mexH*), major transcriptional regulators (*retS*), and siderophore (*pvdD*), cyanide (*cynS*), or quinolone (*phnA*) biosynthesis, respectively.

The major take home message of our endeavor to compare the intraclonal genome diversity of strains of distant geographic origin was the unexpectedly low substitution rate. Statistical analysis provides strong evidence that nucleotide substitutions in coding regions were under purifying selection so that only a low number of substitutions was fixed. This versatile, ubiquitous and phylogenetically ancient organism apparently does not need many *de novo* mutations if it conquers a new habitat. The next step to understand the molecular evolution of intraclonal diversity would be the determination of the relative contributions of *de novo* mutation versus recombination. To accomplish this task, a larger collection of clone PA14 strains than just two isolates will have to be studied (see Spratt, 2004, for an appropriate study design).

PERSPECTIVES

Only four completely sequenced *P. aeruginosa* genomes are officially deposited as finished genomes in GenBank. Draft genomes exist for a five further genomes and several dozen *P. aeruginosa* projects are deposited in the ENA hosted by EMBL-EBI (see text footnote 1). Many of the projects were done for the purpose of (re)sequencing variants of already known strains. Thorough genome assemblies and functional annotations are probably intended only in a minority of cases. But nevertheless an immense increase in *P. aeruginosa* genome data is expected to become available in the near future due to the on-going revolution of sequencing technologies. In particular, the sequencing of strains from environmental habitats should provide us with an unbiased overview of the genetic repertoire of the *P. aeruginosa* population.

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REFERENCES

Aendekerk, S., Diggle, S. P., Song, Z., Høiby, N., Cornelis, P., Williams, P., and Cámara, M. (2005). The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinolone-dependent cell-to-cell communication. *Microbiology* 151(Pt 4), 1113–1125.

Arora, S. K., Wolfgang, M. C., Lory, S., and Ramphal, R. (2004). Sequence polymorphism in the glycosylation island and flagellins of *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 2115–2122.

Battle, S. E., Meyer, F., Rello, J., Kung, V. L., and Hauser, A. R. (2008). Hybrid pathogenicity island PAGI-5 contributes to the highly virulent phenotype of a *Pseudomonas aeruginosa* isolate in mammals. *J. Bacteriol.* 190, 7130–7140.

Battle, S. E., Rello, J., and Hauser, A. R. (2009). Genomic islands of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* 290, 70–78.

Carter, M. Q., Chen, J., and Lory, S. (2010). The *Pseudomonas aeruginosa* pathogenicity island PAPI-1 is transferred via a novel type IV pilus. *J. Bacteriol.* 192, 3249–3258.

Cheng, K., Smyth, R. L., Govan, J. R., Doherty, C., Winstanley, C., Denning, N., Heaf, D. P., van Saene, H., and Hart, C. A. (1996). Spread of beta-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet* 348, 639–642.

Cramer, N., Klockgether, J., Wrasman, K., Schmidt, M., Davenport, C., and Tümmler, B. (2011). Microevolution of the major common

Pseudomonas aeruginosa clones C and PA14 in cystic fibrosis lungs. *Environ. Microbiol.* 13, 1590–1604.

Davenport, C. F., Wiehlmann, L., Reva, O. N., and Tümmler, B. (2009). Visualization of *Pseudomonas* genomic structure by abundant 8-14mer oligonucleotides. *Environ. Microbiol.* 11, 1092–1104.

Dayhoff, M. O. (1978). *Atlas of Protein Sequence and Structure, Observed Frequencies of Amino Acid Replacements Between Closely Related Proteins*, Vol. 5, Suppl. 3. Washington, DC: National Biomedical Research foundation.

Dobrindt, U., Hochhut, B., Hentschel, U., and Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nat. Rev. Microbiol.* 2, 414–424.

Dötsch, A., Klawonn, F., Jarek, M., Scharfe, M., Blöcker, H., and Häussler, S. (2010). Evolutionary conservation of essential and highly expressed genes in *Pseudomonas aeruginosa*. *BMC Genomics* 11, 234. doi: 10.1186/1471-2164-11-234

Erickson, H. P., Anderson, D. E., and Osawa, M. (2010). FtsZ in bacterial cytokinesis: cytoskeleton and force generator all in one. *Microbiol. Mol. Biol. Rev.* 74, 504–528.

Ernst, R. K., D'Argenio, D. A., Ichikawa, J. K., Bangera, M. G., Selgrade, S., Burns, J. L., Hiatt, P., McCoy, K., Brittnacher, M., Kas, A., Spencer, D. H., Olson, M. V., Ramsey, B. W., Lory, S., and Miller, S. I. (2003). Genome mosaicism is conserved but not unique in *Pseudomonas aeruginosa* isolates from the airways of young children with cystic fibrosis. *Environ. Microbiol.* 5, 1341–1349.

Frank, S., Klockgether, J., Hagendorf, P., Geffers, R., Schöck, U., Pohl, T., Davenport, C. F., and Tümmler, B. (2011). *Pseudomonas putida* KT2440 genome update by cDNA sequencing and microarray transcriptomics. *Environ. Microbiol.* 13, 1309–1326.

Gaillard, M., Pernet, N., Vogne, C., Hagenbüchle, O., and van der Meer, J. R. (2008). Host and invader impact of transfer of the clc genomic island into *Pseudomonas aeruginosa* PAO1. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7058–7063.

Giltner, C. L., Rana, N., Lunardo, M. N., Hussain, A. Q., and Burrows, L. L. (2011). Evolutionary and functional diversity of the *Pseudomonas* type IVa pilin island. *Environ. Microbiol.* 13, 250–264.

Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S., and Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev. Cell* 7, 745–754.

Hatfull, G. F. (2008). Bacteriophage genomics. *Curr. Opin. Microbiol.* 11, 447–453.

He, J., Baldini, R. L., Déziel, E., Saucier, M., Zhang, Q., Liberati, N. T., Lee, D., Urbach, J., Goodman, H. M., and Rahme, L. G. (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2530–2535.

Kiewitz, C., Larbig, K., Klockgether, J., Weinel, C., and Tümmler, B. (2000). Monitoring genome evolution ex vivo: reversible chromosomal integration of a 106 kb plasmid at two tRNA(Lys) gene loci in sequential *Pseudomonas aeruginosa* airway isolates. *Microbiology* 146(Pt 10), 2365–2373.

Klockgether, J., Munder, A., Neugebauer, J., Davenport, C. F., Stanke, F., Larbig, K. D., Heeb, S., Schöck, U., Pohl, T. M., Wiehlmann, L., and Tümmler, B. (2010). Genome diversity of *Pseudomonas aeruginosa* PAO1 laboratory strains. *J. Bacteriol.* 192, 1113–1121.

Klockgether, J., Reva, O., Larbig, K., and Tümmler, B. (2004). Sequence analysis of the mobile genome island pKLC102 of *Pseudomonas aeruginosa* C. *J. Bacteriol.* 186, 518–534.

Klockgether, J., Würdemann, D., Reva, O., Wiehlmann, L., and Tümmler, B. (2007). Diversity of the abundant pKLC102/PAGI-2 family of genomic islands in *Pseudomonas aeruginosa*. *J. Bacteriol.* 189, 2443–2459.

Klockgether, J., Würdemann, D., Wiehlmann, L., Binnewies, T. T., Ussery, D. W., and Tümmler, B. (2008). "Genome diversity of *Pseudomonas aeruginosa*," in *Pseudomonas Genomics and Molecular Biology*, ed. P. Cornelis (Norfolk: Caister Academic Press), 19–42.

Kresse, A. U., Dinesh, S. D., Larbig, K., and Römling, U. (2003). Impact of large chromosomal inversions on the adaptation and evolution of *Pseudomonas aeruginosa* chronically colonizing cystic fibrosis lungs. *Mol. Microbiol.* 47, 145–158.

Kulasekara, B. R., Kulasekara, H. D., Wolfgang, M. C., Stevens, L., Frank, D. W., and Lory, S. (2006). Acquisition and evolution of the exoU locus in *Pseudomonas aeruginosa*. *J. Bacteriol.* 188, 4037–4050.

Kung, V. L., Ozer, E. A., and Hauser, A. R. (2010). The accessory genome of *Pseudomonas aeruginosa*. *Microbiol. Mol. Biol. Rev.* 74, 621–641.

Kus, J. V., Tullis, E., Cvitkovitch, D. G., and Burrows, L. L. (2004). Significant differences in type IV pilin allele distribution among *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) versus non-CF patients. *Microbiology* 150(Pt 5), 1315–1326.

Larbig, K. D., Christmann, A., Johann, A., Klockgether, J., Hartsch, T., Merkl, R., Wiehlmann, L., Fritz, H. J., and Tümmler, B. (2002). Gene islands integrated into tRNA(Gly) genes confer genome diversity on a Pseudomonas aeruginosa clone. *J. Bacteriol.* 184, 6665–6680.

Lee, D. G., Urbach, J. M., Wu, G., Liberati, N. T., Feinbaum, R. L., Miyata, S., Diggins, L. T., He, J., Saucier, M., Déziel, E., Friedman, L., Li, L., Grills, G., Montgomery, K., Kucherlapati, R., Rahme, L. G., and Ausubel, F. M. (2006). Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biol.* 7, R90.

Liang, X., Pham, X. Q., Olson, M. V., and Lory, S. (2001). Identification of a genomic island present in the majority of pathogenic isolates of *Pseudomonas aeruginosa*. *J. Bacteriol.* 183, 843–853.

Luque-Almagro, V. M., Huertas, M. J., Sáez, L. P., Luque-Romero, M. M., Moreno-Vivián, C., Castillo, F., Roldán, M. D., and Blasco, R. (2008). Characterization of the *Pseudomonas* pseudoalcaligenes CECT5344 cyanase, an enzyme that is not essential for cyanide assimilation. *Appl. Environ. Microbiol.* 74, 6280–6288.

Mathee, K., Narasimhan, G., Valdes, C., Qiu, X., Matewish, J. M., Koehrsen, M., Rokas, A., Yandava, C. N., Engels, R., Zeng, E., Olavarietta, R., Doud, M., Smith, R. S., Montgomery, P., White, J. R., Godfrey, P. A., Kodira, C., Birren, B., Galagan, J. E., and Lory, S. (2008). Dynamics of *Pseudomonas aeruginosa* genome evolution. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3100–3105.

McCallum, S. J., Gallagher, M. J., Corkill, J. E., Hart, C. A., Ledson, M. J., and Walshaw, M. J. (2002). Spread of an epidemic *Pseudomonas aeruginosa* strain from a patient with cystic fibrosis (CF) to non-CF relatives. *Thorax* 57, 559–560.

Mohd-Zain, Z., Turner, S. L., Cerdeño-Tárraga, A. M., Lilley, A. K., Inzana, T. J., Duncan, A. J., Harding, R. M., Hood, D. W., Peto, T. E., and Crook, D. W. (2004). Transferable antibiotic resistance elements in *Haemophilus influenzae* share a common evolutionary origin with a diverse family of syntenic genomic islands. *J. Bacteriol.* 186, 8114–8122.

Moya, B., Dötsch, A., Juan, C., Blázquez, J., Zamorano, L., Häussler, S., and Oliver, A. (2009). Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog.* 5, e1000353. doi: 10.1371/journal.ppat.1000353

Qiu, X., Gurkar, A. U., and Lory, S. (2006). Interstrain transfer of the large pathogenicity island (PAPI-1) of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19830–19835.

Ramos, J. L. (ed.). (2004). *Pseudomonas Volume 1: Genomics, Life Style and Molecular Architecture*. New York: Kluwer Academics/Plenum Publishers.

Raymond, C. K., Sims, E. H., Kas, A., Spencer, D. H., Kutyavin, T. V., Ivey, R. G., Zhou, Y., Kaul, R., Clendenning, J. B., and Olson, M. V. (2002). Genetic variation at the O-antigen biosynthetic locus in *Pseudomonas aeruginosa*. *J. Bacteriol.* 184, 3614–3622.

Reva, O. N., and Tümmler, B. (2004). Global features of sequences of bacterial chromosomes, plasmids and phages revealed by analysis of oligonucleotide usage patterns. *BMC Bioinformatics* 5, 90. doi: 10.1186/1471-2105-5-90

Reva, O. N., and Tümmler, B. (2005). Differentiation of regions with atypical oligonucleotide composition in bacterial genomes. *BMC Bioinformatics* 6, 251. doi: 10.1186/1471-2105-6-251

Römling, U., Greipel, J., and Tümmler, B. (1995). Gradient of genomic diversity in the *Pseudomonas aeruginosa* chromosome. *Mol. Microbiol.* 17, 323–332.

Römling, U., Schmidt, K. D., and Tümmler, B. (1997). Large genome rearrangements discovered by the detailed analysis of 21 *Pseudomonas aeruginosa* clone C isolates found in environment and disease habitats. *J. Mol. Biol.* 271, 386–404.

Roy, P. H., Tetu, S. G., Larouche, A., Elbourne, L., Tremblay, S., Ren, Q., Dodson, R., Harkins, D., Shay, R., Watkins, K., Mahamoud, Y., and Paulsen, I. T. (2010). Complete genome sequence of the multiresistant taxonomic outlier *Pseudomonas aeruginosa* PA7. *PLoS ONE* 5, e8842. doi: 10.1371/journal.pone.0008842

Schmidt, K. D., Tümmler, B., and Römling, U. (1996). Comparative genome mapping of *Pseudomonas aeruginosa* PAO with *P. aeruginosa* C, which belongs to a major clone in cystic fibrosis patients and aquatic habitats. *J. Bacteriol.* 178, 85–93.

Sharma, C. M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermüller, J., Reinhardt, R., Stadler, P. F., and Vogel, J. (2010). The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* 464, 250–255.

Silby, M. W., Winstanley, C., Godfrey, S. A., Levy, S. B., and Jackson, R. W. (2011). *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol. Rev.* 35, 652–680.

Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P., Moskowitz, S. M., Burns, J. L., Kaul, R., and Olson, M. V. (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8487–8492.

Smith, E. E., Sims, E. H., Spencer, D. H., Kaul, R., and Olson, M. V. (2005). Evidence for diversifying selection at the pyoverdine locus of *Pseudomonas aeruginosa*. *J. Bacteriol.* 187, 2138–2147.

Southey-Pillig, C. J., Davies, D. G., and Sauer, K. (2005). Characterization of temporal protein production in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* 187, 8114–8126.

Spangenberg, C., Heuer, T., Bürger, C., and Tümmler, B. (1996). Genetic diversity of flagellins of *Pseudomonas aeruginosa*. *FEBS Lett.* 396, 213–217.

Spencer, D. H., Kas, A., Smith, E. E., Raymond, C. K., Sims, E. H., Hastings, M., Burns, J. L., Kaul, R., and Olson, M. V. (2003). Whole-genome sequence variation among multiple isolates of *Pseudomonas aeruginosa*. *J. Bacteriol.* 185, 1316–1325.

Spratt, B. G. (2004). Exploring the concept of clonality in bacteria. *Methods Mol. Biol.* 266, 323–352.

Stewart, R. M., Wiehlmann, L., Ashelford, K. E., Preston, S. J., Frimmersdorf, E., Campbell, B. J., Neal, T. J., Hall, N., Tuft, S., Kaye, S. B., and Winstanley, C. (2011). Genetic characterization indicates that a specific subpopulation of *Pseudomonas aeruginosa* is associated with keratitis infections. *J. Clin. Microbiol.* 49, 993–1003.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., and Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959–964.

Verma, A., Schirm, M., Arora, S. K., Thibault, P., Logan, S. M., and Ramphal, R. (2006). Glycosylation of b-type flagellin of *Pseudomonas aeruginosa*: structural and genetic basis. *J. Bacteriol.* 188, 4395–4403.

Wiehlmann, L., Wagner, G., Cramer, N., Siebert, B., Gudowius, P., Morales, G., Köhler, T., van Delden, C., Weinel, C., Slickers, P., and Tümmler, B. (2007). Population structure of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8101–8106.

Winsor, G. L., Van Rossum, T., Lo, R., Khaira, B., Whiteside, M. D., Hancock, R. E., and Brinkman, F. S. (2009). *Pseudomonas* Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes. *Nucleic Acids Res.* 37, D483–D488.

Winstanley, C., Langille, M. G., Fothergill, J. L., Kukavica-Ibrulj, I., Paradis-Bleau, C., Sanschagrin, F., Thomson, N. R., Winsor, G. L., Quail, M. A., Lennard, N., Bignell, A., Clarke, L., Seeger, K., Saunders, D., Harris, D., Parkhill, J., Hancock, R. E., Brinkman, F. S., and Levesque, R. C. (2009). Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool epidemic strain of *Pseudomonas aeruginosa*. *Genome Res.* 19, 12–23.

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Comparisons of two proteomic analyses of non-mucoid and mucoid *Pseudomonas aeruginosa* clinical isolates from a cystic fibrosis patient

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Pseudomonas aeruginosa chronically infects the lungs of cystic fibrosis (CF) patients. The conditions in the CF lung appear to select for *P. aeruginosa* with advantageous phenotypes for chronic infection. However, the mechanisms that allow the establishment of this chronic infection have not been fully characterized. We have previously reported the transcriptional analysis of two CF isolates strains 383 and 2192. Strain 2192 is a mucoid, alginate overproducing strain whereas strain 383 is non-mucoid. Mucoid strains are associated with chronic infection of the CF lung and non-mucoid strains are the typical initially infecting isolates. To elucidate novel differences between these two strains, we employed two methods of shotgun proteomics: isobaric tags for relative and absolute quantitation (iTRAQ) and two-dimensional gel electrophoresis (2-DE). iTRAQ compares the amount of protein between samples and relies on protein abundance, while 2-DE gel electrophoresis depends on selection of separated protein spots. For both these methods, mass spectrometry was then used to identify proteins differentially expressed between the two strains. The compilation of these two proteomic methods along with Western blot analysis revealed proteins of the HSI-I operon of the type 6 secretion system, showed increased expression in 383 compared to 2192, confirming the our previous transcriptional analysis. Proteomic analysis of other proteins did not fully correlate with the transcriptome but other differentially expressed proteins are discussed. Also, differences were noted between the results obtained for the two proteomic techniques. These shotgun proteomic analyses identified proteins that had been predicted only through gene identification; we now refer to these as "proteins of unknown functions" since their existence has now been established however their functional characterization remains to be elucidated.

Keywords: *Pseudomonas aeruginosa*, cystic fibrosis, alginate, iTRAQ, 2-DE, type 6 secretion

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium that is both a ubiquitous environmental organism and an important opportunistic pathogen of humans. *P. aeruginosa* can cause infections in compromised individuals such as those suffering from burns, AIDS, patients undergoing treatment for cancer, or those with cystic fibrosis (CF). *P. aeruginosa* infection of the CF airway leads to high mortality and morbidity.

The mucoid phenotype of *P. aeruginosa* is one of the most extensively researched characteristics of this species (Govan and Deretic, 1996; Ramsey and Wozniak, 2005). Mucoid strains overproduce the exopolysaccharide called alginate (**Figure 1**). Alginate is an unbranched linear polymer of partially acetylated β-D-mannuronic acid and its C5 epimer α-L-guluronic acid. Alginate synthesis begins in the cytoplasm and ends with secretion to the extracellular milieu. The *algD* alginate biosynthetic operon (containing *algD844KEGXLIJFalgA*; Chitnis and Ohman, 1993) and gene *algC* (Zielinski et al., 1991) encode enzymes required for synthesis of alginate. The biosynthetic genes are controlled by the extracytoplasmic function alternative sigma factor σ^{22} (also known as AlgT or AlgU), which is repressed by the transmembrane anti-sigma factor MucA (Martin et al., 1993). Mutation of *mucA* causes unregulated σ^{22} to activate alginate production (Martin et al., 1993). Recently, proteolytic degradation of MucA has also been recognized as a mechanism for alginate production (Qiu et al., 2008; Damron et al., 2009b; Wood and Ohman, 2009; Damron and Yu, 2011). When σ^{22} is free and active, due to *mucA* mutation or MucA proteolytic degradation, alginate overproduction will occur. In addition to increased expression of genes, σ^{22} is also known to repress gene expression as well (Wu et al., 2004; Tart et al., 2005; Jones et al., 2010).

Ineffective clearance of *P. aeruginosa* in the environment in the CF lung has been suggested to result in the conversion to the mucoid, alginate over-expressing phenotype, which provides these bacteria additional protection from antibiotics and phagocytic killing (Govan and Deretic, 1996; Lyczak et al., 2002). Mucoid

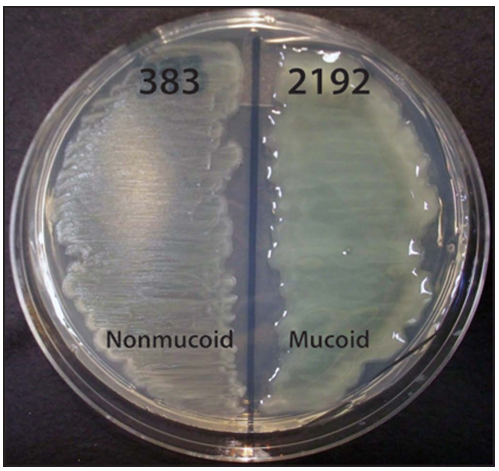

FIGURE 1 | Phenotypes of *P. aeruginosa* CF isolates 383 and 2192 on L agar media. Streaks of strains 383 and 2192 were cultured on L agar for 24 h at 37°C. These two strains were isolated within 2 days apart from a CF patient. Previous data indicated these strains are isogenic (Hanna et al., 2000). The genome of strain 2192 has been sequenced[1] (Mathee et al., 2008).

variants of *P. aeruginosa* effectively establish themselves as dominant pathogens in chronic lung diseases of CF patients (Lyczak et al., 2002). However, many other factors in addition to alginate overproduction have been described which contribute to the lung infection of CF patients (Hogardt and Heesemann, 2010).

Transcriptome analysis of *P. aeruginosa* via microarray has provided valuable insights into gene regulation of *P. aeruginosa* on a global level (Goodman and Lory, 2004). In addition, mass spectrometry provides proteomic analysis. In earlier studies with *P. aeruginosa*, protein identification has been performed using a qualitative approach for the identification of proteins by two-dimensional gel electrophoresis (2-DE) followed by tandem mass spectrometry (MS/MS) method (Quadroni et al., 1999; Malhotra et al., 2000; Sherman et al., 2001; Nouwens et al., 2003; Arevalo-Ferro et al., 2005; Sriramulu et al., 2005; Sharma et al., 2006). A sub-proteomic approach has led to identification and characterization of the periplasmic, membrane, or secreted *P. aeruginosa* proteins as potential virulence factors (Nouwens et al., 2003; Wehmhoner et al., 2003; Blonder et al., 2004; Imperi et al., 2009). Recently, *P. aeruginosa* gene products were identified by using collective data from proteomic, transcriptional profiling, and signature-tagged mutagenesis (Platt et al., 2008). Quantitative proteomics have been developed using isotope labeling of peptides in a method known as isobaric tags for relative and absolute quantitation (iTRAQ; Lacerda et al., 2008). Most transcriptome and proteomic analysis of *P. aeruginosa* have been performed on laboratory strains (Firoved et al., 2002; Wood et al., 2006; Damron et al., 2009a) along with a few studies with CF isolates (Hanna et al., 2000; Rau et al., 2010).

Our laboratory has previously performed proteomic studies of clinical isolates of *P. aeruginosa* strains 383 (non-mucoid) and 2192 (mucoid; **Figure 1**), which were isolated from sputum samples obtained from an individual with CF (Hanna et al., 2000). Using PAO1 GeneChip arrays, we determined at least 188 genes differ in expression between non-mucoid strain 383 and mucoid strain 2192 (Rao et al., 2008). Since that time, the genome of 2192 has been sequenced[2] (Mathee et al., 2008); pangenome analysis has been performed and indicates niche adaptation is the force that drives the composition of *P. aeruginosa* genomes (Mathee et al., 2008).

Our hypothesis is that many other factors or systems, which may contribute to the disease in CF are altered in expression between mucoid and non-mucoid strains. In the present study, we have taken a global approach to detect the novel differences between the strains 383 and 2192 by using the shotgun proteomic methods of both iTRAQ and 2-DE. Total whole cell proteins were subjected to iTRAQ for quantification. In addition, total whole cell lysates and Triton X-114 soluble proteins were separated by 2-DE gel electrophoresis. Our data are consistent with the observations of an inverse relationship between the mucoid phenotype and the type 6 secretion system (T6SS). This suggests that the T6SS may be associated with non-mucoid infection strains but repressed in mucoid infection strains. Furthermore, iTRAQ and 2-DE proteomic analysis detected proteins that had only been predicted by gene identification of open reading frames (ORF) with no known homologs. We now refer to these "hypothetical" and "conserved hypothetical" proteins as "proteins of unknown function" (PUF) and determined that some are differentially expressed in the acute and chronic phenotypic strains of *P. aeruginosa*.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

The bacterial strains used in this study, *P. aeruginosa* strains 383 and 2192, are non-mucoid and mucoid, respectively. These strains were isolated 2 days apart from the sputum of the same CF patient (Hanna et al., 2000). Bacterial cultures were grown overnight in Luria Bertani (LB) medium at 37°C in shaking (200 rpm), diluted to 1:100 in 30 ml of fresh media, and grown to mid-log phase, to an optical density (OD_{600}) of 0.5.

iTRAQ ANALYSIS

Total proteins were extracted from strains 383 and 2192 using Tris–EDTA buffer (10 mM Tris; pH 8.3, 5 mM EDTA). Briefly, 10 ml of each bacterial culture of 0.5 (OD_{600}) were harvested by centrifugation and cell pellets were resuspended in 1.0 ml of Tris–EDTA buffer containing 50 µl of cocktail protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA), and 1 µl (5000 units) of benzonase (Sigma). The cells were disrupted by sonication on ice four to five times for 30 s with a micro-tip probe, then incubated at room temperature for 1 h, followed by centrifugation at 6000 × *g* for 15 min at 4°C. Supernatant solution was transferred to a microfuge tube and the protein concentration was estimated by Bradford method using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Approximately 1 mg of protein was precipitated with

[1]http://www.broad.mit.edu

[2]http://www.broad.mit.edu

10% trichloroacetic acid (TCA) at 4°C for 1 h and centrifuged at 20,800 × *g* for 10 min, followed by washing three times with cold acetone. Analytical replicate samples from each strain were used for iTRAQ labeling [Applied Biosystems Inc. (ABI), Foster City, CA, USA].

One analytical separation was performed with replicate samples of each strain (383 and 2192). Samples were reduced, digested, labeled, and combined per ABI's standard protocol. Pooled samples were subjected to MS/MS for peptide/protein identification after strong cation exchange (SCX) chromatographic fractionation. iTRAQ reagents are isobaric in nature and thus yield similar m/z for the same peptide for the first stage of MS, when labeled with either 114, 115, 116, or 117 tags. Briefly, 100 μg of proteins from each strain were resuspended in dissolution buffer (0.5 M triethyl ammonium bicarbonate, TEAB), and then reduced, alkylated, digested, and labeled according to ABI's standard protocol. Briefly, 20 μl of dissolution buffer, 1 μl of denaturing solution (2% SDS), and 2 μl of the reducing agent (Tris 2-carboxyethyl phosphine) were added to each protein-containing tube and incubated for 1 h at 60°C. At the end of this reduction process, proteins were brought to room temperature and the samples were alkylated by addition of 1 μl of cysteine blocking reagent [200 mM methyl methanethiosulfonate (MTT) in isopropanol] and incubation for 10 min at room temperature. The reduced and alkylated protein samples were digested with sequencing-grade modified porcine trypsin that was reconstituted with Milli Q water; 5 mg trypsin was added to each tube and incubated overnight at 37°C. The peptide samples were labeled as follows: 383-A peptides were labeled with 114, 2192-A with 115, 383-B with 116, and 2192-B with 117. Each labeling was done in 70 μl of ethanol at room temperature for 1 h (according to manufacturer's instructions). Equivalent aliquots (77 μg) of each labeled peptide solution were pooled in a fresh tube and then dried completely in a vacuum centrifuge. These pooled, tryptic-labeled peptide samples were desalted as per manufacturer's protocol prior to SCX chromatographic fractionation. The entire sample was reconstituted in buffer A [10 mM KH_2PO_4, pH 2.93, 25% acetonitrile (ACN)] and loaded onto a Pharmacia Mono S, 5 mm × 50 mm (Pharmacia, Uppsala, Sweden) column, and peptides were eluted with a linear gradient of 0–100% buffer B (10 mM KH_2PO_4, pH 2.73, 25% ACN, and 1 M KCl) over 20 min. For the MS/MS analysis, four fractions (8–11) were selected based on the UV peptide elution profile recorded at absorbance of OD_{280}, and dried samples were stored at −80°C (**Figure S1** in Supplementary Material).

Each SCX fraction was reconstituted in loading buffer (98:2, water/ACN, 0.1% acetic acid) and peptides were separated by reverse phased chromatography. Two microliter of each fraction were loaded onto an in-house LC column packed with 7.5 cm of Gemini 5 μm packing material; the column was washed for 10 min with 1% acetic acid, and the sample was introduced into a QStar mass spectrometer (ABI) via a Tempo nano-LC pumping system. The LC gradient moved from 98% solvent A (0.1 M acetic acid) to 80% solvent B (a mixture of 90% ACN, 10% solvent A) over 120 min, with a flow rate of 250 nL/min. The QStar Elite was run with 3300 V electrospray voltage and a curtain gas setting of 17. The scans were set to collect one MS scan followed by the product ion scans of the three most intense ions from the MS scan.

During MS/MS analysis, peptides are fragmented with appropriate collision energy, and generate a product that corresponded to reporter ions from 114, 115, 116, and 117 m/z and peptide fragment ions, from which amino acid sequences were deduced. This MS/MS spectrum provides the data for protein identification and quantification. The resulting data were analyzed using ProteinPilot software 4.0 (ABI) with the Paragon search engine to search the *Pseudomonas* protein database of strain PAO1[3]. The default search settings used for quantitative processing and protein identification allowed for trypsin cleavage sites lysine and arginine, and the cysteine residues were blocked and alkylated with methyl methanethiosulfonate as described in the iTRAQ protocol. Protein detection threshold was set at 95% peptide confidence level. All four of the data files were concatenated into a single file for analysis and quantitation. Peptide quantification was carried out using ProteinPilot, which computes the area under the peaks at 114, 115, 116, and 117 Da, corresponding to the four iTRAQ reagents. The ratio between proteins was calculated by using each dataset as a denominator and shown in **File S1** in Supplementary Material.

TOTAL PROTEIN EXTRACTIONS AND TRITON SAMPLE PREPARATIONS FOR 2-DE GEL ELECTROPHORESIS

For 2-DE proteome analysis, total cellular proteins were extracted from strains 383 and 2192 by using Chaotropic Membrane Extraction Reagent 2 (Sigma; 5.0 M Urea, 2.0 M thiourea 40 mM Trizma™ base, 2.0% CHAPS, and 2.0% SB3-10, pH 10.1), followed by reduction and alkylation procedures, as previously described (Herbert et al., 1998; Molloy et al., 1998). Briefly, 10 ml of 0.5 (OD_{600}) cultures were harvested by centrifugation and cell pellets were resuspended in 2.0 ml of Chaotropic Membrane Extraction Reagent 2 containing 100 μl of protease inhibitors (Sigma), 1 μl of benzonase (Sigma), and 80 μl of tributylphosphine (TBP; Sigma) to get a final concentration of 5 mM in the total volume. Cell suspensions were disrupted by sonication with a micro-tip probe for 4–5 min on ice, followed by centrifugation at 6000 × *g* for 15 min at 4°C for supernatant collection, and removal of unbroken cells. Further, the reduced proteins were alkylated by adding 23 μl of iodoacetamide (Sigma) to a final concentration of 15 mM. This mixture was incubated for 90 min at room temperature in the dark. Protein concentration was determined by the Bradford method, and sample aliquots were stored at −20°C until use. Triton X-114 extractions were performed as described previously (Bordier, 1981), with slight modifications. Two ml bacterial pellets from 0.5 OD_{600} cultures were collected from strains 383 and 2192. Proteins were extracted by solubilizing in 2 ml of 1.7% Triton X-114 (Sigma) in 1× phosphate buffered saline (pH 7.4; PBS) with 100 μl of protease inhibitors (Sigma) by gentle rocking at 4°C for 2 h. Insoluble cell debris was removed by centrifugation at 6000 × *g* for 15 min at 4°C and the Triton X-114 solubilized proteins were collected by transferring supernatants to fresh microcentrifuge tubes, adding PBS to reduce the concentration of Triton X-114 to 1%, and incubating at 30°C for 3 min. The resulting cloudy solutions were partitioned into aqueous and detergent phases by centrifugation at 300 × *g* for 3 min

[3]http://www.pseudomonas.com

at room temperature. The upper aqueous phase was transferred to a fresh tube and re-extracted three times following the above procedure. The lower partitioned detergent phase was pooled from each extract and washed with cold 1× PBS. Finally, total extract, aqueous partitioned, and detergent partitioned proteins were precipitated with cold acetone (1:10, v/v) overnight at −70°C. Acetone precipitated proteins were collected after centrifugation at 20,800 × *g* for 15 min at 4°C and protein samples were subjected to 2-DE gel electrophoresis. The protein samples were prepared for electrophoresis in Chaotropic Membrane Extraction Reagent 2 (Sigma) by reduction and alkylation procedures performed as described earlier. Protein concentrations of each sample were determined by the Bradford method and samples were stored at −20°C until use.

All protein preparations were subjected to 2-DE analysis by using PROTEAN® IEF Cell (Bio-Rad). One hundred fifty microgram of proteins were solubilized in rehydration buffer (Bio-Rad) containing 9 M urea, 2% Triton X-100, 2% Pharmalyte pH 3–10 (Pharmacia), 2% β-mercaptoethanol, bromophenol blue, and TBP (20 mM) was added. Total proteins were applied on each immobilized pH gradient (IPG) strip (isoelectric point [pI] of 3–10 NL, 11 cm) from Bio-Rad, and active rehydration was carried out for 12–15 h under low-viscosity paraffin oil. Proteins were first separated by IEF with the following voltage/time profile: a linear step for 0–500 V for 15 min, 500–8000 V up to a total of 30,000 V/h. After completing the isoelectrofocusing (IEF), strips were incubated with equilibration buffer (3.7 g of Tris base, 150 ml of 10% SDS, 57.5 ml of glycerol, 7.71 g of DTT and 0.2 g of bromophenol blue, and made to 500 ml distilled H_2O) for 15–30 min. For the second dimension, proteins were resolved on 8–16% SDS-PAGE (Criterion gels) with Dodeca cell system from Bio-Rad at 100 V. Gels were stained with silver or with Bio-Safe Coomassie (Bio-Rad). Stained gels were scanned using HP scanner and images were stored as tagged image files (TIFF) files. All the cored spots from silver and Coomassie were characterized by either peptide mass mapping (PMM) or tandem mass spectrometry (MS/MS) for protein identification.

PROTEIN IDENTIFICATION BY TANDEM MASS SPECTROMETRY (MS/MS)

The selected individual protein spots were cut out of the stained gels and were submitted to the W. M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia Biomedical Research Facility for analysis, as described elsewhere (Hanna et al., 2000). Data was analyzed by using the SEQUEST (ThermoFinnigan) search algorithm against *Pseudomonas* genome deposited with National Center for Biotechnology Information (NCBI).

GENERATION OF T6SS ANTIBODIES

Rabbit polyclonal antibodies were raised and purified against synthetic peptides CLASAPKPKDDEPQA for TssB1 (PA0083), CVDGDPAHSTETTKA for TssC1 (PA0084), KYIDKSTPNLMMAC for Hcp1 (PA0085; GenScript, Piscataway, NJ, USA).

WESTERN BLOT ANALYSIS OF T6SS PROTEINS

Pseudomonas aeruginosa strains (383 and 2192) were grown on L agar overnight at 37°C. Cells were scraped of the plates and suspended in L broth. Starting cultures were matched to an OD_{600} of 0.1 and then diluted 1:1 with L broth. Five milliliter of culture were rotated at 100 rpm 37°C for 3 h until the strains were at OD_{600} of 0.5. Cultures were streaked onto L agar to ensure non-mucoid and mucoid phenotypes were maintained during log growth. Cell suspensions were matched and diluted in 2× Laemmli buffer. Samples were frozen at −80°C until separated on 12% polyacrylamide SDS-PAGE and then transferred to 0.2 μm nitrocellulose. Blocking and probing of membranes were performed with 5% skim milk in phosphate buffered saline. Polyclonal antibodies for T6SS proteins were diluted 1:500 and monoclonal antibodies to RpoA (Neoclone). Secondary antibodies with horseradish peroxidase were diluted 1:3000. Amersham Advanced ECL chemiluminescent substrate utilized for T6SS blots and Amersham ECL was used for RpoA blots. Amersham ECL hyper film was exposed for 1 min to probed membranes and developed.

RESULTS AND DISCUSSION

iTRAQ ANALYSIS OF THE PROTEOMES OF STRAINS 383 AND 2192

Early *P. aeruginosa* isolates taken from the CF airway are generally non-mucoid and resemble environmental strains. Strains 383 and 2192 have been classified as isogenic (Hanna et al., 2000). Strain 383 is non-mucoid and thus represents an initially infecting isolate, while 2192 is mucoid and represents a chronically infecting isolate. To discover the proteomic differences in these two CF isolates, iTRAQ analysis and quantitation was performed. This approach is a "gel-free" mass spectrometry technique that uses isobaric amine-specific tags to compare the peptide intensities between samples and infer quantitative values for corresponding proteins. Two analytical replicates of protein preparations from each strain were subjected to digestion and iTRAQ labeling, followed by MS/MS analyses. Expression data were compiled by scoring of peptides with 99% confidence using ProteinPilot software version 4. Proteins were identified on the basis of having at least one distinct peptide with threshold of 1% false positive rate using the ProteinPilot search algorithm against all bacterial and non-redundant database entries deposited with NCBI. In this study we report the identification of 399 proteins (above 95% confidence) from 5690 peptides. The relative quantification data were obtained for 297 proteins between strains 383 and 2192. Of the quantified 297, 81 proteins showed significant *p* values in either one or both iTRAQ replicates (**File S1** in Supplementary Material). In comparison with earlier studies of other *P. aeruginosa* strains (Guina et al., 2003; Wu et al., 2005), here we have observed similar numbers differentially expressed proteins.

Isobaric tags for relative and absolute quantitation analysis of the proteomes of 383 and 2192 identified 297 proteins differentially regulated (**Table S1A** and **File S1** in Supplementary Material). The 297 quantified proteins can be grouped into three categories: (1) 67 proteins (23%) showed higher expression in the non-mucoid strain, 383, (2) 77 proteins (26%) in showed higher expression in the mucoid strain, 2192, and (3) 153 proteins (51%) showed no difference in relative expression between the non-mucoid and mucoid strains (**Table S1A** in Supplementary Material).

Bioinformatics analysis of the 297 proteins recognized from iTRAQ analysis allowed functional classifications as defined by the

Pseudomonas Genome Database[4] (Winsor et al., 2009; **Table 1** and **File S2** in Supplementary Material). The distribution of proteins observed showed representatives of almost all of the functional classes identified suggest iTRAQ analysis. However, this may represent a shallow sampling of the proteome and may also define the limits of detection. No proteins corresponding to the functional classes of antibiotic resistance and susceptibility, chemotaxis, or phage proteins were detected in the analysis, but proteins assigned to the other 23 functional classes were identified. Proteins representing 16% the amino acid biosynthesis and metabolism class were observed. Interestingly most of the proteins of this class were expressed at equal levels between the two strains. We would expect that a mucoid *P. aeruginosa* strain, such as 2192 would have increased expression of proteins corresponding to the cell wall/LPS/capsule class. iTRAQ analysis indicated four proteins of this class were upregulated in strain 2192 (**Table 1**). Furthermore the mucoid phenotype would place a demand on metabolism pathways to overproduce alginate. Eight proteins of the central intermediary metabolism class were upregulated in mucoid strain 2192 (**Table 1**). However, the protein class of energy metabolism has an equal number of increased and decreased proteins and 13 proteins equally expressed. If iTRAQ analysis properly represents the proteome, it would be expected that some classes of proteins would not be discordant. Seven proteins of the associated with DNA maintenance were equal expressed between the two strains (**Table 1**).

The bacterial membrane is the largest single structure of a bacterium. Moreover, homeostasis is maintained by upholding the integrity of this structure. In our iTRAQ analysis of the two CF isolates, we noted a total of 12 proteins (19.4% of the entire class), which correspond to the fatty acid and phospholipid metabolism class (**Table 1**). Since membrane integrity is paramount for bacteria, we would predict this class would be highly represented in a distribution of the total proteome. Another validation of the iTRAQ analysis was the identification of differential expression of transcriptional regulators and transcriptional machinery (**Table 1**). Transcriptional regulators are rarely expressed at high levels. Therefore, identification of these regulators via iTRAQ suggests the analysis was able to detect at least some proteins that may be of relatively low abundance. Conversely translational machinery would likely represent a high amount of the peptide concentration within a total protein lysate. iTRAQ analysis

[4]www.pseudomonas.com

Table 1 | Distribution and PseudoCAP functional classification of proteins identified in CF strains 383 and 2192 by iTRAQ analysis.

PseudoCAP functional class (total no. in class)	2192/383 iTRAQ expression			Total proteins identified	Percentage of functional class
	Increased expression	Decreased expression	Equal expression		
Adaptation and protection (179)	1	0	6	7	3.9
Amino acid biosynthesis and metabolism (242)	10	9	20	39	16.1
Antibiotic resistance and susceptibility (55)	0	0	0	0	0
Biosynthesis of cofactors (160)	1	2	6	9	5.6
Carbon compound catabolism (172)	1	1	4	6	3.5
Cell division (29)	3	0	1	4	13.8
Cell wall/LPS/capsule (182)	4	0	2	6	3.3
Central intermediary metabolism (99)	8	0	7	15	15.2
Chaperones and heat shock proteins (56)	3	2	2	7	12.5
Chemotaxis (64)	0	0	0	0	0
DNA replication, recombination, modification, and repair (88)	2	1	7	10	11.4
Energy metabolism (208)	3	3	13	19	9.1
Fatty acid and phospholipid metabolism (62)	3	4	5	12	19.4
Hypothetical, unclassified, unknown (2002)	16	7	9	32	1.6
Membrane proteins (676)	2	4	2	8	1.2
Nucleotide biosynthesis and metabolism (87)	2	1	7	10	11.5
Protein secretion/export apparatus (124)	0	2	3	5	4.0
Putative enzymes (474)	2	1	1	4	0.8
Related to phage, transposon, or plasmid (65)	0	0	0	0	0
Secreted factors: toxins, enzymes, alginate (105)	2	1	0	3	2.9
Transcription, RNA processing, and degradation (55)	1	6	6	13	23.6
Transcriptional regulators (478)	3	0	5	8	1.7
Translation, post-translational modification, degradation (197)	8	17	46	71	36.0
Transport of small molecules (587)	0	3	5	8	1.3
Two-component regulatory systems (121)	0	0	1	1	0.8

identified 36% of the PseudoCAP functional class of translation, post-translational modification and degradation. Seventy-one of the 197 proteins belonging to this class were identified. However, most (46) were equally expressed by the two strains (**Table 1**).

We have also compared the proteins detected by iTRAQ with the location of the genes on the chromosome. As anticipated by our microarray analysis (Rao et al., 2008), in some cases proteins expression seemed to correspond to genes organized in operons. A particularly prominent region of the chromosome from PA4239 to PA4274, which encodes most of the 50S and 30S ribosomal subunits, were identified in our iTRAQ analysis (**Table S1B** in Supplementary Material). *In vitro* growth analysis, as determined by ribosomal content, has previously suggested that *P. aeruginosa* isolated from chronically infected patients grow as much as three-fold slower than laboratory strains such as PAO1 (Yang et al., 2008). Here in our iTRAQ analysis, we observed similar down-regulation of ribosomal proteins in strain 2192 (**Table S1A** in Supplementary Material).

The high detection of ribosomal proteins in both samples likely decreases the depth of detection of the total proteome and is a limitation of the current study. In light of this, one way to increase the depth of iTRAQ analysis may be to deplete the amount of translation associated proteins in a sample. Fractionations that remove the translational machinery may result in more unique analysis of a proteome. This idea is reminiscent of the recent push to deplete rRNA from total RNA samples for RNA-seq analysis. With the majority of a total RNA sample being comprised of rRNA, removal of rRNA can provide more unique sequence analysis. Application of ribosomal depletion to total protein samples may increase the depth of analysis of a proteome and is currently being investigated in our laboratory.

Table 2 indicates selected proteins identified in iTRAQ analysis. Additional data representing the entire iTRAQ analysis is supplied in **Files S1** and **S2** in Supplementary Material. Since strain 2192 is mucoid, we would expect to see proteins such as those that play a role in alginate overproduction. Our proteomic analysis indicated that alginate motility regulator Z (AmrZ), AlgF, AlgC, and AlgD were upregulated in mucoid strain 2192 (**Table 2**). AmrZ, is a ribbon–helix–helix DNA-binding protein (Baynham et al., 2006) that can act as a transcriptional repressor (Tart et al., 2006) or enhancer of *algD* expression (Baynham et al., 1999). It was expected that AmrZ would be present as we had also observed its increased expression in our previous transcriptome analysis (Rao et al., 2008). AlgC is a phosphomannomutase enzyme which catalyzes the second step in the alginate pathway (Zielinski et al., 1991). AlgC converts mannose 6-phosphate into mannose 1-phosphate. Furthermore, AlgC is a phosphoglucomutase involved in LPS synthesis (Coyne et al., 1994). Here we observed AlgC significantly upregulated in strain 2192 (**Table 2**). AlgD, GDP-mannose 6-dehydrogenase, converts GDP-mannose into GDP-mannuronate which is the precursor of polymannuronic acid (Tatnell et al., 1994). Another alginate enzyme, AlgF was also upregulated by not identified as significant due to low number of peptide identifications (**Table 2**). AlgF is one of three proteins that function to acetylate O-2 and or O-3 of the mannuronic acid residues (Franklin and Ohman, 2002). Acetylation changes the physical and immunological properties of the alginate (Pier et al., 2001).

In addition to identifying σ^{22}-dependent alginate production proteins, outer-membrane protein F (PA1777) was also upregulated in strain 2192 (**Table 2**). Previous studies have shown that OprF is upregulated in mucoid strains (Hanna et al., 2000; Malhotra et al., 2000) and is directly controlled by σ^{22} (Firoved et al., 2002). Thus, our shotgun proteomic analysis was validated by the identification of some known alginate biosynthesis proteins and AlgU-dependent proteins such as OprF that were upregulated in the mucoid 2192 strain.

When *P. aeruginosa* overproduces alginate, there would be high demands on the central metabolism to produce metabolic intermediates and such to fuel the high energy demand for alginate overproduction. In **Table 2**, a number of proteins are shown that have direct roles in metabolism. Additionally, we noticed a number of hypothetical proteins upregulated in 2192 some of which are shown in **Table 2**.

Several heat shock proteins were downregulated in strain 2192 (**Table 2**). GroES, HtpG, and GroEL were significantly downregulated. Previous work has shown that CF patients produce antibodies to GroEL (Ulanova et al., 1997). Other recent work has shown that heat shock proteins such as GroES and HtpG are actually downregulated in biofilms (Patrauchan et al., 2007). It is interesting that heat shock proteins may be differentially expressed depending on the growth conditions such as planktonic versus biofilm modes of growth.

Many total transcriptome analyses have examined the effect of σ^{22} expression (Firoved et al., 2002, 2004a,1991; Firoved and Deretic, 2003; Wood et al., 2006; Wood and Ohman, 2009). However, here unlike other studies, we were also interested in proteins that have decreased expression in mucoid strains. In our iTRAQ analysis, three proteins which are components of one (HIS-I) of the three T6SS (Mougous et al., 2006) were downregulated in the mucoid strain. These proteins, TssB1 (PA0083), TssC1 (PA0084), Hcp1 (PA0085), have greater expression levels in non-mucoid strain 383 than in mucoid strain 2192 (**Table 2**). Hcp1 is secreted by the T6SS and has been found in CF sputum (Mougous et al., 2006). CF patients also have antibodies in their serum to Hcp1 (Mougous et al., 2006). Although the exact role of Hcp1 is not clear, it has been suggested that Hcp1 may form a channel through which macromolecules might pass and may be used for communication with the host (Mougous et al., 2006). Recently, the HSI-I system was shown to secrete Tse2 (PA2702) which is a toxin that arrests both prokaryotes and eukaryotes; however, Tse2 is targeted toward prokaryotes (Hood et al., 2010). We do not observe expression of Tse2 in either of our proteomic analysis or differential expression in transcriptomic analysis (Rao et al., 2008); however, *tse2* is present in the 2192 genome[5].

RetS is a hybrid sensor kinase that controls expression of the T6SS in *P. aeruginosa*. Without the *retS*, all of the HSI-I genes are highly expressed (Goodman et al., 2004; Mougous et al., 2006; Ventre et al., 2006; **Figure 2**). Comparison of our previous transcriptome analysis to the transcriptome analysis of Δ*retS* is shown

[5]http://www.broad.mit.edu

Table 2 | Selected proteins with differential expression between strains 383 and 2192 as observed by iTRAQ analysis.

N[a]	PA loci	Name	Peptides[b]	2192-A/383-A		2192-B/383-B		Average AB[c]
				115:114	*p* Value 115:114	117:116	*p* Value 117:116	
285	PA3902	Hypothetical protein	1	6.75	0.0983	24.47		15.61
305	PA3819	Hypothetical protein	1	18.87	0.0927	9.32		14.09
283	PA3550	Alginate o-acetyltransferase AlgF	1	6.31		17.41		11.86
221	PA0315	Hypothetical protein	2	8.40		4.85		6.63
317	PA3031	Hypothetical protein	1	5.52		7.29		6.41
110	PA3385	Alginate and motility regulator AmrZ	5	9.90	0.0001	6.08	0.0066	7.99
32	PA3540	GDP-mannose 6-dehydrogenase AlgD	9	4.26	0.0003	7.27	0.0001	5.76
54	PA5322	Phosphomannomutase AlgC	7	5.91	0.0020	5.54	3.09E-05	5.73
134	PA3755	Ecotin precursor Eco	4	3.19	0.0409	2.75	0.0047	2.97
132	PA5060	Polyhydroxyalkanoate synthesis protein PhaF	4	3.07	0.0085	2.83	0.0017	2.95
164	PA1155	Ribonucleoside reductase, small chain NrdB	3	2.70	0.0014	1.52	0.0347	2.11
215	PA4922	Azurin precursor Azu	2	2.33	0.0096	1.79	0.0227	2.06
194	PA3471	Probable malic enzyme	2	2.22	0.0447	1.51	0.1020	1.87
34	PA0555	Fructose-1,6-bisphosphate aldolase Fda	9	1.89	0.0115	1.73	0.0004	1.81
88	PA0552	Phosphoglycerate kinase Pgk	5	1.74	0.0555	1.83	0.0016	1.78
119	PA3040	Probable DNA-binding protein	5	1.90	0.0051	1.47	0.0000	1.68
61	PA0962	Probable DNA-binding stress protein	8	1.56	0.0562	1.40	0.0018	1.48
128	PA1777	Outer-membrane protein OprF precursor	4	1.61	0.0397	1.34	0.0184	1.47
78	PA0625	Succinate-semialdehyde dehydrogenase GabD	6	1.62	0.0533	1.32	0.0347	1.47
44	PA3001	Probable glyceraldehyde-3-phosphate dehydrogenase	9	1.29	0.0247	1.55	0.0004	1.42
70	PA1165	Ribonucleoside reductase, large chain NrdA	7	0.96	0.6108	1.73	0.0010	1.35
18	PA1588	Succinyl-CoA synthetase beta chain SucC	12	1.27	0.0396	1.34	0.0067	1.30
20	PA2950	Hypothetical protein	12	1.12	0.2466	1.40	0.0001	1.26
45	PA4386	GroES protein	11	0.98	0.8852	0.84	0.0355	0.91
91	PA1586	Dihydrolipoamide succinyltransferase SucB	6	1.00	0.9967	0.77	0.0230	0.88
120	PA4315	Transcriptional regulator MvaT, P16 subunit	5	1.00	0.9881	0.70	0.0003	0.85
11	PA1596	Heat shock protein HtpG	20	0.84	0.0727	0.82	0.0056	0.83
142	PA4232	Single-stranded DNA-binding protein	4	0.65	0.0185	1.01	0.9193	0.83
273	PA5253	Regulatory protein AlgP	1	0.86	0.7890	0.66	0.1443	0.76
47	PA5016	Dihydrolipoamide acetyltransferase AceF	8	0.75	0.0127	0.80	0.0273	0.77
2	PA4385	GroEL protein	49	0.75	0.0000	0.69	0.0000	0.72
181	PA2853	Outer-membrane lipoprotein OprI precursor	3	0.69	0.1906	0.69	0.0376	0.69
38	PA1587	Lipoamide dehydrogenase-glc	8	0.46	0.0130	0.85	0.1952	0.66
206	PA3836	Hypothetical protein	2	0.65	0.1557	0.52	0.0231	0.59
58	PA1092	Flagellin type B FliC	7	0.48	0.0275	0.68	0.0110	0.58
103	PA0085	T6SS protein Hcp1	4	0.31	0.0008	0.30	0.0002	0.30
244	PA0083	T6SS protein TssB1	2	0.54	0.2784	0.39	0.2798	0.46
248	PA0084	T6SS protein TssC1	2	0.42		0.61	0.1531	0.51

[a]*This column indicates the protein number which corresponds to the raw iTRAQ* ***File S1*** *in Supplementary Material.*

[b]*This column indicates the number of peptides at 95% confidence.*

[c]*This column indicates the average of the two replicates. Proteins that show at least one replicate with a significant p value (less than 0.05) are colored red if the protein was upregulated or green if the protein was downregulated.*

in **Figure 2**. It seems than non-mucoid strain 383 has increased expression of most of the HIS-I operon and the HSI-II genes *hsiA2*, *hsiC2*, and *hsiF1* (**Figure 2**). Furthermore, as mentioned, T6SS proteins TssB1, TssC1, Hcp1 were detected in iTRAQ analysis (**Table 2**). Also of interest, HSI-II genes were not affected by deletion of *retS*, but strain 383 has increased HSI-II expression of *hsiA2*, *hsiC2*, and *hsiF2* (**Figure 2**). These data suggest that mucoidy may suppress expression of Type 6 secretion machinery in *P. aeruginosa*. To further corroborate this, examination of the literature revealed that inactivation of σ^{22} in a *mucA* mutant strain caused increased expression of HSI-I and HSI-II genes (Tart et al., 2005). In a recent study, extensive transcriptome analysis of early and late CF isolates revealed that, *tssB1*, *tssC1*, and *hcp1* were upregulated in non-mucoid derivatives of mucoid CF strains (Rau et al., 2010). Collectively, our data along with others suggested an inverse relationship between the T6SS and the mucoid phenotype of

FIGURE 2 | Type 6 secretion operons and their expression in strain 383 and 2192. The relative expression of the type 6 secretion system (T6SS) genes are indicated for the comparisons of strain 383 to 2192 from Rao et al. (2008) and also the relative expression from a *retS* deletion mutant where T6SS genes are uniformly upregulated (Goodman et al., 2004; Mougous et al., 2006; Ventre et al., 2006). Genes are named and colored according to *Pseudomonas* Genome Database. **(A,B)** Indicates the *P. aeruginosa* HSI-I and HSI-II operons of T6SS genes, respectively.

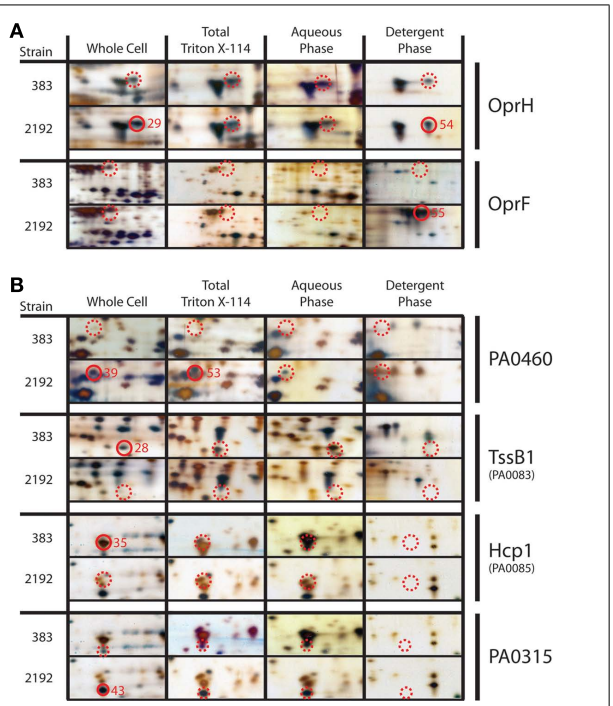

FIGURE 3 | Two-dimensional gel protein analysis of strains 383 and 2192. (A) Indicates outer-membrane proteins that were analyzed and **(B)** indicates other proteins of interest. Samples are designated as follows: whole cell total soluble extract, total Triton X-114 soluble extract, aqueous phase extract and detergent phase extract. Approximately 15 μg of total protein was focused in a 3–10 (NL) IPG strips using a PROTEAN IEF cell (Bio-Rad). Second dimension, focused IPG strips were placed onto 8–16% SDS-PAGE Criterion gel and proteins were separated by molecular size. Following electrophoresis gels were silver stained and stored in distilled water with 0.5% acetic acid. Closed red circles represent cored spots with the core numbers indicated whereas dashed red circles indicate the location of spots for comparison that were not cored or MS identified. Closed and dashed red circles are indicated smaller on PA0315 panels to indicate the protein spot precisely due to a closely co-migrating spot. All cored protein spots indicated were the most abundant protein in the sample as determined by peptide mass mapping (**Table S2** in Supplementary Material) except spot 43, which corresponds to PA0315.

P. aeruginosa. It has been previously theorized that the T6SS may be used to out compete other bacteria and increase the fitness of *P. aeruginosa* for infection of the CF lung (Hood et al., 2010). The addition of mucoid phenotype to this model paints a picture whereby early non-mucoid isolates compete to initiate infection and then mucoid phenotype emerges, after which chronic infection can be established, negating the necessity of T6SS.

2-DE ELECTROPHORESIS WITH MS DETECTION OF TOTAL PROTEIN SAMPLES FROM STRAINS 383 AND 2192

One inherent drawback to the use of chromatography-based proteomics is potential bias due to association and elution of peptides during liquid separation. Theoretically, some peptides could lost due to interactions during separation. The advantage of 2-DE analysis is that it gives the size and pI of the isolated proteins, whereas only protein fragments are detected by iTRAQ. To visualize protein differences between the non-mucoid strain 383 and the mucoid strain 2192, independent biological samples for each strain, 383 and 2192, were separated and representative examples are shown in **Figure S2** in Supplementary Material. Spots with differential expression were visualized (**Figure S3** in Supplementary Material) and quantified for each strain. The predicted size and pI were compared to that of the full-length protein sequences (**Table S2A** in Supplementary Material). A protein spot on 2-DE can contain multiple co-migrating proteins (**Table S2A** in Supplementary Material). To determine the proteins that make up a 2-DE spot, either PMM or mass spectral analysis or both were performed (**Table S2A** in Supplementary Material). PMM indicates the proteins that are the most abundant in a cored spot (**Table S2B** in Supplementary Material). MS/MS does not suggest a protein is the most abundant but rather identifies peptides that may correspond to the any protein in the cored spot. As expected half of the protein spots analyzed contained multiple proteins and some proteins were identified in more than one location (**Table S2A** in Supplementary Material).

Total protein lysates, as well as membrane, and extracellular protein enriched preparations, were utilized for 2-DE analysis. For membrane/extracellular preparations, Triton X-114 treatments as indicated in Section "Materials and Methods" were also employed to enrich for detergent-soluble hydrophobic membrane proteins (Bordier, 1981). This method has been used in other bacterial proteomic studies (Sinha et al., 2005). 2-DE analysis revealed two outer-membrane proteins, OprH and OprF were differentially expressed between strains 383 and 2192 (**Figure 3A**). OprH and OprF spots were visible in the Triton X-114 enrichment as expected (**Figure 3A**). Previously, it has been shown that OprH protein is expressed in both mucoid and non-mucoid strains (Kelly et al., 1990; Hanna et al., 2000). Here, an OprH protein spot was observed in both strains but was visually more abundant in the mucoid 2192 strain than non-mucoid strain 383 (**Figure 3A**) however our densitometry did not corroborate this observation (**Table 3**).

One differential stained protein spot between mucoid strain 2192 and non-mucoid strain 383 (whole cell extract spot #39 and #53 Triton X-114 preparation) was a conserved hypothetical protein (PA0460; **Figure 3B**). Little to no information is available for PA0460 except that it has been shown to be five-fold upregulated

when the sensor kinase *phoQ* is deleted from strain PAO1 (Gooderham et al., 2009). Based on our data presented here (**Figure 3B**), PA0460 may be a secreted protein, as it was predicted with signal peptide at N-terminal 21 amino acids followed by a cleavage site (data not shown). Spot #43 from mucoid strain (2192) whole cell extract identified PA0315, another conserved hypothetical protein (**Figure 3B**). PA0315 was upregulated in 2192 and our densitometry corroborated this observation. Recent data suggests PA0315 may be a homolog to a quorum sensing regulated protein of *Burkholderia cepacia* (Riedel et al., 2003).

TYPE 6 SECRETION PROTEIN EXPRESSION IN STRAINS 383 AND 2192

Our previous transcriptome analysis had indicated a differential expression of T6SS proteins as described above. Furthermore, iTRAQ analysis revealed a significant decrease in Hcp1 in strain 2192. As expected, 2-DE gel electrophoresis confirmed our iTRAQ analysis (**Table 2**) that Hcp1 and TssB1 (PA0083) were increased in expression in 383 (**Figure 3B** and **Table 3**). However, more direct confirmation of this observation was necessary. For this, antibodies specific to TssB1 (PA0083), TssC1 (PA0084), and Hcp1 (PA0085) were used to quantify expression of the T6SS in CF strains 383 and 2192. In **Figure 4**, Western blot analysis is shown for TssB1, TssC1, and Hcp1. Antibodies specific for the alpha subunit of RNA polymerase (RpoA) were utilized as a loading control for the analysis. Western blot analysis confirmed iTRAQ and 2-DE and showed that TssB1 and Hcp1 were more highly expressed in the non-mucoid strain 383 than mucoid strain 2192 (**Figure 4**). Of interest antibodies for TssC1 (PA0084) suggested that TssC1 correlates with TssB1 and Hcp1 expression. These data suggest that strain 383 has increased T6SS protein expression than strain 2192 (**Figure 4**). Ultimately our data corroborate both the microarray and the two shotgun proteomic methods and show that the T6SS is differentially regulated in CF strains 383 and 2192.

CONCLUSION AND FUTURE DIRECTIONS

The goal of this study was to obtain the proteomic profiles of *P. aeruginosa* non-mucoid strain 383 and mucoid strain 2192 to discover novel differences. To achieve this goal, we utilized a two different complementary proteomic techniques (iTRAQ and 2-DE) followed by MS analysis and correlated these results with our previous transcriptome analysis (Rao et al., 2008). Microarray analysis elucidates the relative gene expression profiles between RNA samples; proteomic approaches such as iTRAQ and 2-DE/MS can validate microarray analysis and provide insightful information. As we expected these techniques identified proteins differentially regulated between strains 383 and 2192. However, we have also noted some limitations of these proteomic approaches.

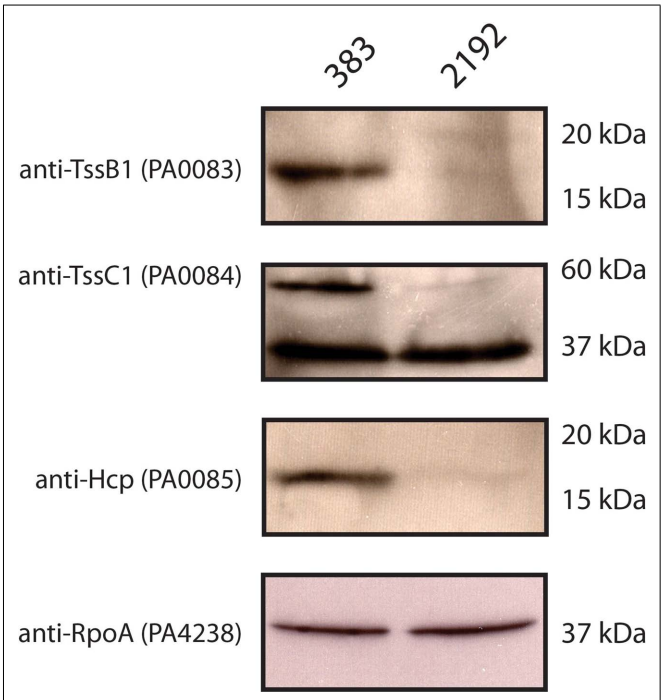

FIGURE 4 | Western blot analysis of Type 6 secretion system (T6SS) proteins in strains 383 and 2192. L broth cultures were inoculated with the strains 383 and 2192 and grow at 37°C into log phase (OD_{600} 0.5). Log phase whole cells suspended in L broth were added to equal volumes of 2× Laemmli buffer and subjected to separation by 12% SDS-PAGE and electro-transferred to 0.2 μm nitrocellulose membranes. Membranes were probed with the antibodies indicated. Molecular weight markers are indicated showing the relative migration of proteins detected. Shown is a representative of three independent experiments.

Table 3 | Selected proteins quantified from cored protein spots of 2-DE.

PA no.	Protein	2-DE spot no.	Fold increase 383/2192 2-DE[a,b]	Fold increase 383/2192 iTRAQ[a,c]
PA0083	TssB1	28	4.06	2.2 (NS)
PA0085	Hcp1	35	1.14	3.3
PA0315	Hypothetical protein	43	ND[e]	0.61 (NS)
PA0460	Hypothetical protein	53	ND[d]	NI
PA1159	Cold-shock protein	46	1.1	1.1
PA1178	Outer-membrane H1	29	2.46	NI
PA1777	OprF	55	ND	0.7

[a] *All the values are represented in log 2 scale and additional data can be found in (**Table S2** in Supplementary Material).*

[b] *ND indicates that protein spot intensity was not measured in 2-DE gels.*

[c] *NS indicates not significant by iTRAQ analysis or NI indicates not identified.*

[d] *PA0460 fold increase is not indicated because PMM was not performed on spot 39, which contains other proteins (**Table S2A** in Supplementary Material).*

[e] *PA0315 fold increase is not indicated because PMM was not performed on spot 43, which contains other proteins (**Table S2A** in Supplementary Material).*

For example, proteomic techniques such as iTRAQ and 2-DE currently do not give a complete protein profile. Our iTRAQ proteomic analysis gave expression data for only 4.8% of the ORF of strain 2192 consistent with other studies. In spite of this limitation, we were able to identify novel biological differences between isolates of *P. aeruginosa* from a single CF patient. iTRAQ and 2-DE analysis revealed a differential expression of the T6SS in strains 383 and 2192. In non-mucoid strain 383, proteins TssB1 and Hcp1 were observed with increased spot intensities. iTRAQ quantification data also indicated an increase in TssB1 and Hcp1. iTRAQ and 2-DE results were corroborated by Western blot analysis using T6SS antibodies for TssB1, TssC1, Hcp1 (**Figure 4**). Most of T6SS analysis has been performed with laboratory strains. Our data suggest 383 and 2192 may be useful prototype strains to further elucidate T6SS. Collectively, our data indicates an inverse relationship between the T6SS and the chronic mucoid phenotype which is controlled by σ^{22}.

Previously, among the 99 proteins that we identified in our proteomic analysis of PAO1, 9% were "hypothetical" and 6% were "conserved hypothetical" (Sherman et al., 2001). Furthermore, the *P. aeruginosa* pangenome (Mathee et al., 2008) and recent genome sequencing efforts indicate there are many ORF left to be characterized. Here among the proteins identified at least 25% were "hypothetical" or "conserved hypothetical". We now refer to these as PUFs; since they have been confirmed by mass spectral analysis, the existence of these proteins are no longer hypothetical. This intermediate classification between hypothetical and characterized protein is important because it confirms the annotation of an organism. The *P. aeruginosa* reference strain, PAO1, currently contains 2002 hypothetical ORF. It is likely many of these proteins have been identified by proteomic approaches by various studies (66 were identified here) but searching the literature for each hypothetical can be difficult given the body of data in the public domain. We believe that one potential way to facilitate the elucidation of PUFs into characterized proteins is to begin database compilation of the PUFs and the studies in which they were identified.

In conclusion, iTRAQ, 2-DE, and Western blot analyses indicated differential expression of many proteins between these two CF isolates. Multiple approaches directed focus on a differential regulation of T6SS proteins between the non-mucoid strain 383 and the mucoid strain 2192. We recognized that one limitation of this approach was the use of standard laboratory growth conditions. Future studies could utilize synthetic growth media that better mimic CF conditions.

Our study suggests that iTRAQ analysis provides insightful proteomic data, but we suggest it may be possible in the future to deplete translational machinery from the protein sample to increase the depth of analysis. Since proteomic analysis by iTRAQ or 2-DE requires known protein sequences, the recent explosion of deep sequencing may quickly increase power of shotgun proteomic analysis, which may assist in the determination of the functions of many PUFs in organisms such as *P. aeruginosa*.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/cellular_and_infection_microbiology/10.3389/fmicb.2011.00162/abstract

REFERENCES

Arevalo-Ferro, C., Reil, G., Gorg, A., Eberl, L., and Riedel, K. (2005). Biofilm formation of *Pseudomonas putida* IsoF: the role of quorum sensing as assessed by proteomics. *Syst. Appl. Microbiol.* 28, 87–114.

Baynham, P. J., Brown, A. L., Hall, L. L., and Wozniak, D. J. (1999). *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and *algD* transcriptional activation. *Mol. Microbiol.* 33, 1069–1080.

Baynham, P. J., Ramsey, D. M., Gvozdyev, B. V., Cordonnier, E. M., and Wozniak, D. J. (2006). The *Pseudomonas aeruginosa* ribbon-helix-helix DNA-binding protein AlgZ (AmrZ) controls twitching motility and biogenesis of type IV pili. *J. Bacteriol.* 188, 132–140.

Blonder, J., Goshe, M. B., Xiao, W., Camp, D. G. II, Wingerd, M., Davis, R. W., and Smith, R. D. (2004). Global analysis of the membrane subproteome of *Pseudomonas aeruginosa* using liquid chromatography-tandem mass spectrometry. *J. Proteome Res.* 3, 434–444.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256, 1604–1607.

Chitnis, C. E., and Ohman, D. E. (1993). Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Mol. Microbiol.* 8, 583–593.

Coyne, M. J. Jr., Russell, K. S., Coyle, C. L., and Goldberg, J. B. (1994). The *Pseudomonas aeruginosa algC* gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. *J. Bacteriol.* 176, 3500–3507.

Damron, F. H., Napper, J., Teter, M. A., and Yu, H. D. (2009a). Lipotoxin F of *Pseudomonas aeruginosa* is an AlgU-dependent and alginate-independent outer membrane protein involved in resistance to oxidative stress and adhesion to A549 human lung epithelia. *Microbiology* 155, 1028–1038.

Damron, F. H., Qiu, D., and Yu, H. D. (2009b). The *Pseudomonas aeruginosa* sensor kinase KinB negatively controls alginate production through AlgW-dependent MucA proteolysis. *J. Bacteriol.* 191, 2285–2295.

Damron, F. H., and Yu, H. D. (2011). *Pseudomonas aeruginosa* MucD regulates alginate pathway through activation of MucA degradation via MucP proteolytic activity. *J. Bacteriol.* 193, 286–291.

Firoved, A. M., Boucher, J. C., and Deretic, V. (2002). Global genomic analysis of AlgU (sigma(E))-dependent promoters (sigmulon) in *Pseudomonas aeruginosa* and implications for inflammatory processes in cystic fibrosis. *J. Bacteriol.* 184, 1057–1064.

Firoved, A. M., and Deretic, V. (2003). Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.* 185, 1071–1081.

Firoved, A. M., Ornatowski, W., and Deretic, V. (2004a). Microarray analysis reveals induction of lipoprotein genes in mucoid *Pseudomonas aeruginosa*: implications for inflammation in cystic fibrosis. *Infect. Immun.* 72, 5012–5018.

Firoved, A. M., Wood, S. R., Ornatowski, W., Deretic, V., and Timmins, G. S. (2004b). Microarray analysis and functional characterization of the nitrosative stress response in nonmucoid and mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 4046–4050.

Franklin, M. J., and Ohman, D. E. (2002). Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. *J. Bacteriol.* 184, 3000–3007.

Gooderham, W. J., Gellatly, S. L., Sanschagrin, F., McPhee, J. B., Bains, M.,

Cosseau, C., Levesque, R. C., and Hancock, R. E. (2009). The sensor kinase PhoQ mediates virulence in *Pseudomonas aeruginosa*. *Microbiology* 155, 699–711.

Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S., and Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev. Cell* 7, 745–754.

Goodman, A. L., and Lory, S. (2004). Analysis of regulatory networks in *Pseudomonas aeruginosa* by genomewide transcriptional profiling. *Curr. Opin. Microbiol.* 7, 39–44.

Govan, J. R., and Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol. Rev.* 60, 539–574.

Guina, T., Wu, M., Miller, S. I., Purvine, S. O., Yi, E. C., Eng, J., Goodlett, D. R., Aebersold, R., Ernst, R. K., and Lee, K. A. (2003). Proteomic analysis of *Pseudomonas aeruginosa* grown under magnesium limitation. *J. Am. Soc. Mass Spectrom.* 14, 742–751.

Hanna, S. L., Sherman, N. E., Kinter, M. T., and Goldberg, J. B. (2000). Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry. *Microbiology* 146, 2495–2508.

Herbert, B. R., Molloy, M. P., Gooley, A. A., Walsh, B. J., Bryson, W. G., and Williams, K. L. (1998). Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. *Electrophoresis* 19, 845–851.

Hogardt, M., and Heesemann, J. (2010). Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. *Int. J. Med. Microbiol.* 300, 557–562.

Hood, R. D., Singh, P., Hsu, F., Guvener, T., Carl, M. A., Trinidad, R. R., Silverman, J. M., Ohlson, B. B., Hicks, K. G., Plemel, R. L., Li, M., Schwarz, S., Wang, W. Y., Merz, A. J., Goodlett, D. R., and Mougous, J. D. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe* 7, 25–37.

Imperi, F., Ciccosanti, F., Perdomo, A. B., Tiburzi, F., Mancone, C., Alonzi, T., Ascenzi, P., Piacentini, M., Visca, P., and Fimia, G. M. (2009). Analysis of the periplasmic proteome of *Pseudomonas aeruginosa*, a metabolically versatile opportunistic pathogen. *Proteomics* 9, 1901–1915.

Jones, A. K., Fulcher, N. B., Balzer, G. J., Urbanowski, M. L., Pritchett, C. L., Schurr, M. J., Yahr, T. L., and Wolfgang, M. C. (2010). Activation of the *Pseudomonas aeruginosa* AlgU regulon through *mucA* mutation inhibits cyclic AMP/Vfr signaling. *J. Bacteriol.* 192, 5709–5717.

Kelly, N. M., Macdonald, M. H., Martin, N., Nicas, T., and Hancock, R. E. (1990). Comparison of the outer membrane protein and lipopolysaccharide profiles of mucoid and non-mucoid *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 28, 2017–2021.

Lacerda, C. M., Xin, L., Rogers, I., and Reardon, K. F. (2008). Analysis of iTRAQ data using Mascot and Peaks quantification algorithms. *Brief. Funct. Genomic. Proteomic.* 7, 119–126.

Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* 15, 194–222.

Malhotra, S., Silo-Suh, L. A., Mathee, K., and Ohman, D. E. (2000). Proteome analysis of the effect of mucoid conversion on global protein expression in *Pseudomonas aeruginosa* strain PAO1 shows induction of the disulfide bond isomerase, DsbA. *J. Bacteriol.* 182, 6999–7006.

Martin, D. W., Schurr, M. J., Mudd, M. H., Govan, J. R., Holloway, B. W., and Deretic, V. (1993). Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc. Natl. Acad. Sci. U.S.A.* 90, 8377–8381.

Mathee, K., Narasimhan, G., Valdes, C., Qiu, X., Matewish, J. M., Koehrsen, M., Rokas, A., Yandava, C. N., Engels, R., Zeng, E., Olavarietta, R., Doud, M., Smith, R. S., Montgomery, P., White, J. R., Godfrey, P. A., Kodira, C., Birren, B., Galagan, J. E., and Lory, S. (2008). Dynamics of *Pseudomonas aeruginosa* genome evolution. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3100–3105.

Molloy, M. P., Herbert, B. R., Walsh, B. J., Tyler, M. I., Traini, M., Sanchez, J. C., Hochstrasser, D. F., Williams, K. L., and Gooley, A. A. (1998). Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. *Electrophoresis* 19, 837–844.

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordonez, C. L., Lory, S., Walz, T., Joachimiak, A., and Mekalanos, J. J. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312, 1526–1530.

Nouwens, A. S., Beatson, S. A., Whitchurch, C. B., Walsh, B. J., Schweizer, H. P., Mattick, J. S., and Cordwell, S. J. (2003). Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. *Microbiology* 149, 1311–1322.

Patrauchan, M. A., Sarkisova, S. A., and Franklin, M. J. (2007). Strain-specific proteome responses of *Pseudomonas aeruginosa* to biofilm-associated growth and to calcium. *Microbiology* 153, 3838–3851.

Pier, G. B., Coleman, F., Grout, M., Franklin, M., and Ohman, D. E. (2001). Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. *Infect. Immun.* 69, 1895–1901.

Platt, M. D., Schurr, M. J., Sauer, K., Vazquez, G., Kukavica-Ibrulj, I., Potvin, E., Levesque, R. C., Fedynak, A., Brinkman, F. S., Schurr, J., Hwang, S. H., Lau, G. W., Limbach, P. A., Rowe, J. J., Lieberman, M. A., Barraud, N., Webb, J., Kjelleberg, S., Hunt, D. F., and Hassett, D. J. (2008). Proteomic, microarray, and signature-tagged mutagenesis analyses of anaerobic *Pseudomonas aeruginosa* at pH 6.5, likely representing chronic, late-stage cystic fibrosis airway conditions. *J. Bacteriol.* 190, 2739–2758.

Qiu, D., Damron, F. H., Mima, T., Schweizer, H. P., and Yu, H. D. (2008). PBAD-based shuttle vectors for functional analysis of toxic and highly-regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. *Appl. Environ. Microbiol.* 74, 7422–7426.

Quadroni, M., James, P., Dainese-Hatt, P., and Kertesz, M. A. (1999). Proteome mapping, mass spectrometric sequencing and reverse transcription-PCR for characterization of the sulfate starvation-induced response in *Pseudomonas aeruginosa* PAO1. *Eur. J. Biochem.* 266, 986–996.

Ramsey, D. M., and Wozniak, D. J. (2005). Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Mol. Microbiol.* 56, 309–322.

Rao, J., Digiandomenico, A., Unger, J., Bao, Y., Polanowska-Grabowska, R. K., and Goldberg, J. B. (2008). A novel oxidized low-density lipoprotein-binding protein from *Pseudomonas aeruginosa*. *Microbiology* 154, 654–665.

Rau, M. H., Hansen, S. K., Johansen, H. K., Thomsen, L. E., Workman, C. T., Nielsen, K. F., Jelsbak, L., Hoiby, N., Yang, L., and Molin, S. (2010). Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. *Environ. Microbiol.* 12, 1643–1658.

Riedel, K., Arevalo-Ferro, C., Reil, G., Gorg, A., Lottspeich, F., and Eberl, L. (2003). Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics. *Electrophoresis* 24, 740–750.

Sharma, S., Sundaram, C. S., Luthra, P. M., Singh, Y., Sirdeshmukh, R., and Gade, W. N. (2006). Role of proteins in resistance mechanism of *Pseudomonas fluorescens* against heavy metal induced stress with proteomics approach. *J. Biotechnol.* 126, 374–382.

Sherman, N. E., Stefansson, B., Fox, J. W., and Goldberg, J. B. (2001). *Pseudomonas aeruginosa* and a proteomic approach to bacterial pathogenesis. *Dis. Markers* 17, 285–293.

Sinha, S., Kosalai, K., Arora, S., Namane, A., Sharma, P., Gaikwad, A. N., Brodin, P., and Cole, S. T. (2005). Immunogenic membrane-associated proteins of *Mycobacterium tuberculosis* revealed by proteomics. *Microbiology* 151, 2411–2419.

Sriramulu, D. D., Nimtz, M., and Romling, U. (2005). Proteome analysis reveals adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment. *Proteomics* 5, 3712–3721.

Tart, A. H., Blanks, M. J., and Wozniak, D. J. (2006). The AlgT-dependent transcriptional regulator AmrZ (AlgZ) inhibits flagellum biosynthesis in mucoid, nonmotile *Pseudomonas aeruginosa* cystic fibrosis isolates. *J. Bacteriol.* 188, 6483–6489.

Tart, A. H., Wolfgang, M. C., and Wozniak, D. J. (2005). The alternative sigma factor AlgT represses *Pseudomonas aeruginosa* flagellum biosynthesis by inhibiting expression of *fleQ J. Bacteriol.* 187, 7955–7962.

Tatnell, P. J., Russell, N. J., and Gacesa, P. (1994). GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies. *Microbiology* 140, 1745–1754.

Ulanova, M., Petersen, T. D., Ciofu, O., Jensen, P., Hahn-Zoric, M., Hanson, L. A., and Hoiby, N. (1997). The clonal antibody response to *Pseudomonas aeruginosa* heat shock protein is highly diverse in cystic fibrosis patients. *APMIS* 105, 449–456.

Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S., and Filloux, A. (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 103, 171–176.

Wehmhoner, D., Haussler, S., Tummler, B., Jansch, L., Bredenbruch, F., Wehland, J., and Steinmetz, I. (2003). Inter- and intraclonal diversity of the *Pseudomonas aeruginosa* proteome manifests within the secretome. *J. Bacteriol.* 185, 5807–5814.

Winsor, G. L., Van Rossum, T., Lo, R., Khaira, B., Whiteside, M. D., Hancock, R. E., and Brinkman, F. S. (2009). *Pseudomonas* Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes. *Nucleic Acids Res.* 37, D483–D488.

Wood, L. F., Leech, A. J., and Ohman, D. E. (2006). Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. *Mol. Microbiol.* 62, 412–426.

Wood, L. F., and Ohman, D. E. (2009). Use of cell wall stress to characterize sigma 22 (AlgT/U) activation by regulated proteolysis and its regulon in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 72, 183–201.

Wu, M., Guina, T., Brittnacher, M., Nguyen, H., Eng, J., and Miller, S. I. (2005). The *Pseudomonas aeruginosa* proteome during anaerobic growth. *J. Bacteriol.* 187, 8185–8190.

Wu, W., Badrane, H., Arora, S., Baker, H. V., and Jin, S. (2004). MucA-mediated coordination of type III secretion and alginate synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 7575–7585.

Yang, L., Haagensen, J. A., Jelsbak, L., Johansen, H. K., Sternberg, C., Hoiby, N., and Molin, S. (2008). In situ growth rates and biofilm development of *Pseudomonas aeruginosa* populations in chronic lung infections. *J. Bacteriol.* 190, 2767–2776.

Zielinski, N. A., Chakrabarty, A. M., and Berry, A. (1991). Characterization and regulation of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase. *J. Biol. Chem.* 266, 9754–9763.

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Regulation and function of versatile aerobic and anaerobic respiratory metabolism in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a ubiquitously distributed opportunistic pathogen that inhabits soil and water as well as animal-, human-, and plant-host-associated environments. The ubiquity would be attributed to its very versatile energy metabolism. *P. aeruginosa* has a highly branched respiratory chain terminated by multiple terminal oxidases and denitrification enzymes. Five terminal oxidases for aerobic respiration have been identified in the *P. aeruginosa* cells. Three of them, the cbb_3-1 oxidase, the cbb_3-2 oxidase, and the aa_3 oxidase, are cytochrome *c* oxidases and the other two, the bo_3 oxidase and the cyanide-insensitive oxidase, are quinol oxidases. Each oxidase has a specific affinity for oxygen, efficiency of energy coupling, and tolerance to various stresses such as cyanide and reactive nitrogen species. These terminal oxidases are used differentially according to the environmental conditions. *P. aeruginosa* also has a complete set of the denitrification enzymes that reduce nitrate to molecular nitrogen via nitrite, nitric oxide (NO), and nitrous oxide. These nitrogen oxides function as alternative electron acceptors and enable *P. aeruginosa* to grow under anaerobic conditions. One of the denitrification enzymes, NO reductase, is also expected to function for detoxification of NO produced by the host immune defense system. The control of the expression of these aerobic and anaerobic respiratory enzymes would contribute to the adaptation of *P. aeruginosa* to a wide range of environmental conditions including in the infected hosts. Characteristics of these respiratory enzymes and the regulatory system that controls the expression of the respiratory genes in the *P. aeruginosa* cells are overviewed in this article.

Keywords: respiration, terminal oxidase, denitrification, nitric oxide, *Pseudomonas aeruginosa*

INTRODUCTION

The opportunistic pathogen *Pseudomonas aeruginosa* has a remarkable ability to grow under a variety of environmental conditions, including soil and water as well as animal-, human-, and plant-host-associated environments. It is responsible for severe nosocomial infections in immunocompromised patients. In particular, it causes life-threatening chronic lung infection in patients with the inherited disease cystic fibrosis (CF; Lyczak et al., 2002). The genome of *P. aeruginosa* is relatively large (6.3 Mb) and carries a large number of genes for utilization of various carbon sources, energy metabolisms, and regulatory systems, which might contribute to the environmental adaptability of this bacterium (Stover et al., 2000). The main energy producing system of *P. aeruginosa* is respiration, which utilizes a proton motive force for ATP synthesis. In the case of eukaryotic respiration in mitochondria, the electron transfer pathway consists of four complexes, NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), a cytochrome bc_1 complex (complex III), and a cytochrome *c* oxidase (complex IV). Protons are pumped across the membrane during electron transfer through complexes I, III, and IV, producing the proton gradient. On the other hand, *P. aeruginosa* as well as many other bacterial species use a variety of electron donors and acceptors for respiration and therefore have far more complex and flexible electron transfer pathways. At least 17 respiratory dehydrogenases that are predicted to be responsible for feeding electrons from respiratory substrates into the quinone pool, including three types of NADH dehydrogenases and a succinate dehydrogenase, have been annotated in the genome of *P. aeruginosa* (Williams et al., 2007). *P. aeruginosa* has five terminal oxidases that catalyze the four-electron reduction of molecular oxygen to water (Matsushita et al., 1982, 1983; Fujiwara et al., 1992; Cunningham and Williams, 1995; Cunningham et al., 1997; Stover et al., 2000; Comolli and Donohue, 2002, 2004). Three of them are cytochrome *c* oxidases that receive electrons via the cytochrome bc_1 complex and *c*-type cytochromes. The other two are quinol oxidases that receive electrons directly from ubiquinol (**Figure 1**). The respiratory chain is also branched to the denitrification enzymes that reduce nitrogen oxides. These alternative respiratory branches enable *P. aeruginosa* to grow under anaerobic conditions in the presence of nitrate or nitrite (Zumft, 1997). *P. aeruginosa* also has the ability to ferment arginine and pyruvate anaerobically. A fundamental understanding of the respiratory systems and the physiology of aerobic and anaerobic energy metabolism would be necessary for better comprehension of the ubiquity and pathogenicity of *P. aeruginosa*. Some excellent reviews on the aerobic and anaerobic respiration of *P. aeruginosa* are now available (Williams et al., 2007; Schobert and Jahn, 2010; Schobert and Tielen, 2010). This article will additionally focus on some recent information on the transcriptional regulation of the aerobic and anaerobic respiratory genes.

MULTIPLE TERMINAL OXIDASES FOR AEROBIC RESPIRATION

Pseudomonas aeruginosa has five terminal oxidases for aerobic respiration (**Figure 1**; Matsushita et al., 1982, 1983; Fujiwara et al., 1992; Cunningham and Williams, 1995; Cunningham et al., 1997;

Stover et al., 2000; Comolli and Donohue, 2002, 2004). Three of them, the cbb_3-1 oxidase (Cbb3-1), the cbb_3-2 oxidase (Cbb3-2), and the aa_3 oxidase (Aa3), are cytochrome *c* oxidases. The other two, the cytochrome bo_3 oxidase (Cyo) and the cyanide-insensitive oxidase (CIO), are quinol oxidases. These terminal oxidases are expected to have their specific affinity for oxygen, efficiency of proton-translocation, and resistance to various stresses such as cyanide and reactive nitrogen species. We have constructed five kinds of quadruple mutant strains, which lack four out of the five terminal oxidase gene clusters, and used them to characterize the features of each terminal oxidase (unpublished data). The K_m values of Aa3, CIO, and Cyo for oxygen were high, whereas those of Cbb3-1 and Cbb3-2 were one order lower than those of the other three terminal oxidases, indicating that Aa3, CIO, and Cyo are low affinity enzymes and Cbb3-1 and Cbb3-2 are high affinity enzymes. The analysis of the proton-pumping activity (H^+/O ratio) using the quadruple mutant strains showed that the electron transport complex terminated by Aa3 has the highest efficiency to create a proton gradient across the cell membrane, whereas that by CIO has the lowest efficiency. Carrying multiple terminal oxidases of such different characteristics and the differential use of them under different conditions must contribute to the ubiquity of *P. aeruginosa* in various environmental niches. Two redox-responsive transcriptional regulators, ANR (anaerobic regulation of arginine deiminase and nitrate reduction) and RoxSR, mainly regulate the expression of the terminal oxidase genes. ANR is a direct oxygen sensor and functions as a global regulator for anaerobic gene expression of *P. aeruginosa* (Zimmermann et al., 1991). RoxSR is a two-component transcriptional regulator consisting of the membrane-bound sensor kinase RoxS and the response regulator RoxR. RoxSR corresponds to PrrBA of *Rhodobacter sphaeroides* and RegBA of *Rhodobacter capsulatus*, which are the principal regulators controlling the expression of the genes for photosynthesis, carbon dioxide fixation, nitrogen fixation, and hydrogen metabolism, as well as numerous other functions in these purple photosynthetic bacteria (Dubbs and Tabita, 2004; Elsen et al., 2004; Eraso et al., 2008). The function and regulatory features of each terminal oxidase of *P. aeruginosa* are described below and in **Figure 2**.

THE CYTOCHROME cbb_3 OXIDASES

The cbb_3-type cytochrome *c* oxidase is phylogenetically the most distant member of the heme–copper oxidase superfamily and exclusively found in bacteria (Pitcher and Watmough, 2004). The X-ray structure of the enzyme from *Pseudomonas stutzeri* was reported

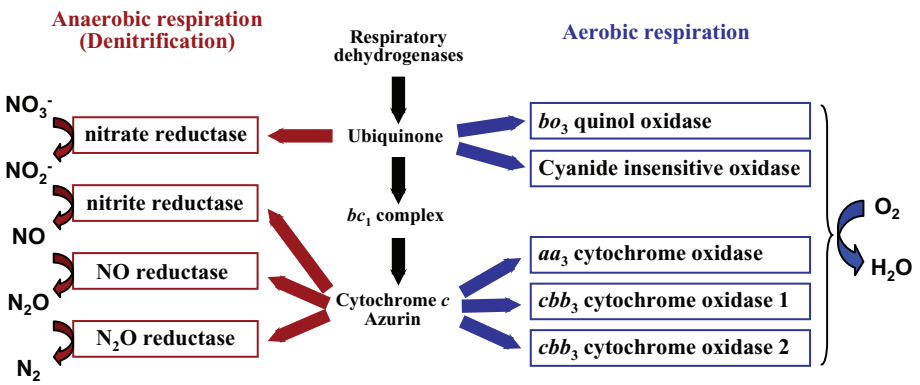

FIGURE 1 | Branched respiratory chain of *P. aeruginosa*. Under aerobic conditions, oxygen is utilized as a terminal electron acceptor and reduced to water by five terminal oxidases. Two quinol oxidases, the bo_3 oxidase and the cyanide-insensitive oxidase, receive electrons directly from quinol. Three cytochrome *c* oxidases, the aa_3 oxidase and the two cbb_3 oxidases, receive electrons via the cytochrome bc_1 complex and *c*-type cytochromes or a small blue-copper protein azurin. Under anaerobic conditions, electrons are transferred to nitrogen oxides via the denitrification enzymes.

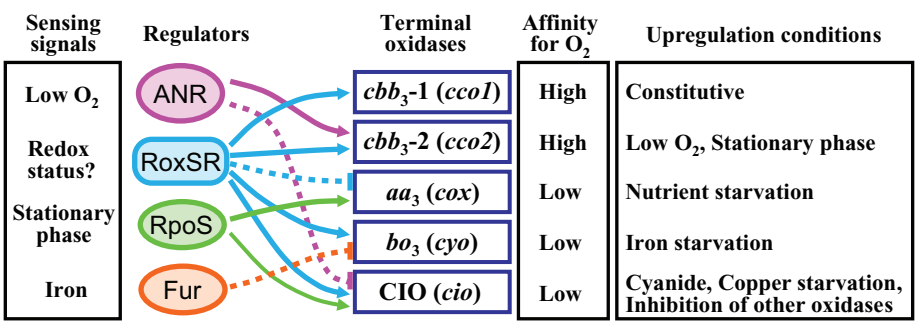

FIGURE 2 | Schematic model of the regulatory network controlling the multiple terminal oxidases in *P. aeruginosa*. The sensing signals for the regulators are shown in the left column. Affinity for oxygen and upregulation conditions of the terminal oxidases are shown in the right columns. Activation is indicated by arrows. Inhibition is indicated by bars with dotted lines.

recently (Buschmann et al., 2010). This type of enzyme is known to have very high affinity for oxygen and low proton-translocation efficiency. The K_m values for oxygen were determined to be 7 and 40 nM for the enzymes from *Bradyrhizobium japonicum* and *Campylobacter jejuni*, respectively (Preisig et al., 1996; Jackson et al., 2007). The cbb_3 oxidase is known to be induced under low oxygen conditions in many bacteria, such as *Paracoccus denitrificans*, *R. sphaeroides*, and *R. capsulatus* (Mouncey and Kaplan, 1998; Otten et al., 2001; Swem and Bauer, 2002). In the symbiotic nitrogen fixation bacterium *B. japonicum*, the cbb_3 oxidase is essential for nitrogen fixation in hypoxic root nodules, in which the oxygen concentration is extremely low (~20 nM; Preisig et al., 1993, 1996). The cbb_3 oxidase is also known to be functioning in the obligately microaerophilic bacteria *Helicobacter pylori* and *C. jejuni* (Nagata et al., 1996; Jackson et al., 2007). From these observations, the cbb_3 oxidase is recognized as the major player under the low oxygen environments.

The cbb_3 oxidase is also known to have a repressive role in the PrrBA-dependent expression of the photosynthesis genes in *R. sphaeroides* (O'Gara et al., 1998; Oh and Kaplan, 1999, 2000). The photosynthesis genes are usually induced under low oxygen or anaerobic conditions in non-oxygenic photosynthetic bacteria. However, disruption of the cbb_3 oxidase genes or inhibition of the cbb_3 oxidase activity results in expression of the photosynthesis genes even under aerobic conditions in *R. sphaeroides*. It has been proposed that the cbb_3 oxidase senses the electron flow through the respiratory chain and generates an inhibitory signal that prevents the activation of PrrBA (Oh et al., 2004).

The cbb_3 oxidase is encoded by a tetracistronic operon *ccoNOQP* (*fixNOQP*). The *ccoN* (*fixN*) product is the catalytic subunit that contains the binuclear center consisting of a high spin heme b_3 and Cu_B. The *ccoO* (*fixO*) and *ccoP* (*fixP*) genes encode transmembrane monoheme and diheme cytochrome *c* subunits, respectively. The gene product of *ccoQ* (*fixQ*) is thought to be required for stabilization of the core complex of the cbb_3-type oxidases (Zufferey et al., 1996; Oh and Kaplan, 2002; Peters et al., 2008).

Two complete sets of the genes encoding the cbb_3 cytochrome oxidases (Cbb3-1 and Cbb3-2) are tandemly clustered in the genome of *P. aeruginosa* (Stover et al., 2000). Cbb3-1 and Cbb3-2 are encoded by the *ccoN1O1Q1P1* genes (PA1552–1554) and the *ccoN2O2Q2P2* genes (PA1555–1557), respectively. The small *ccoQ1* and *ccoQ2* genes were not annotated in the genome previously (Stover et al., 2000). Tandem repeats of the genes for two cbb_3-type cytochrome oxidases are also found in the genomes of other pseudomonads, such as *Pseudomonas putida*, *Pseudomonas fluorescens*, and *P. stutzeri*. In addition to the two complete sets of the *cco* gene clusters, two orphan *ccoN*-like genes (PA1856 and PA4133), which are highly similar to the main subunit genes of the cbb_3 oxidases, have been identified in the *P. aeruginosa* genome (Stover et al., 2000). Both of these *ccoN*-like genes are followed by small genes (PA1855 and PA4134) that have similarity with the *ccoQ* genes. These extra *ccoNQ*-like gene clusters might have complementary functions for aerobic respiration.

The *cco1* genes for Cbb3-1 were constitutively expressed at a high level even under high oxygen conditions and slightly downregulated at the stationary phase (Comolli and Donohue, 2004; Alvarez-Ortega and Harwood, 2007; Kawakami et al., 2010). Cbb3-1 seemed to be the dominant oxidase, especially under the normal aerobic growth conditions at exponential phase in LB medium. Disruption of the *cco1* genes slightly affected the aerobic growth (Comolli and Donohue, 2004; Alvarez-Ortega and Harwood, 2007). Mutation of the *cco1* genes also showed a small colony phenotype (Schurek et al., 2008). In contrast, the *cco2* genes for Cbb3-2 were upregulated under low oxygen conditions or at the stationary phase. The expression level of Cbb3-2 surpassed that of Cbb3-1 under those induced conditions (Kawakami et al., 2010). ANR is involved in the transcriptional activation of the *cco2* genes under low oxygen conditions (Comolli and Donohue, 2004; Kawakami et al., 2010). The induction of the *cco2* genes at the stationary phase likely occurred because the oxygen concentration in the medium was depleted to the level that induces the activation by ANR. RoxSR is also involved in the induction of the *cco2* genes (Kawakami et al., 2010). A similar RoxSR-dependent regulation was also reported in the case of *cco1* (corresponding to *cco2* of *P. aeruginosa*) in *P. putida* (Fernández-Piñar et al., 2008). A *cco1 cco2* double mutant of *P. aeruginosa* showed very slow growth under a 2% oxygen condition and failed to grow under a 0.4% oxygen condition (Alvarez-Ortega and Harwood, 2007). These results indicate that Cbb3-1 plays a primary role in aerobic growth irrespective of oxygen concentration and that Cbb3-2 plays a compensatory or supplementary role under the oxygen-depleted conditions.

THE CYTOCHROME aa_3 OXIDASE

The aa_3-type cytochrome *c* oxidase is a major member of the heme–copper oxidase superfamily and is distributed in a wide range of bacteria. This type of heme–copper oxidase enzyme is closely related to the mitochondrial terminal oxidase and has a high proton-pumping activity. The aa_3-type oxidases have low affinity for oxygen and usually play a dominant role under high oxygen conditions in many bacteria, such as *P. denitrificans*, *B. japonicum*, *R. sphaeroides*, and *Bacillus subtilis* (Bosma et al., 1987; Gabel and Maier, 1993; Flory and Donohue, 1997; Winstedt and von Wachenfeldt, 2000; Arai et al., 2008).

In *P. aeruginosa*, the aa_3 oxidase (Aa3) is encoded by the *coxBA*-PA0107-*coIII* (*coxC*) gene cluster (PA0105–0108; Stover et al., 2000). The *coxA*, *coxB*, and *coIII* (*coxC*) genes encode subunits I, II, and III, respectively. Subunit I carries the heme a_3–Cu_B binuclear catalytic center. Subunit II has a binding site of Cu_A, which is the electron transfer site with cytochrome *c*. PA0107 encodes a putative cytochrome *c* oxidase assembly protein involved in the insertion of copper into subunit I. The expression level of the *cox* genes is kept very low under normal laboratory growth conditions even at high oxygen tension, although these genes are slightly upregulated at the stationary phase in *P. aeruginosa* (Schuster et al., 2004; Alvarez-Ortega and Harwood, 2007). We recently found that the *cox* genes were significantly induced under starvation of carbon, nitrogen, or iron (Kawakami et al., 2010). The *cox* promoter was found to be dependent on a stationary phase sigma factor RpoS and repressed by RoxSR (Schuster et al., 2004; Kawakami et al., 2010). At the stationary phase, RpoS is highly expressed and activates the *cox* promoter, but the promoter is simultaneously repressed by RoxSR, which is expected to be active in the hypoxic high-cell-density stationary phase culture. Therefore, the expression level of the *cox* genes might be kept low under any nutrient-rich conditions.

The *cco1 cco2 cio cyo* quadruple mutant, which is deficient in the four terminal oxidase gene clusters other than the *cox* gene cluster, was not able to grow under aerobic conditions. However, suppressor mutants that are able to grow aerobically emerged after aerobic incubation for several weeks (unpublished data). The *cox* structural genes had no mutation, but the *roxS* gene was inactivated and the RpoS-dependent promoter region of the *cox* genes was altered in the suppressor mutants, confirming the tight regulation of the *cox* genes by RoxSR and RpoS. The respiratory chain terminated by Aa3 has the highest ability to create a proton gradient across the membrane (the H^+/O ratio is 6) among the five terminal oxidases of *P. aeruginosa* (unpublished data). Therefore, it is reasonable that Aa3 is utilized only under starvation conditions for efficient energy production. Utilization of Aa3 might be advantageous for proliferation in natural oligotrophic environments. However, if Aa3 were highly expressed under the nutrient-rich laboratory growth conditions, its high proton-pumping activity might cause an imbalance of energy and redox homeostasis in *P. aeruginosa* cells.

THE bo_3 QUINOL OXIDASE

The bo_3-type quinol oxidase is also a member of the heme–copper oxidase superfamily. This type of the terminal oxidase is homologous to the aa_3-type cytochrome oxidase but contains heme *b* and heme *o*. It lacks the Cu_A-binding site, which is involved in the interaction with cytochrome *c*, and receives electrons from ubiquinol. The bo_3 oxidase of *Escherichia coli* is known to have a low affinity for oxygen and functions under high oxygen conditions (Cotter et al., 1990; D'Mello et al., 1995). It is encoded by the *cyoABCDE* genes in *E. coli* (Nakamura et al., 1997). The *cyoA*, *cyoB*, and *cyoC* genes encode the subunits corresponding to the subunits II, I, and III of the aa_3-type cytochrome oxidase, respectively. The *cyoD* gene encodes subunit IV, which is proposed to assist the Cu_B-binding to subunit I during biosynthesis or assembly of the oxidase complex (Saiki et al., 1996). The *cyoE* encodes a protoheme IX farnesyltransferase (heme *o* synthetase), which is required for production of heme *o* from heme *b* (Saiki et al., 1992; Mogi et al., 1994).

The *cyoABCDE* genes (PA1317–1321) of the *P. aeruginosa* bo_3 oxidase (Cyo) are highly homologous with the corresponding genes of *E. coli*. Cyo was identified as a quinol oxidase and shown to have a high K_m value for oxygen by using the *cco1 cco2 cox cio* quadruple mutant (unpublished data). The H^+/O ratio of Cyo was speculated to be 4, which was comparable to that of the bo_3 oxidase from *E. coli* (Puustinen et al., 1989). The *cyo* genes were downregulated at the stationary phase or under the low oxygen conditions in *P. aeruginosa* cells (Alvarez-Ortega and Harwood, 2007; Kawakami et al., 2010). Considering that the affinity for oxygen is low, Cyo might function under high oxygen conditions as in the case of *E. coli*. However, because the expression level of the *cyo* genes was significantly lower than that of the *cco1* genes without stresses, Cyo might make a minor contribution to the cell growth under normal laboratory growth conditions. The *cyo* genes were found to be significantly induced by iron starvation or in the presence of a nitric oxide (NO)-generating reagent, *S*-nitrosoglutathione (GSNO; Ochsner et al., 2002; Kawakami et al., 2010). Cyo is likely responsible for respiration under iron-limiting conditions and the expression of the *cyo* genes is regulated by the transcriptional regulator Fur (ferric uptake regulator), which is known to bind to DNA in the presence of Fe^{2+} and represses the iron-regulated promoters (Vasil and Ochsner, 1999; Vasil, 2007). Because the predicted number of iron atoms sequestered in the Cyo complex is lower than those of the other terminal oxidase complexes and the respiratory complex terminated by Cyo does not require the iron-containing cytochrome bc_1 complex and soluble cytochrome *c* (Fujiwara et al., 1992; Thöny-Meyer, 1997), the demand for iron might be lower when Cyo is utilized. The *cyo* genes were upregulated by GSNO, but the role of Cyo in nitrosative stress resistance is not certain. Because Fur is known to lose its DNA-binding ability in the presence of NO (D'Autréaux et al., 2002), the upregulation by GSNO might be merely due to the inactivation of Fur by NO. An obligately aerobic bacterium, *P. putida*, also has five sets of terminal oxidases corresponding to those of *P. aeruginosa* (Ugidos et al., 2008). In *P. putida*, inactivation of the *cyo* genes relieves the catabolite repression of the phenol- or alkane-degradation genes (Petruschka et al., 2001; Dinamarca et al., 2002). The *cyo* mutation also leads to a significant change in the transcriptome profile, and the absence of Cyo in *P. putida* was compensated for by upregulation of CIO and one of the cbb_3 oxidases corresponding to Cbb3-2 of *P. aeruginosa* (Morales et al., 2006). In contrast to *P. aeruginosa*, Cyo might make a major contribution to the aerobic growth of this obligately aerobic bacterium, although it remains to be investigated whether inactivation of Cyo has a significant influence in *P. aeruginosa*.

THE CYANIDE-INSENSITIVE OXIDASE

The cyanide-insensitive quinol oxidase (CIO) has been found and described from *P. aeruginosa* and *C. jejuni* (Matsushita et al., 1983; Cunningham et al., 1997; Jackson et al., 2007). While the Cbb3-1, Cbb3-2, Aa3, and Cyo oxidases belong to the heme–copper oxidase superfamily, CIO is the only copper-free terminal oxidase in *P. aeruginosa*. It consists of two subunits encoded by the *cioAB* genes, which are highly homologous to the *cydAB* genes for the cytochrome *bd*-type quinol oxidases (Cunningham et al., 1997; Jünemann, 1997; Jackson et al., 2007). The *bd* oxidases have high affinity for oxygen and contain low-spin heme b_{558}, high spin heme b_{595}, and heme *d* (D'mello et al., 1996; Jünemann, 1997). *E. coli* has two *bd* oxidases (Bekker et al., 2009), one of which is known to be induced and predominant under oxygen-limited conditions (Rice and Hempfling, 1978; Cotter et al., 1990). In the free-living nitrogen-fixing bacterium *Azotobacter vinelandii*, the *bd* oxidase rapidly consumes oxygen and protects the oxygen-sensitive nitrogenase complex (Kelly et al., 1990; Poole and Hill, 1997). This phenomenon is called respiratory protection.

Although the *cioAB* genes for CIO are highly homologous to the *cydAB* genes for the *bd* oxidase, CIOs from *P. aeruginosa* and *C. jejuni* lack the spectral features for heme b_{595} and heme *d* (Matsushita et al., 1983; Cunningham and Williams, 1995; Cunningham et al., 1997; Jackson et al., 2007). It had been considered that the hemes of the *bd* oxidase are replaced by other unknown redox centers in CIO. However, recent analysis using the membrane vesicle of a CIO-overproducing *Gluconobacter oxydans* revealed that CIO carries all of the hemes b_{558}, b_{595}, and *d*. The absence of the spectroscopic properties was predicted to be caused by the unique ligand-binding properties of CIO (Mogi et al., 2009). A distinctive feature that could be used easily to discriminate CIO from the *bd* oxidase is that the conserved sequence of the periplasmic loop (Q-loop) that

contains the putative quinol oxidizing site is significantly shorter in CioA than in CydA (Cunningham et al., 1997). CIO is known to have higher resistance to cyanide than Cyo (Mogi et al., 2009). In contrast to the finding that the *bd* oxidases have high affinity for oxygen, CIO of *C. jejuni* was reported to have low affinity for oxygen, with a K_m value of 0.8 μM (Jackson et al., 2007). It has been recognized that CIO of *P. aeruginosa* has high affinity for oxygen because the *cco1 cco2 cio* triple mutant, which lacks Cbb3-1, Cbb3-2, and CIO, could not grow microaerobically under a 2% O_2 concentration (Alvarez-Ortega and Harwood, 2007). However, our recent analysis using the *cco1 cco2 cox cyo* quadruple mutant revealed that the K_m value of CIO of *P. aeruginosa* for oxygen is significantly higher than those of Cbb3-1 and Cbb3-2 and comparable to those of Aa3 and Cyo, indicating that CIO of *P. aeruginosa* is a low affinity enzyme like CIO of *C. jejuni* (unpublished data).

Pseudomonas aeruginosa produces a respiratory chain inhibitor, hydrogen cyanide, at concentrations up to 300 μM under low oxygen conditions. The concentrations are high enough to inhibit the activity of the heme–copper oxidases. Therefore, CIO is believed to have a role as an electron sink under cyanogenic conditions (Cunningham and Williams, 1995; Cunningham et al., 1997; Blumer and Haas, 2000). The reason why the *cco1 cco2 cio* triple mutant could not grow under the 2% O_2 condition (Alvarez-Ortega and Harwood, 2007) was probably because of the inhibition of Aa3 and Cyo by the endogenous cyanide. Cyanide is reported to be the primary toxic factor responsible for the paralytic killing of *Caenorhabditis elegans* by *P. aeruginosa* (Gallagher and Manoil, 2001). CIO appeared to be required for this pathogenicity because the mutant of the *cioAB* genes (PA3929–3930) had lower killing activity against the nematoda (Zlosnik et al., 2006).

The *cioAB* genes are upregulated at stationary phase or at very low ambient oxygen concentration (0.4–0.5%) in *P. aeruginosa* (Cooper et al., 2003; Alvarez-Ortega and Harwood, 2007; Kawakami et al., 2010). Respiratory chain inhibitors, cyanide and sodium nitroprusside (SNP), significantly induce the *cio* genes (Comolli and Donohue, 2002; Cooper et al., 2003; Kawakami et al., 2010). The *cio* genes are also induced by copper starvation or disruption of the *senC* gene (PA0114), which encodes a putative copper chaperone for the heme–copper oxidases (Frangipani et al., 2008; Frangipani and Haas, 2009). Since CIO is the only non-heme–copper oxidase in *P. aeruginosa*, CIO must be important for respiration under copper-restricted conditions. The *cio* genes are positively regulated by RoxSR and disruption of the *cco1* genes for Cbb3-1 causes upregulation of the *cio* promoter (Comolli and Donohue, 2004). Regulation of the RoxSR activity by the electron flow through the cbb_3 oxidase might be operative in *P. aeruginosa* as in the case of PrrBA in *R. sphaeroides* (Oh and Kaplan, 2000). The expression of CIO is negatively regulated by ANR and an *anr* mutant has been shown to express a high level of CIO, especially under low oxygen concentrations (Cooper et al., 2003). It seems likely that ANR prevents the overproduction of CIO when it is not necessary. The stationary phase sigma factor RpoS also activates the expression of the *cio* genes, although its contribution is minor when ANR or RoxSR is operative (Kawakami et al., 2010). The regulation of the *cio* genes in *P. aeruginosa* is thus complicated, but CIO seems to be controlled so as to be expressed when the other terminal oxidases of the heme–copper superfamily are not functioning.

REGULATORY FACTORS CONTROLLING THE MULTIPLE TERMINAL OXIDASES

Two redox-responsive transcriptional regulators, ANR and RoxSR, and a stationary phase sigma factor RpoS play dominant roles in the control of the multiple terminal oxidases in *P. aeruginosa* (**Figure 2**). ANR functions as a global regulator for anaerobic gene expression in response to oxygen depletion (Galimand et al., 1991; Sawers, 1991; Zimmermann et al., 1991). Approximately 170 transcription units are predicted to belong to the ANR regulon (Trunk et al., 2010). ANR is an analog of *E. coli* FNR (fumarate nitrate reductase regulator), which senses intracellular oxygen levels by an oxygen-sensitive $[4Fe–4S]^{2+}$ cluster bound to N-terminal Cys residues (Kiley and Beinert, 1998; Unden et al., 2002).

RoxSR is an analog of PrrBA/RegBA of purple photosynthetic bacteria, which activates expression of the photosynthesis genes under low oxygen conditions (Dubbs and Tabita, 2004; Elsen et al., 2004; Eraso et al., 2008). It has been proposed that PrrBA of *R. sphaeroides* receives an inhibitory signal from the electron flow through the cbb_3 oxidase because the PrrBA-dependent genes are upregulated even under aerobic conditions when the cbb_3 oxidase activity is blocked by inhibitors or mutations (O'Gara et al., 1998; Oh and Kaplan, 1999, 2000; Kim et al., 2007). The activity of RegBA of *R. capsulatus* and *Rhodospirillum rubrum* is proposed to be controlled by the redox status of ubiquinones (Grammel and Ghosh, 2008; Wu and Bauer, 2010). The sensing signal of RoxSR of *P. aeruginosa* is not certain at present. Mutation of the *cco1* genes for Cbb3-1 caused upregulation of the RoxSR-dependent expression of CIO in *P. aeruginosa* PAK, suggesting the possibility that RoxSR senses the electron flow through Cbb3-1 (Comolli and Donohue, 2004). However, because Cbb3-1 is the dominant terminal oxidase under normal aerobic growth conditions, deletion of Cbb3-1 might greatly influence the redox status of the ubiquinone pool. In the case of *P. putida*, in which Cyo might make a major contribution under aerobic conditions, the absence of Cyo causes upregulation of CIO as well as a significant change in the transcriptome profile (Morales et al., 2006). In any case, the activity of RoxSR of *Pseudomonas* species is expected to be controlled, directly or indirectly, by the redox status of the respiratory chain.

ANR activates the expression of Cbb3-2, which is a high affinity enzyme and predicted to be dominant under low oxygen conditions (Ray and Williams, 1997; Comolli and Donohue, 2004). ANR represses the expression of CIO (Cunningham et al., 1997; Cooper et al., 2003; Comolli and Donohue, 2004), which was recently found to be a low affinity enzyme (unpublished data). Another low affinity enzyme, Cyo, is probably repressed in an indirect manner by ANR (Kawakami et al., 2010), which is in contrast to the direct repression of Cyo by ANR in *P. putida* (Ugidos et al., 2008). RoxSR regulates the expression of all five terminal oxidases, though whether directly or indirectly is not certain (Comolli and Donohue, 2002; Kawakami et al., 2010). Aa3 is repressed and the other four terminal oxidases are activated by RoxSR. Some other genes related to respiratory function, such as *hemB* and *nuoAL*, are under the control of RoxSR, indicating that RoxSR plays an extensive role in the regulation of respiration in *P. aeruginosa* (Kawakami et al., 2010). Cbb3-2, Aa3, and CIO are upregulated at the stationary phase. RpoS plays a significant role in the expression of Aa3, but make a minor contribution to the regulation of CIO and no contribution to that of

Cbb3-2 (Cooper et al., 2003; Schuster et al., 2004; Kawakami et al., 2010). RpoS is known to be necessary for survival under carbon starvation in *P. aeruginosa* (Jørgensen et al., 1999; Suh et al., 1999). Therefore, the induction of Aa3 under nutrient starvation conditions might be mainly the effect of the function of RpoS.

Regulation of the terminal oxidases not only by peripheral oxygen tension by ANR but also by the redox status of the respiratory chain by RoxSR might be a sophisticated mechanism for fine tuning of multiple enzymes with different characteristics, because the redox status of the respiratory components is significantly affected by nutritional conditions and respiratory stressors as well as oxygen availability. Availability of nitrogen oxides that act as alternative electron acceptors for anaerobic respiration might also influence the redox status of the respiratory chain under low oxygen conditions.

MICROAEROBIC PHYSIOLOGY OF *P. AERUGINOSA*

The cbb_3 oxidases are dominantly expressed and function even under the aerobic conditions in *P. aeruginosa*. This feature is unique to *P. aeruginosa*, because the cbb_3 oxidases are known to have very high affinity for oxygen and are usually repressed under high oxygen conditions in other bacterial species (Preisig et al., 1996; Mouncey and Kaplan, 1998; Otten et al., 2001; Swem and Bauer, 2002; Jackson et al., 2007). Our preliminary experiment revealed that both of the two cbb_3 oxidases of *P. aeruginosa* also have high affinity for oxygen (unpublished data). The low affinity enzymes are highly induced only under starvation or stressed conditions (Kawakami et al., 2010). This is in contrast to the case of the non-pathogenic obligately aerobic bacterium *P. putida*, which is phylogenetically close to *P. aeruginosa* and has the same set of five terminal oxidases, but in which Cyo might make a major contribution under aerobic conditions (Morales et al., 2006; Fernández-Piñar et al., 2008; Ugidos et al., 2008).

The dominant expression and function of the high affinity enzymes might indicate that the *P. aeruginosa* cells are maintained in a microaerobic state even under aerobic conditions. *P. aeruginosa* actively produces a microaerobic environment even at high aeration rates by reducing the transfer rate of oxygen from the gas phase into the liquid phase (Sabra et al., 2002). This phenomenon was predicted to be due to the production of biosurfactants such as rhamnolipid. *P. aeruginosa* strains infected in the CF airway show the mucoid phenotype by overproduction of the exopolysaccharide alginate, which is known to act as a physical barrier to oxygen entering the cell. Even the non-mucoid strain PAO1 forms a mucoid polysaccharide capsule, which might consist primarily of alginate, on the cell surface as a response to oxidative stress (Sabra et al., 2002). Alginate is also reported to restrict the diffusion of oxygen (Hassett, 1996). A nitrogen-fixing bacterium, *A. vinelandii*, which is closely related to *Pseudomonas*, is also known to produce alginate as a barrier to protect oxygen-sensitive nitrogenase from oxygen (Sabra et al., 2000).

The microaerobic physiology of *P. aeruginosa* might be significantly related to its pathogenesis. During pulmonary infection, *P. aeruginosa* is exposed to reactive oxygen species produced by the host immune cells. The physical blockage of oxygen transfer by production of the polysaccharide layer and reduction of the oxygen diffusion rate would be advantageous for *P. aeruginosa* to survive under such oxidative stress conditions. Moreover, release of virulence factors, such as elastase and pyocyanin, into the culture supernatant of *P. aeruginosa* has been reported to be enhanced by the microaerobic culture conditions (Sabra et al., 2002).

ANAEROBIC ENERGY METABOLISM

Pseudomonas aeruginosa had been considered as an obligately aerobic bacterium previously, but it is now recognized to be highly adapted to anaerobic conditions. Because the *P. aeruginosa*-infected mucus in the CF airway is depleted of oxygen, the anaerobic physiology of *P. aeruginosa* is believed to be important for its pathogenesis (Yoon et al., 2002; Schobert and Jahn, 2010; Schobert and Tielen, 2010). In the absence of oxygen, *P. aeruginosa* can grow by dissimilatory nitrate respiration by using nitrogen oxides as alternative terminal electron acceptors of the respiratory chain. This process is called denitrification because soluble nitrate and nitrite are reduced to and released as gaseous nitrous oxide (N_2O) or dinitrogen (N_2; Zumft, 1997). This pathway is ecologically important because it is one of the very few routes for generating atmospheric N_2. Complete denitrification consists of four sequential steps to reduce nitrate to N_2 via nitrite, nitric oxide (NO), and N_2O. Each step of the pathway is catalyzed by individual metalloenzymes, i.e., nitrate reductase, nitrite reductase, NO reductase, and N_2O reductase (**Figure 3**).

DENITRIFICATION ENZYMES AND GENES

Nitrate reductase catalyzes the first step of denitrification, reduction of nitrate to nitrite. Three types of nitrate reductases, Nar, Nap, and Nas, which are localized to the cytoplasmic membrane, periplasm, and cytoplasm, respectively, are encoded in the genome of *P. aeruginosa* (Berks et al., 1995; Stover et al., 2000). They all contain a molybdopterin guanine dinucleotide cofactor. The membrane-bound Nar is the enzyme responsible for anaerobic nitrate respiration of *P. aeruginosa* in CF sputum (Palmer et al., 2007). Nar is encoded in the *narK1K2GHJI* gene cluster (PA3872–3877). NarG, NarH, and NarI are the structural subunits of the enzyme and NarJ is required for the assembly of the functional enzyme (Philippot and Højberg, 1999). The *narK1* and *narK2* genes encode putative nitrate/nitrite transporters homologous to each other, but only *narK2* has been reported to be required for denitrifying growth (Sharma et al., 2006). The *narXL* genes (PA3878–3879) encoding a two-component transcriptional regulator, NarXL, which is required for the nitrate-responsive expression of the *narK1K2GHJI* operon, is located upstream from the *narK1* gene (Schreiber et al., 2007). Reduction of nitrate by Nar is coupled to quinol oxidation and consumes two protons from the cytoplasm, thereby contributing to creation of a proton gradient across the membrane (Zumft, 1997).

The periplasmic enzyme Nap and related proteins are encoded in the *napEFDABC* gene cluster (PA1172–1177). Nap is a quinol oxidase, but nitrate reduction in the periplasm by Nap does not contribute to generation of the proton gradient. The physiological function of Nap in *P. aeruginosa* is not certain at present. In *R. sphaeroides*, Nap is regulated by both nitrate and electron supply and predicted to be involved in redox balancing by using nitrate as an ancillary oxidant to dissipate excess reductant (Gavira et al., 2002). The cytoplasmic enzyme Nas is exclusively involved in nitrate assimilation. The *nasC* gene (PA1779) for Nas is clustered with the *nirBD* genes (PA1780–1781) encoding assimilatory nitrite reductase, which catalyzes the reduction of nitrite to ammonium (Berks et al., 1995).

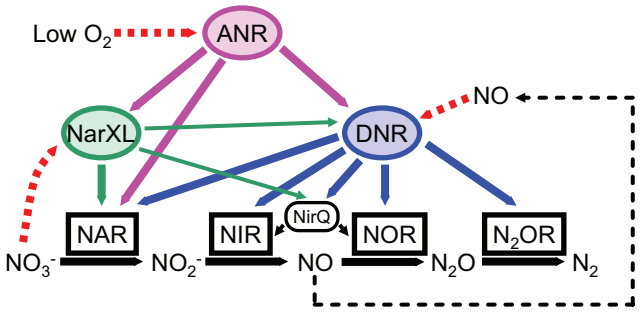

FIGURE 3 | Schematic model of the regulatory network controlling the denitrification genes in *P. aeruginosa*. ANR activates the expression of DNR under anaerobic or low oxygen conditions. DNR activates the expression of all denitrification genes in response to nitric oxide. A two-component nitrate sensing regulator, NarXL is required for expression of the *nar* genes encoding nitrate reductase. Both ANR and DNR can activate the *nar* gene expression. NirQ is predicted to be involved in the fine tuning of the activities of nitrite reductase and nitric oxide reductase. NAR, NIR, NOR, and N_2OR indicate nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, respectively.

Nitrite reductase catalyzes the second step of denitrification, reduction of nitrite to NO. Two types of dissimilatory nitrite reductases, the copper-containing type and the cytochrome cd_1-type, have been reported so far and *P. aeruginosa* has the latter type (Zumft, 1997). Both types are located in the periplasm. The cytochrome cd_1 nitrite reductase consists of two identical subunits, each containing a covalently attached heme *c* and a non-covalently bound heme d_1, which is a prosthetic group unique to this type of nitrite reductase (Silvestrini et al., 1994). In *P. aeruginos*a, nitrite reductase is encoded in the *nirSMCFDLGHJEN* gene cluster (PA0509–0519). *nirS* is the structural gene for the enzyme (Silvestrini et al., 1989). *nirM* encodes cytochrome *c*-551 and *nirC* encodes another monoheme cytochrome *c*, and both these cytochromes mediate electron transfer from the cytochrome bc_1 complex to nitrite reductase (Arai et al., 1990; Nordling et al., 1990; Hasegawa et al., 2001, 2003). The *nirFDLGHJE* genes are necessary for the biosynthesis of heme d_1 (Kawasaki et al., 1995, 1997). *nirN* encodes a *c*-type cytochrome, which is similar to *nirS*, but its function is not certain (Hasegawa et al., 2001).

Reduction of NO to N_2O is catalyzed by NO reductase (Hendriks et al., 2000; Zumft, 2005). Two types of bacterial NO reductases, designated cNOR and qNOR, have been characterized. cNOR is a membrane-bound cytochrome *bc* complex and receives electrons from soluble cytochrome *c*. qNOR lacks the cytochrome *c* component and receives electrons from quinol. *P. aeruginosa* and many denitrification bacteria have cNOR. qNOR has been found in bacteria and cyanobacteria such as *Ralstonia eutropha*, *Neisseria gonorrhoeae*, and *Synechocystis* sp. PCC6803 (Cramm et al., 1999; Householder et al., 2000; Büsch et al., 2002). Bacterial NO reductase shows similarity to the main subunit of cytochrome *c* oxidases of the heme–copper superfamily and is predicted to be the ancestor of oxygen-based respiratory enzymes (Saraste, 1994; Saraste and Castresana, 1994; Zumft, 2005). NO reductases have no proton-pumping activity, but cNOR contributes to generation of the proton gradient across the membrane because the electrons for its reaction are supplied through the cytochrome bc_1 complex via soluble cytochrome *c*. The NO reductase of *P. aeruginosa* has been purified and characterized and its crystal structure has been reported recently (Kumita et al., 2004; Hino et al., 2010). NO reductase is encoded in the *norCBD* operon (PA0523–0525), which is clustered with the *nir* genes for nitrite reductase in the genome of *P. aeruginosa* (Arai et al., 1995a). *norC* and *norB* encode cytochrome *c* and cytochrome *b* subunits of the enzyme, respectively. *norD* encodes a soluble protein predicted to be required for production of the active enzyme. NorB carries the binuclear catalytic center consisting of heme b_3 and non-heme Fe_B. NorC mediates electron transfer from soluble cytochrome *c* to NorB (Zumft, 2005). NO reductase functions not only for anaerobic energy conservation as a respiratory enzyme, but also for detoxification of exogenous NO. The machinery for detoxification of NO and its derivative reactive nitrogen species is necessary because the infected *P. aeruginosa* cells are subjected to nitrosative stress by the attack of the host immune system. The NO reductase-deficient mutant of *P. aeruginosa* shows a reduced survival rate in NO-producing macrophages (Kakishima et al., 2007). *P. aeruginosa* has another NO-detoxification enzyme, flavohemoglobin, which is encoded by the *fhp* gene (PA2664; Arai et al., 2005). Flavohemoglobin is known to exhibit NO dioxygenase activity under aerobic conditions and NO reductase activity under anaerobic conditions (Poole and Hughes, 2000). However, flavohemoglobin of *P. aeruginosa* is necessary for detoxification of NO under aerobic conditions but does not support denitrifying growth by complementing the function of NO reductase under anaerobic conditions (Arai et al., 2005). The *fhp* gene is regulated by the NO-responsive transcriptional regulator FhpR and transcribed as an operon with the following two genes, *ppyR* (PA2663) and *nnrS* (PA2662), which encode putative membrane proteins. Disruption of these *ppyR* and *nnrS* genes has no effect on the aerobic tolerance to reactive nitrogen species, but the *nnrS* mutant shows very poor growth under anaerobic denitrification conditions (unpublished data). The *ppyR* gene has been reported to have a role in the formation of biofilm (Attila et al., 2008).

The *nirQOP* operon (PA0520–0522) is located between the structural genes for nitrite reductase (*nirS*) and NO reductase (*norCB*; Arai et al., 1994, 1996, 1998). The activities of nitrite reductase and NO reductase should be coordinately regulated in order to avoid accumulation of highly cytotoxic intermediate, NO. The function of NirQ is predicted to be the fine tuning of the expression and activation of nitrite reductase and NO reductase (Jüngst and Zumft, 1992; Arai et al., 1996). The *nirO* and *nirP* genes encode transmembrane proteins. NirO has sequence similarity to subunit III of cytochrome *c* oxidases. The function of the *nirOP* genes is not certain, but it has been proposed that these genes are involved in efficient energy conservation under anaerobic conditions (Arai et al., 1996, 1998).

The final step of the denitrification pathway, reduction of N_2O to N_2, is catalyzed by N_2O reductase. This enzyme has been intensively studied in *P. stutzeri* and *P. denitrificans* (Zumft, 1997; Zumft and Kroneck, 2007) and has also been purified and characterized from *P. aeruginosa* (SooHoo and Hollocher, 1991). N_2O reductase is a periplasmic enzyme and predicted to receive electrons from the cytochrome bc_1 complex via soluble

cytochrome *c* or pseudoazurin. It has two types of copper centers, the mixed-valent dinuclear Cu_A species at the electron entry site and the tetranuclear Cu_Z center at the catalytically active site (Zumft and Kroneck, 2007). The Cu_A site of N_2O reductase shows similarity to the corresponding electron entry site in subunit II of cytochrome oxidases of the heme–copper superfamily, suggesting an evolutionary relationship between the two enzymes for anaerobic and aerobic respiration (Saraste, 1994; Saraste and Castresana, 1994). In *P. aeruginosa*, N_2O reductase is encoded in the *nosRZDFYL* operon (PA3391–3396; Arai et al., 2003). The structural gene for the enzyme is *nosZ*. The *nosDFY* gene products are thought to be involved in the processing and insertion of copper into the enzyme. The *nosL* gene product is proposed to be an outer membrane disulfide isomerase. *nosR* encodes a membrane protein of unknown function. Many denitrifying bacteria can grow on N_2O as the only electron acceptor under anaerobic conditions. However, *P. aeruginosa* cannot grow on exogenous N_2O as the only electron acceptor, although it can utilize endogenous N_2O for the generation of energy for growth during denitrification. This is probably because the *nosR* promoter is regulated by NO and exogenous N_2O does not induce the *nos* genes (SooHoo and Hollocher, 1990; Arai et al., 2003).

TRANSCRIPTIONAL REGULATION OF THE DENITRIFICATION GENES

Denitrification enzymes are induced under anaerobic or low oxygen conditions in the presence of nitrate or nitrite (Arai et al., 1991a,b; **Figure 3**). Two transcriptional regulators, ANR and DNR (dissimilatory nitrate respiration regulator), are required for full expression of all denitrification genes (Arai et al., 1994, 1995b, 1997, 1999, 2003; Ye et al., 1995; Schreiber et al., 2007). NarXL regulates some of the denitrification promoters, such as *narK1*, *nirQ*, and *dnr* (Schreiber et al., 2007). Denitrification is also regulated by quorum-sensing signal molecules (Yoon et al., 2002; Toyofuku et al., 2007, 2008).

The *dnr* gene (PA0527) encoding DNR is clustered with the *nir–nor* genes for nitrite reductase and NO reductase. Both ANR and DNR belong to the CRP/FNR superfamily of transcriptional regulators and activate a synthetic promoter that has a consensus FNR-binding motif (TTGAT----ATCAA; Hasegawa et al., 1998). However, the promoters of the denitrification genes, which have sequences similar to the FNR-binding motif, are activated only by DNR, but not by ANR. Expression of DNR is under the control of ANR. Thus, the ANR-mediated anaerobic induction of the denitrification genes is an indirect event that occurs by way of DNR (Arai et al., 1997). The *hemN*, *hemF*, and *narK1* promoters are known to be recognized by both ANR and DNR (Rompf et al., 1998; Schreiber et al., 2007). It is not certain how ANR and DNR distinguish their target promoters. Transcriptome analysis of the ANR and DNR regulons revealed that DNR specifically regulates the denitrification genes (Trunk et al., 2010). DNR specifically senses NO and does not respond to CO (Arai et al., 2003). Heme is required for the *in vivo* activity of DNR (Castiglione et al., 2009). The crystal structures of the apo-forms of full-length and truncated DNR have been solved recently (Giardina et al., 2008, 2009, 2011). The structural analyses showed that DNR undergoes a very large conformation rearrangement on activation.

ANAEROBIC FERMENTATION

Pseudomonas aeruginosa is able to grow in the absence of oxygen and nitrogen oxides, although very slowly, by using arginine as an energy source in rich medium (Shoesmith and Sherris, 1960; Vander Wauven et al., 1984). Anaerobic degradation of arginine to ornithine through the arginine deiminase (ADI) pathway is a non-redox process but produces 1 mol of ATP per arginine by a substrate-level phosphorylation. The ADI pathway enzymes are encoded in the *arcDABC* operon (PA5170–5173; Lüthi et al., 1990). The *arcABC* genes encode arginine deiminase, catabolic ornithine carbamoyltransferase, and carbamate kinase, respectively. *arcD* encodes an arginine-ornithine antiporter. The *arc* genes are induced by ANR in response to oxygen depletion (Gamper et al., 1991). An arginine-responsive regulator, ArgR, which is involved in the regulation of many arginine metabolic pathways, enhances the *arcD* promoter activity (Lu et al., 1999). The expression of the *arc* genes is partially repressed by NarXL in the presence of nitrate, indicating that the more energetically efficient denitrification is preferred over arginine fermentation (Benkert et al., 2008).

Pyruvate fermentation allows long-term anaerobic survival of *P. aeruginosa* under stationary conditions, although it does not sustain significant anaerobic growth (Eschbach et al., 2004). Acetate kinase and phosphotransacetylase encoded by the *ackA-pta* operon (PA0835–0836) and NADH-dependent lactate dehydrogenase encoded by *ldhA* (PA0927) are responsible for pyruvate fermentation. ATP is produced by the acetate kinase activity. Lactate dehydrogenase functions for reoxidation of NADH produced by the pyruvate dehydrogenase activity. ANR and the integration host factor (IHF) are required for the expression of the *ackA-pts* operon (Eschbach et al., 2004).

ROLE OF PYOCYANIN IN ANAEROBIC SURVIVAL

Pseudomonas aeruginosa produces a small redox-active phenazine compound, pyocyanin. Pyocyanin generates reactive oxygen species and acts as an antibiotic agent in the soil or a virulence factor during infection. Recently, Newman's group proposed that pyocyanin has additional roles for maintenance of redox homeostasis and control of multicellular behavior (Price-Whelan et al., 2007; Dietrich et al., 2008; Ramos et al., 2010; Wang et al., 2010; **Figure 4**). Pyocyanin decreases the intracellular NADH levels under energy starvation conditions, indicating that the reoxidation of NADH could be coupled to the reduction of pyocyanin. Because pyocyanin is auto-oxidized by oxygen, it could function as an extracellular electron shuttle. When the *P. aeruginosa* cells form a biofilm or colony, a steep oxygen gradient is present in the cell community. The cells at the bottom are limited for oxygen but pyocyanin could serve as an alternative electron acceptor. The reduced pyocyanin could be reoxidized after diffusion to the oxygen-rich surface. Thus, pyocyanin contributes to the redox homeostasis and survival of the cells in the deeper anaerobic niches. Further experimental evidence would be required to certify this hypothesis. Pyocyanin is also reported to stimulate pyruvate excretion by decreasing the carbon flux through the central metabolic pathway in *P. aeruginosa* PA14 at the late stationary phase (Price-Whelan et al., 2007). The pyruvate secreted by the cells at the aerobic niches, such as the surfaces of biofilms, might support the survival of the cells at anaerobic niches of the community as a substrate of anaerobic pyruvate fermentation.

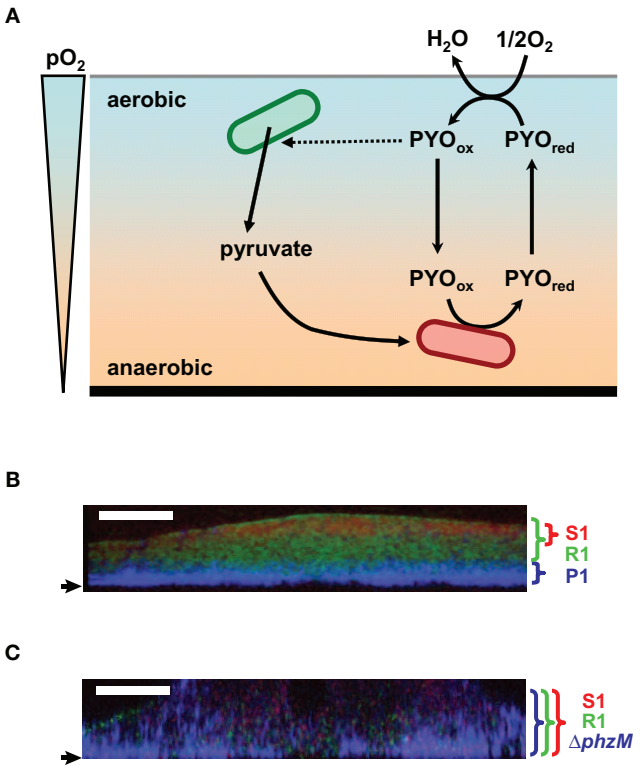

FIGURE 4 | Hypothetical model of the role of pyocyanin in anaerobic survival in biofilm and construction of the multilayered structure of the multispecies biofilm. (A) Pyocyanin is predicted to act as an electron acceptor for the anaerobic cells and shuttle electrons between anaerobic and aerobic niches. Pyocyanin stimulates excretion of pyruvate. The secreted pyruvate is expected to be utilized for the anaerobic pyruvate fermentation. PYO_{red} and PYO_{ox} indicate the reduced- and oxidized-forms of pyocyanin, respectively. pO_2 indicates partial oxygen pressure. **(B,C)** FISH images of three-species biofilms in vertical sections. A pyocyanin-overproducing *P. aeruginosa* strains P1 (P1) and its pyocyanin-non-producing derivative (Δ*phzM*) appear blue with a Cy3-labeled probe, A pyocyanin-resistant *Raoultella* strain (R1) and a pyocyanin-sensitive *Brevibacillus* strain (S1) appear green and red with the FITC- and Cy5-labeled probes, respectively. Strain P1 forms a multilayered biofilm with strains R1 and S1 **(B)**. An intermingled biofilm is formed when strain Δ*phzM* is used **(C)**. Small arrows indicate the bottom of biofilms. White bars indicate 50 μm.

Another example of the relationship between pyocyanin and biofilm lifestyle has been reported in a mixed-species biofilm (Narisawa et al., 2008; **Figures 4B,C**). The pyocyanin-overproducing *P. aeruginosa* strain P1 forms a multilayered multispecies biofilm when cocultured with a pyocyanin-resistant *Raoultella* strain R1 and a pyocyanin-sensitive *Brevibacillus* strain S1. The layer of strain P1 at the bottom was covered with the layers of resistant and sensitive strains. The sensitive strain was separated from strain P1 by the layer of the resistant strain. This multilayered structure is expected to be advantageous for strain P1 for protection from antibiotics, grazing, desiccation, and many other stresses, although the bottom of the biofilm is disadvantageous for oxygen acquisition. A pyocyanin non-producing derivative of strain P1 forms an intermingled multispecies biofilm with the pyocyanin-resistant and -sensitive strains. *P. aeruginosa* PAO1, which poorly produces pyocyanin under anaerobic conditions, does not construct the multilayered biofilm. These results indicate that pyocyanin has the ability to construct the organized layered structure in the multispecies biofilm. Pyocyanin might also serve as an electron acceptor for the anaerobic preservation of the *P. aeruginosa* cells at the oxygen-depleted bottom of the multilayered biofilm.

CONCLUSIONS AND FUTURE PERSPECTIVES

The respiratory chain of *P. aeruginosa* is highly branched and terminated either by multiple terminal oxidases of different characteristics or by the denitrification enzymes that reduce nitrogen oxides. This versatile respiratory function as well as fermentative energy generating systems contribute to the ubiquitous distribution and persistence of *P. aeruginosa* in various environments under both aerobic and anaerobic conditions. One of the characteristic features of the respiratory chain of *P. aeruginosa* is that high affinity terminal oxidases of the cbb_3-type are dominant even under aerobic conditions. *P. aeruginosa* seems to actively produce microaerobic environments by itself. The microaerobic and anaerobic physiology of *P. aeruginosa* is significantly related to its pathogenicity. All terminal oxidases for aerobic respiration are directly or indirectly regulated by RoxSR, which is predicted to sense the redox status of the respiratory chain either by the redox status of the ubiquinone pool or by the electron flow through the terminal oxidases. Because ubiquinone is located at the pivotal point of the divergent electron transport chain of both aerobic and anaerobic respiration (**Figure 1**), its redox status must be important for the traffic control of the electron flow. The oxygen sensing global regulator ANR directly regulates a subset of the terminal oxidases and indirectly induces all denitrification enzymes. The NO-sensing regulator DNR directly regulates the expression of all denitrification enzymes. Thus, the respiratory function of *P. aeruginosa* is efficiently controlled by oxygen, NO, and the redox status of the respiratory chain.

In recent years, bacteria-specific energy metabolism has been garnering increasing attention as a therapeutic target (Hurdle et al., 2011). Most of the classical antibiotics target the bioprocesses of actively growing bacteria, such as biosynthesis of proteins, DNA, and peptidoglycan. However, these antibiotics are not effective for eradicating persistent infections, in which most of the bacterial cells are under slow-growing and/or non-growing conditions. Because maintenance of the cellular energy and redox homeostasis is necessary even for the non-growing cells to maintain viability, inhibition of the energy metabolism is expected to be effective to kill the persisting cells. The problem is the versatility of the bacterial respiratory chains when considering the respiratory components as drug targets. Actually, inactivation of a subset of the multiple terminal oxidases of *P. aeruginosa* has no or only minor effect. In contrast to the rich variety of the enzymes of energy metabolism, the regulatory machinery for the energy metabolism is less variable, with many bacterial species sharing common regulatory systems. Therefore, the regulatory systems might be worth considering as a possible drug target.

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REFERENCES

Alvarez-Ortega, C., and Harwood, C. S. (2007). Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Mol. Microbiol.* 65, 153–165.

Arai, H., Hayashi, M., Kuroi, A., Ishii, M., and Igarashi, Y. (2005). Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of *Pseudomonas aeruginosa*. *J. Bacteriol.* 187, 3960–3968.

Arai, H., Igarashi, Y., and Kodama, T. (1991a). Anaerobically induced expression of the nitrite reductase cytochrome c-551 operon from *Pseudomonas aeruginosa*. *FEBS Lett.* 280, 351–353.

Arai, H., Igarashi, Y., and Kodama, T. (1991b). Nitrite activates the transcription of the *Pseudomonas aeruginosa* nitrite reductase and cytochrome c-551 operon under anaerobic conditions. *FEBS Lett.* 288, 227–228.

Arai, H., Igarashi, Y., and Kodama, T. (1994). Structure and ANR-dependent transcription of the nir genes for denitrification from *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.* 58, 1286–1291.

Arai, H., Igarashi, Y., and Kodama, T. (1995a). The structural genes for nitric oxide reductase from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta* 1261, 279–284.

Arai, H., Igarashi, Y., and Kodama, T. (1995b). Expression of the nir and nor genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. *FEBS Lett.* 371, 73–76.

Arai, H., Kawasaki, S., Igarashi, Y., and Kodama, T. (1996). "Arrangement and transcriptional regulation of the denitrification genes in *Pseudomonas aeruginosa*," in *Molecular Biology of Pseudomonads*, eds T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (Washington DC: ASM Press), 298–308.

Arai, H., Kodama, T., and Igarashi, Y. (1997). Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 25, 1141–1148.

Arai, H., Kodama, T., and Igarashi, Y. (1998). The role of the nirQOP genes in energy conservation during anaerobic growth of *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.* 62, 1995–1999.

Arai, H., Kodama, T., and Igarashi, Y. (1999). Effect of nitrogen oxides on expression of the nir and nor genes for denitrification in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* 170, 19–24.

Arai, H., Mizutani, M., and Igarashi, Y. (2003). Transcriptional regulation of the nos genes for nitrous oxide reductase in *Pseudomonas aeruginosa*. *Microbiology* 149, 29–36.

Arai, H., Roh, J. H., and Kaplan, S. (2008). Transcriptome dynamics during the transition from anaerobic photosynthesis to aerobic respiration in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.* 190, 286–299.

Arai, H., Sanbongi, Y., Igarashi, Y., and Kodama, T. (1990). Cloning and sequencing of the gene encoding cytochrome *c*-551 from *Pseudomonas aeruginosa*. *FEBS Lett.* 261, 196–198.

Attila, C., Ueda, A., and Wood, T. K. (2008). PA2663 (PpyR) increases biofilm formation in *Pseudomonas aeruginosa* PAO1 through the psl operon and stimulates virulence and quorum-sensing phenotypes. *Appl. Microbiol. Biotechnol.* 78, 293–307.

Bekker, M., de Vries, S., Ter Beek, A., Hellingwerf, K. J., and Teixeira de Mattos, M. J. (2009). Respiration of *Escherichia coli* can be fully uncoupled via the nonelectrogenic terminal cytochrome bd-II oxidase. *J. Bacteriol.* 191, 5510–5517.

Benkert, B., Quäck, N., Schreiber, K., Jaensch, L., Jahn, D., and Schobert, M. (2008). Nitrate-responsive NarX-NarL represses arginine-mediated induction of the *Pseudomonas aeruginosa* arginine fermentation arcDABC operon. *Microbiology* 154, 3053–3060.

Berks, B. C., Ferguson, S. J., Moir, J. W. B., and Richardson, D. J. (1995). Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta* 1232, 97–173.

Blumer, C., and Haas, D. (2000). Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Arch. Microbiol.* 173, 170–177.

Bosma, G., Braster, M., Stouthamer, A. H., and van Verseveld, H. W. (1987). Isolation and characterization of ubiquinol oxidase complexes from *Paracoccus denitrificans* cells cultured under various limiting growth conditions in the chemostat. *Eur. J. Biochem.* 165, 657–663.

Büsch, A., Friedrich, B., and Cramm, R. (2002). Characterization of the norB gene, encoding nitric oxide reductase, in the nondenitrifying cyanobacterium *Synechocystis* sp. strain PCC6803. *Appl. Environ. Microbiol.* 68, 668–672.

Buschmann, S., Warkentin, E., Xie, H., Langer, J. D., Ermler, U., and Michel, H. (2010). The structure of cbb3 cytochrome oxidase provides insights into proton pumping. *Science* 329, 327–330.

Castiglione, N., Rinaldo, S., Giardina, G., and Cutruzzolà, F. (2009). The transcription factor DNR from *Pseudomonas aeruginosa* specifically requires nitric oxide and haem for the activation of a target promoter in *Escherichia coli*. *Microbiology* 155, 2838–2844.

Comolli, J. C., and Donohue, T. J. (2002). *Pseudomonas aeruginosa* RoxR, a response regulator related to *Rhodobacter sphaeroides* PrrA, activates expression of the cyanide-insensitive terminal oxidase. *Mol. Microbiol.* 45, 755–768.

Comolli, J. C., and Donohue, T. J. (2004). Differences in two *Pseudomonas aeruginosa* cbb3 cytochrome oxidases. *Mol. Microbiol.* 51, 1193–1203.

Cooper, M., Tavankar, G. R., and Williams, H. D. (2003). Regulation of expression of the cyanide-insensitive terminal oxidase in *Pseudomonas aeruginosa*. *Microbiology* 149, 1275–1284.

Cotter, P. A., Chepuri, V., Geniss, R. B., and Gunsalus, R. P. (1990). Cytochrome o (cyoABCDE) and d (cydAB) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the fnr gene product. *J. Bacteriol.* 172, 6333–6338.

Cramm, R., Pohlmann, A., and Friedrich, B. (1999). Purification and characterization of the single-component nitric oxide reductase from *Ralstonia eutropha* H16. *FEBS Lett.* 460, 6–10.

Cunningham, L., Pitt, M., and Williams, H. D. (1997). The cioAB genes from *Pseudomonas aeruginosa* code for a novel cyanide-insensitive terminal oxidase related to the cytochrome bd quinol oxidases. *Mol. Microbiol.* 24, 579–591.

Cunningham, L., and Williams, H. D. (1995). Isolation and characterization of mutants defective in the cyanide-insensitive respiratory pathway of *Pseudomonas aeruginosa*. *J. Bacteriol.* 177, 432–438.

D'Autréaux, B., Touati, D., Bersch, B., Latour, J.-M., and Michaud-Soret, I. (2002). Direct inhibition by nitric oxide of the transcriptional ferric uptake regulation protein via nitrosylation of the iron. *Proc. Natl. Acad. Sci. U.S.A.* 99, 16619–16624.

Dietrich, L. E. P., Teal, T. K., Price-Whelan, A., and Newman, D. K. (2008). Redox-active antibiotics control gene expression and community behavior in divergent bacteria. *Science* 321, 1203–1206.

Dinamarca, M. A., Ruiz-Manzano, A., and Rojo, F. (2002). Inactivation of cytochrome o ubiquinol oxidase relieves catabolic repression of the *Pseudomonas putida* GPo1 alkane degradation pathway. *J. Bacteriol.* 184, 3785–3793.

D'Mello, R., Hill, S., and Poole, R. K. (1995). The oxygen affinity of cytochrome bo' in *Escherichia coli* determined by the deoxygenation of oxyleghemoglobin and oxymyoglobin: Km values for oxygen are in the submicromolar range. *J. Bacteriol.* 177, 867–870.

D'mello, R., Hill, S., and Poole, R. K. (1996). The cytochrome bd quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity in vivo by oxygen inhibition. *Microbiology* 142, 755–763.

Dubbs, J. M., and Tabita, F. R. (2004). Regulators of nonsulfur purple phototrophic bacteria and the interactive control of CO2 assimilation, nitrogen fixation, hydrogen metabolism and energy generation. *FEMS Microbiol. Rev.* 28, 353–376.

Elsen, S., Swem, L. R., Swem, D. L., and Bauer, C. E. (2004). RegB/RegA, a highly conserved redox-responding global two-component regulatory system. *Microbiol. Mol. Biol. Rev.* 68, 263–279.

Eraso, J. M., Roh, J. H., Zeng, X., Callister, S. J., Lipton, M. S., and Kaplan, S. (2008). Role of the global transcriptional regulator PrrA in *Rhodobacter sphaeroides* 2.4.1: combined transcriptome and proteome analysis. *J. Bacteriol.* 190, 4831–4848.

Eschbach, M., Schreiber, K., Trunk, K., Buer, J., Jahn, D., and Schobert, M. (2004). Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. *J. Bacteriol.* 186, 4596–4604.

Fernández-Piñar, R., Ramos, J. L., Rodríguez-Herva, J. J., and Espinosa-Urgel, M. (2008). A two-component regulatory system integrates redox state and population density sensing in *Pseudomonas putida*. *J. Bacteriol.* 190, 7666–7674.

Flory, J. E., and Donohue, T. J. (1997). Transcriptional control of several aerobically induced cytochrome structural genes in *Rhodobacter sphaeroides*. *Microbiology* 143, 3101–3110.

Frangipani, E., and Haas, D. (2009). Copper acquisition by the SenC protein regulates aerobic respiration in *Pseudomonas aeruginosa* PAO1. *FEMS Microbiol. Lett.* 298, 234–240.

Frangipani, E., Slaveykova, V. I., Reimmann, C., and Haas, D. (2008). Adaptation of aerobically growing *Pseudomonas aeruginosa* to copper starvation. *J. Bacteriol.* 190, 6706–6717.

Fujiwara, T., Fukumori, Y., and Yamanaka, T. (1992). A novel terminal oxidase, cytochrome baa3 purified from aerobically grown *Pseudomonas aeruginosa*: it shows a clear difference between resting state and pulsed state. *J. Biochem.* 112, 290–298.

Gabel, C., and Maier, R. J. (1993). Oxygen-dependent transcriptional regulation of cytochrome aa3 in *Bradyrhizobium japonicum*. *J. Bacteriol.* 175, 128–132.

Galimand, M., Gamper, M., Zimmermann, A., and Haas, D. (1991). Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. *J. Bacteriol.* 173, 1598–1606.

Gallagher, L. A., and Manoil, C. (2001). *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J. Bacteriol.* 183, 6207–6214.

Gamper, M., Zimmermann, A., and Haas, D. (1991). Anaerobic regulation of transcription initiation in the arcDABC operon of *Pseudomonas aeruginosa*. *J. Bacteriol.* 173, 4742–4250.

Gavira, M., Roldán, M. D., Castillo, F., and Moreno-Vivián, C. (2002). Regulation of nap gene expression and periplasmic nitrate reductase activity in the phototrophic bacterium *Rhodobacter sphaeroides* DSM158. *J. Bacteriol.* 184, 1693–1702.

Giardina, G., Castiglione, N., Caruso, M., Cutruzzolà, F., and Rinaldo, S. (2011). The *Pseudomonas aeruginosa* DNR transcription factor: light and shade of nitric oxide-sensing mechanisms. *Biochem. Soc. Trans.* 39, 294–298.

Giardina, G., Rinaldo, S., Castiglione, N., Caruso, M., and Cutruzzolà, F. (2009). A dramatic conformational rearrangement is necessary for the activation of DNR from *Pseudomonas aeruginosa*. Crystal structure of wild-type DNR. *Proteins* 77, 174–180.

Giardina, G., Rinaldo, S., Johnson, K. A., Di Matteo, A., Brunori, M., and Cutruzzolà, F. (2008). NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. *J. Mol. Biol.* 378, 1002–1015.

Grammel, H., and Ghosh, R. (2008). Redox-state dynamics of ubiquinone-10 imply cooperative regulation of photosynthetic membrane expression in *Rhodospirillum rubrum*. *J. Bacteriol.* 190, 4912–4921.

Hasegawa, N., Arai, H., and Igarashi, Y. (1998). Activation of a consensus FNR-dependent promoter by DNR of *Pseudomonas aeruginosa* in response to nitrite. *FEMS Microbiol. Lett.* 166, 213–217.

Hasegawa, N., Arai, H., and Igarashi, Y. (2001). Two c-type cytochromes, NirM and NirC, encoded in the nir gene cluster of *Pseudomonas aeruginosa* act as electron donors for nitrite reductase. *Biochem. Biophys. Res. Commun.* 288, 1223–1230.

Hasegawa, N., Arai, H., and Igarashi, Y. (2003). Need for cytochrome bc1 complex for dissimilatory nitrite reduction of *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.* 67, 121–126.

Hassett, D. J. (1996). Anaerobic production of alginate by *Pseudomonas aeruginosa*: alginate restricts diffusion of oxygen. *J. Bacteriol.* 178, 7322–7325.

Hendriks, J., Oubrie, A., Castresana, J., Urbani, A., Gemeinhardt, S., and Saraste, M. (2000). Nitric oxide reductases in bacteria. *Biochim. Biophys. Acta* 1459, 266–273.

Hino, T., Matsumoto, Y., Nagano, S., Sugimoto, H., Fukumori, Y., Murata, T., Iwata, S., and Shiro, Y. (2010). Structural basis of biological N_2O generation by bacterial nitric oxide reductase. *Science* 330, 1666–1670.

Householder, T. C., Fozo, E. M., Cardinale, J. A., and Clark, V. L. (2000). Gonococcal nitric oxide reductase is encoded by a single gene, norB, which is required for anaerobic growth and is induced by nitric oxide. *Infect. Immun.* 68, 5241–5246.

Hurdle, J. G., O'Neill, A. J., Chopra, I., and Lee, R. E. (2011). Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat. Rev. Microbiol.* 9, 62–75.

Jackson, R. J., Elvers, K. T., Lee, L. J., Gidley, M. D., Wainwright, L. M., Lightfoot, J., Park, S. F., and Poole, R. K. (2007). Oxygen reactivity of both respiratory oxidases in *Campylobacter jejuni*: the cydAB genes encode a cyanide-resistant, low-affinity oxidase that is not of the cytochrome bd type. *J. Bacteriol.* 189, 1604–1615.

Jørgensen, F., Bally, M., Chapon-Herve, V., Michel, G., Lazdunski, A., Williams, P., and Stewart, G. S. A. B. (1999). RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. *Microbiology* 145, 835–844.

Jünemann, S. (1997). Cytochrome bd terminal oxidase. *Biochim. Biophys. Acta* 1321, 107–127.

Jüngst, A., and Zumft, W. G. (1992). Interdependence of respiratory NO reduction and nitrite reduction revealed by mutagenesis of nirQ, a novel gene in the denitrification gene cluster of *Pseudomonas stutzeri*. *FEBS Lett.* 314, 308–314.

Kakishima, K., Shiratsuchi, A., Taoka, A., Nakanishi, Y., and Fukumori, Y. (2007). Participation of nitric oxide reductase in survival of *Pseudomonas aeruginosa* in LPS-activated macrophages. *Biochem. Biophys. Res. Commun.* 355, 587–591.

Kawakami, T., Kuroki, M., Ishii, M., Igarashi, Y., and Arai, H. (2010). Differential expression of multiple terminal oxidases for aerobic respiration in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 12, 1399–1412.

Kawasaki, S., Arai, H., Igarashi, Y., and Kodama, T. (1995). Sequencing and characterization of the downstream region of the genes encoding nitrite reductase and cytochrome c-551 (nirSM) from *Pseudomonas aeruginosa*: identification of the gene necessary for biosynthesis of heme d1. *Gene* 167, 87–91.

Kawasaki, S., Arai, H., Kodama, T., and Igarashi, Y. (1997). Gene cluster for dissimilatory nitrite reductase (nir) from *Pseudomonas aeruginosa*: sequencing and identification of a locus for heme d1 biosynthesis. *J. Bacteriol.* 179, 235–242.

Kelly, M. J. S., Poole, R. K., Yates, M. G., and Kennedy, C. (1990). Cloning and mutagenesis of genes encoding the cytochrome bd terminal oxidase complex in *Azotobacter vinelandii*: mutants deficient in the cytochrome d complex are unable to fix nitrogen in air. *J. Bacteriol.* 172, 6010–6019.

Kiley, P. J., and Beinert, H. (1998). Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. *FEMS Microbiol. Rev.* 22, 341–352.

Kim, Y.-J., Ko, I.-J., Lee, J.-M., Kang, H.-Y., Kim, Y. M., Kaplan, S., and Oh, J.-I. (2007). Dominant role of the cbb3 oxidase in regulation of photosynthesis gene expression through the PrrBA system in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.* 189, 5617–5625.

Kumita, H., Matsuura, K., Hino, T., Takahashi, S., Hori, H., Fukumori, Y., Morishima, I., and Shiro, Y. (2004). NO reduction by nitric-oxide reductase from denitrifying bacterium *Pseudomonas aeruginosa*: characterization of reaction intermediates that appear in the single turnover cycle. *J. Biol. Chem.* 279, 55247–55254.

Lu, C. D., Winteler, H., Abdelal, A., and Haas, D. (1999). The ArgR regulatory protein, a helper to the anaerobic regulator ANR during transcriptional activation of the arcD promoter in *Pseudomonas aeruginosa*. *J. Bacteriol.* 181, 2459–2464.

Lüthi, E., Baur, H., Gamper, M., Brunner, F., Villeval, D., Mercenier, A., and Haas, D. (1990). The arc operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa* contains an additional gene, arcD, encoding a membrane protein. *Gene* 87, 37–43.

Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* 15, 194–222.

Matsushita, K., Shinagawa, E., Adachi, O., and Ameyama, M. (1982). o-type cytochrome oxidase in the membrane of aerobically grown *Pseudomonas aeruginosa*. *FEBS Lett.* 139, 255–258.

Matsushita, K., Yamada, M., Shinagawa, E., Adachi, O., and Ameyama, M. (1983). Membrane-bound respiratory chain of *Pseudomonas aeruginosa* grown aerobically. A KCN-insensitive alternate oxidase chain and its energetics. *J. Biochem.* 93, 1137–1144.

Mogi, T., Ano, Y., Nakatsuka, T., Toyama, H., Muroi, A., Miyoshi, H., Migita, C. T., Ui, H., Shiomi, K., Omura, S., Kita, K., and Matsushita, K. (2009). Biochemical and spectroscopic properties of cyanide-insensitive quinol oxidase from *Gluconobacter oxydans*. *J. Biochem.* 146, 263–271.

Mogi, T., Saiki, K., and Anraku, Y. (1994). Biosynthesis and functional role of haem O and haem A. *Mol. Microbiol.* 14, 391–398.

Morales, G., Ugidos, A., and Rojo, F. (2006). Inactivation of the *Pseudomonas putida* cytochrome o ubiquinol oxidase leads to a significant change in the transcriptome and to increased expression of the CIO and cbb3-1 terminal oxidases. *Environ. Microbiol.* 8, 1764–1774.

Mouncey, N. J., and Kaplan, S. (1998). Oxygen regulation of the ccoN gene encoding a component of the cbb3 oxidase in *Rhodobacter sphaeroides* 2.4.1T: involvement of the FnrL protein. *J. Bacteriol.* 180, 2228–2231.

Nagata, K., Tsukita, S., Tamura, T., and Sone, N. (1996). A cb-type cytochrome-c oxidase terminates the respiratory chain in *Helicobacter pylori*. *Microbiology* 142, 1757–1763.

Nakamura, H., Saiki, K., Mogi, T., and Anraku, Y. (1997). Assignment and functional roles of the cyoABCDE gene products required for the *Escherichia coli* bo-type quinol oxidase. *J. Biochem.* 122, 415–421.

Narisawa, N., Haruta, S., Arai, H., Ishii, M., and Igarashi, Y. (2008). Coexistence of antibiotic-producing and antibiotic-sensitive bacteria in biofilms is mediated by resistant bacteria. *Appl. Environ. Microbiol.* 74, 3887–3894.

Nordling, M., Young, S., Karlsson, B. G., and Lundberg, L. G. (1990). The structural gene for cytochrome c551 from *Pseudomonas aeruginosa*. The nucleotide sequence shows a location downstream of the nitrite reductase gene. *FEBS Lett.* 259, 230–232.

Ochsner, U. A., Wilderman, P. J., Vasil, A. I., and Vasil, M. L. (2002). GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel

pyoverdine biosynthesis genes. *Mol. Microbiol.* 45, 1277–1287.

O'Gara, J. P., Eraso, J. M., and Kaplan, S. (1998). A redox-responsive pathway for aerobic regulation of photosynthesis gene expression in *Rhodobacter sphaeroides* 2.4.1. *J. Bacteriol.* 180, 4044–4050.

Oh, J. I., and Kaplan, S. (1999). The cbb3 terminal oxidase of *Rhodobacter sphaeroides* 2.4.1: structural and functional implications for the regulation of spectral complex formation. *Biochemistry* 38, 2688–2696.

Oh, J. I., and Kaplan, S. (2000). Redox signaling: globalization of gene expression. *EMBO J.* 19, 4237–4247.

Oh, J. I., and Kaplan, S. (2002). Oxygen adaptation – The role of the CcoQ subunit of the cbb3 cytochrome c oxidase of *Rhodobacter sphaeroides* 2.4.1. *J. Biol. Chem.* 277, 16220–16228.

Oh, J. I., Ko, I. J., and Kaplan, S. (2004). Reconstitution of the *Rhodobacter sphaeroides* cbb3-PrrBA signal transduction pathway in vitro. *Biochemistry* 43, 7915–7923.

Otten, M. F., Stork, D. M., Reijnders, W. N., Westerhoff, H. V., and Van Spanning, R. J. M. (2001). Regulation of expression of terminal oxidases in *Paracoccus denitrificans*. *Eur. J. Biochem.* 268, 2486–2497.

Palmer, K. L., Brown, S. A., and Whiteley, M. (2007). Membrane-bound nitrate reductase is required for anaerobic growth in cystic fibrosis sputum. *J. Bacteriol.* 189, 4449–4455.

Peters, A., Kulajta, C., Pawlik, G., Daldal, F., and Koch, H.-G. (2008). Stability of the cbb3-type cytochrome oxidase requires specific CcoQ–CcoP interactions. *J. Bacteriol.* 190, 5576–5586.

Petruschka, L., Burchhardt, G., Müller, C., Weihe, C., and Herrmann, H. (2001). The cyo operon of *Pseudomonas putida* is involved in catabolite repression of phenol degradation. *Mol. Genet. Genomics* 266, 199–206.

Philippot, L., and Højberg, O. (1999). Dissimilatory nitrate reductases in bacteria. *Biochim. Biophys. Acta* 1446, 1–23.

Pitcher, R. S., and Watmough, N. J. (2004). The bacterial cytochrome cbb3 oxidases. *Biochim. Biophys. Acta* 1655, 388–399.

Poole, R. K., and Hill, S. (1997). Respiratory protection of nitrogenase activity in *Azotobacter vinelandii* – roles of the terminal oxidases. *Biosci. Rep.* 17, 303–317.

Poole, R. K., and Hughes, M. N. (2000). New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Mol. Microbiol.* 36, 775–783.

Preisig, O., Anthamatten, D., and Hennecke, H. (1993). Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 90, 3309–3313.

Preisig, O., Zufferey, R., Thöny-Meyer, L., Appleby, C. A., and Hennecke, H. (1996). A high-affinity cbb3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. *J. Bacteriol.* 178, 1532–1538.

Price-Whelan, A., Dietrich, L. E. P., and Newman, D. K. (2007). Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* 189, 6372–6381.

Puustinen, A., Finel, M., Virkki, M., and Wikström, M. (1989). Cytochrome o (bo) is a proton pump in *Paracoccus denitrificans* and *Escherichia coli*. *FEBS Lett.* 249, 163–167.

Ramos, I., Dietrich, L. E. P., Price-Whelan, A., and Newman, D. K. (2010). Phenazines affect biofilm formation by *Pseudomonas aeruginosa* in similar ways at various scales. *Res. Microbiol.* 161, 187–191.

Ray, A., and Williams, H. D. (1997). The effects of mutation of the anr gene on the aerobic respiratory chain of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* 156, 227–232.

Rice, C. W., and Hempfling, W. P. (1978). Oxygen-limited continuous culture and respiratory energy conservation in *Escherichia coli*. *J. Bacteriol.* 134, 115–124.

Rompf, A., Hungerer, C., Hoffman, T., Lindenmeyer, M., Römling, U., Groβ, U., Doss, M. O., Arai, H., Igarashi, Y., and Jahn, D. (1998). Regulation of *Pseudomonas aeruginosa* hemF and hemN by the dual action of the redox response regulators Anr and Dnr. *Mol. Microbiol.* 29, 985–997.

Sabra, W., Kim, E.-J., and Zeng, A.-P. (2002). Physiological responses of *Pseudomonas aeruginosa* PAO1 to oxidative stress in controlled microaerobic and aerobic cultures. *Microbiology* 148, 3195–3202.

Sabra, W., Zeng, A.-P., Lünsdorf, H., and Deckwer, W.-D. (2000). Effect of oxygen on formation and structure of *Azotobacter vinelandii* alginate and its role in protecting nitrogenase. *Appl. Environ. Microbiol.* 66, 4037–4044.

Saiki, K., Mogi, T., and Anraku, Y. (1992). Heme O biosynthesis in *Escherichia coli*: the cyoE gene in the cytochrome bo operon encodes a protoheme IX farnesyltransferase. *Biochem. Biophys. Res. Commun.* 189, 1491–1497.

Saiki, K., Nakamura, H., Mogi, T., and Anraku, Y. (1996). Probing a role of subunit IV of the *Escherichia coli* botype ubiquinol oxidase by deletion and cross-linking analyses. *J. Biol. Chem.* 271, 15336–15340.

Saraste, M. (1994). Structure and evolution of cytochrome oxidase. *Antonie Van Leeuwenhoek* 65, 285–287.

Saraste, M., and Castresana, J. (1994). Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Lett.* 341, 1–4.

Sawers, R. G. (1991). Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli*. *Mol. Microbiol.* 5, 1469–1481.

Schobert, M., and Jahn, D. (2010). Anaerobic physiology of *Pseudomonas aeruginosa* in the cystic fibrosis lung. *Int. J. Med. Microbiol.* 300, 549–556.

Schobert, M., and Tielen, P. (2010). Contribution of oxygen-limiting conditions to persistent infection of *Pseudomonas aeruginosa*. *Future Microbiol.* 5, 603–621.

Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M., and Jahn, D. (2007). The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J. Bacteriol.* 189, 4310–4314.

Schurek, K. N., Marr, A. K., Taylor, P. K., Wiegand, I., Semenec, L., Khaira, B. K., and Hancock, R. E. W. (2008). Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 52, 4213–4219.

Schuster, M., Hawkins, A. C., Harwood, C. S., and Greenberg, E. P. (2004). The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol. Microbiol.* 51, 973–985.

Sharma, V., Noriega, C. E., and Rowe, J. J. (2006). Involvement of NarK1 and NarK2 proteins in transport of nitrate and nitrite in the denitrifying bacterium *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* 72, 695–701.

Shoesmith, J. H., and Sherris, J. C. (1960). Studies on the mechanism of arginine-activated motility in a *Pseudomonas* strain. *J. Gen. Microbiol.* 22, 10–24.

Silvestrini, M. C., Falcinelli, S., Ciabatti, I., Cutruzzolà, F., and Brunori, M. (1994). *Pseudomonas aeruginosa* nitrite reductase (or cytochrome oxidase): an overview. *Biochimie* 76, 641–654.

Silvestrini, M. C., Galeotti, C. L., Gervais, M., Schininà, E., Barra, D., Bossa, F., and Brunori, M. (1989). Nitrite reductase from *Pseudomonas aeruginosa*: sequence of the gene and the protein. *FEBS Lett.* 254, 33–38.

SooHoo, C. K., and Hollocher, T. C. (1990). Loss of nitrous oxide reductase in *Pseudomonas aeruginosa* cultured under N_2O as determined by rocket immunoelectrophoresis. *Appl. Environ. Microbiol.* 56, 3591–3592.

SooHoo, C. K., and Hollocher, T. C. (1991). Purification and characterization of nitrous oxide reductase from *Pseudomonas aeruginosa* strain P2. *J. Biol. Chem.* 266, 2203–2209.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K.-S., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E. W., Lory, S., and Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 406, 959–964.

Suh, S.-J., Silo-Suh, L., Woods, D. E., Hassett, D. J., West, S. E. H., and Ohman, D. E. (1999). Effect of rpoS mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J. Bacteriol.* 181, 3890–3897.

Swem, D. L., and Bauer, C. E. (2002). Coordination of ubiquinol oxidase and cytochrome cbb3 oxidase expression by multiple regulators in *Rhodobacter capsulatus*. *J. Bacteriol.* 184, 2815–2820.

Thöny-Meyer, L. (1997). Biogenesis of respiratory cytochromes in bacteria. *Microbiol. Mol. Biol. Rev.* 61, 337–376.

Toyofuku, M., Nomura, N., Fujii, T., Takaya, N., Maseda, H., Sawada, I., Nakajima, T., and Uchiyama, H. (2007). Quorum sensing regulates denitrification in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 189, 4969–4972.

Toyofuku, M., Nomura, N., Kuno, E., Tashiro, Y., Nakajima, T., and Uchiyama, H. (2008). Influence of the *Pseudomonas* quinolone signal on denitrification in *Pseudomonas aeruginosa*. *J. Bacteriol.* 190, 7947–7956.

Trunk, K., Benkert, B., Quäck, N., Münch, R., Scheer, M., Garbe, J., Jänsch, L., Trost, M., Wehland, J., Buer, J., Jahn, M., Schobert, M., and Jahn, D. (2010). Anaerobic adaptation in *Pseudomonas aeruginosa*: definition of the Anr and Dnr regulons. *Environ. Microbiol.* 12, 1719–1733.

Ugidos, A., Morales, G., Rial, E., Williams, H. D., and Rojo, F. (2008). The coordinate regulation of multiple terminal

oxidases by the *Pseudomonas putida* ANR global regulator. *Environ. Microbiol.* 10, 1690–1702.

Unden, G., Achebach, S., Holighaus, G., Tran, H. G., Wackwitz, B., and Zeuner, Y. (2002). Control of FNR function of *Escherichia coli* by O2 and reducing conditions. *J. Mol. Microbiol. Biotechnol.* 4, 263–268.

Vander Wauven, C., Piérard, A., Kley-Raymann, M., and Haas, D. (1984). *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. *J. Bacteriol.* 160, 928–934.

Vasil, M. L. (2007). How we learnt about iron acquisition in *Pseudomonas aeruginosa*: a series of very fortunate events. *Biometals* 20, 587–601.

Vasil, M. L., and Ochsner, U. A. (1999). The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol. Microbiol.* 34, 399–413.

Wang, Y., Kern, S. E., and Newman, D. K. (2010). Endogenous phenazine antibiotics promote anaerobic survival of *Pseudomonas aeruginosa* via extracellular electron transfer. *J. Bacteriol.* 192, 365–369.

Williams, H. D., Zlosnik, J. E., and Ryall, B. (2007). Oxygen, cyanide and energy generation in the cystic fibrosis pathogen *Pseudomonas aeruginosa*. *Adv. Microb. Physiol.* 52, 1–71.

Winstedt, L., and von Wachenfeldt, C. (2000). Terminal oxidases of *Bacillus subtilis* strain 168: one quinol oxidase, cytochrome aa3 or cytochrome bd, is required for aerobic growth. *J. Bacteriol.* 182, 6557–6564.

Wu, J., and Bauer, C. E. (2010). RegB kinase activity is controlled in part by monitoring the ratio of oxidized to reduced ubiquinones in the ubiquinone pool. *MBio* 1, e00272–10.

Ye, R. W., Haas, D., Ka, J.-O., Krishnapillai, V., Zimmermann, A., Baird, C., and Tiedje, J. M. (1995). Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J. Bacteriol.* 177, 3606–3609.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H. L., DeKievit, T. R., Gardner, P. R., Schwab, U., Rowe, J. J., Iglewski, B. H., McDermott, T. R., Mason, R. P., Wozniak, D. J., Hancock, R. E. W., Parsek, M. R., Noah, T. L., Boucher, R. C., and Hassett, D. J. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev. Cell* 3, 593–603.

Zimmermann, A., Reimmann, C., Galimand, M., and Haas, D. (1991). Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on anr, a regulatory gene homologous with fnr of *Escherichia coli*. *Mol. Microbiol.* 5, 1483–1490.

Zlosnik, J. E. A., Tavankar, G. R., Bundy, J. G., Mossialos, D., O'Toole, R., and Williams, H. D. (2006). Investigation of the physiological relationship between the cyanide-insensitive oxidase and cyanide production in *Pseudomonas aeruginosa*. *Microbiology* 152, 1407–1415.

Zufferey, R., Preisig, O., Hennecke, H., and Thöny-Meyer, L. (1996). Assembly and function of the cytochrome cbb3 oxidase subunits in *Bradyrhizobium japonicum*. *J. Biol. Chem.* 271, 9114–9119.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* 61, 533–616.

Zumft, W. G. (2005). Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *J. Inorg. Biochem.* 99, 194–215.

Zumft, W. G., and Kroneck, P. M. H. (2007). Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by Bacteria and Archaea. *Adv. Microb. Physiol.* 52, 107–227.

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Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl

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Pseudomonas aeruginosa thrives in many aqueous environments and is an opportunistic pathogen that can cause both acute and chronic infections. Environmental conditions and host defenses cause differing stresses on the bacteria, and to survive in vastly different environments, *P. aeruginosa* must be able to adapt to its surroundings. One strategy for bacterial adaptation is to self-encapsulate with matrix material, primarily composed of secreted extracellular polysaccharides. *P. aeruginosa* has the genetic capacity to produce at least three secreted polysaccharides; alginate, Psl, and Pel. These polysaccharides differ in chemical structure and in their biosynthetic mechanisms. Since alginate is often associated with chronic pulmonary infections, its biosynthetic pathway is the best characterized. However, alginate is only produced by a subset of *P. aeruginosa* strains. Most environmental and other clinical isolates secrete either Pel or Psl. Little information is available on the biosynthesis of these polysaccharides. Here, we review the literature on the alginate biosynthetic pathway, with emphasis on recent findings describing the structure of alginate biosynthetic proteins. This information combined with the characterization of the domain architecture of proteins encoded on the *Psl* and *Pel* operons allowed us to make predictive models for the biosynthesis of these two polysaccharides. The results indicate that alginate and Pel share certain features, including some biosynthetic proteins with structurally or functionally similar properties. In contrast, Psl biosynthesis resembles the EPS/CPS capsular biosynthesis pathway of *Escherichia coli*, where the Psl pentameric subunits are assembled in association with an isoprenoid lipid carrier. These models and the environmental cues that cause the cells to produce predominantly one polysaccharide over the others are subjects of current investigation.

Keywords: *Pseudomonas aeruginosa*, alginate, Psl polysaccharide, Pel polysaccharide, Rossmann fold, glycosyltransferase

INTRODUCTION

Pseudomonas aeruginosa is a versatile organism that is able to adapt to a wide range of environments. The bacterium grows in aqueous environments but can also colonize and cause opportunistic infections in both plants and mammals (Lyczak et al., 2000, 2002; Silo-Suh et al., 2002). With its repertoire of virulence factors, *P. aeruginosa* can even cause death to worms and insects (Tan et al., 1999; Hendrickson et al., 2001; Sibley et al., 2008). The ability of *P. aeruginosa* to adapt to different environments is due, at least in part, to the high percentage of transcriptional regulators that allow the cells to adapt rapidly to changing environmental conditions (Stover et al., 2000). One strategy that *P. aeruginosa* possesses that provides the organism with additional survival advantages during changing environmental conditions is the production of several different types of extracellular polysaccharides. The polysaccharides provide the cells with enhanced tolerance to desiccation, oxidizing agents, and host defensive processes (Berry et al., 1989; DeVault et al., 1990; Govan and Deretic, 1996; Pier et al., 2001; Friedman and Kolter, 2004b; Jackson et al., 2004). Production of extracellular polysaccharides during growth on laboratory media provides a visually striking demonstration of the capacity of *P. aeruginosa* to adapt to its environment. For example, *P. aeruginosa* isolates obtained from chronic pulmonary infections of patients with cystic fibrosis (CF) are often mucoid. These isolates secrete large amounts of the polysaccharide, alginate. In contrast, some *P. aeruginosa* CF isolates have a rugose, small colony variant (RSCV) phenotype (Starkey et al., 2009). RSCV isolates autoaggregate during culture in liquid media due to the production of exopolysaccharides that have been termed Pel and Psl. The O-antigen of lipopolysaccharides (LPS) also provides many other adaptive advantages to *P. aeruginosa*. As LPS has been extensively reviewed elsewhere (Kintz and Goldberg, 2008; King et al., 2009), this review will discuss only the secreted exopolysaccharides, alginate, Pel, and Psl.

The importance of alginate in the pathology of CF disease led to it being the first exopolysaccharide described and it remains

the best characterized *P. aeruginosa* exopolysaccharide in terms of our understanding of its biosynthesis and regulatory mechanisms (Hay et al., 2009). However, alginate is generally not produced by strains isolated from environments other than the CF lung. Isolates obtained from environments other than CF pulmonary tissue produce two different exopolysaccharides, which were identified as a consequence of the complete *P. aeruginosa* genome sequence becoming available, and through the use of high throughput transposon screening. The Psl polysaccharide was identified using reverse genetics of a putative polysaccharide gene cluster (PA2231–PA2245 of the *P. aeruginosa* PAO1 genome; Jackson et al., 2004). Mutation of the various *Psl* genes resulted in clones that were impaired in their ability to form surface-attached communities of microorganisms, termed biofilms (Ma et al., 2006). Pel was identified by screening transposon mutant libraries of the *P. aeruginosa* strain PA14 for the lack of structured microbial mats that form at the air-water interface of stagnant cultures, termed pellicles (Friedman and Kolter, 2004a).

Alginate and Psl exopolysaccharides have distinct chemical structures (**Figure 1**). While the structure of Pel has not been fully characterized, it likely has a structure that differs from alginate and Psl (Coulon et al., 2010). Each exopolysaccharide provides differing physiological properties to the cells and the biofilm matrix. In general, individual *P. aeruginosa* strains, produce predominantly one secreted polysaccharide at any given time (Ohman, 1986; Friedman and Kolter, 2004b; Jackson et al., 2004), although the sequenced strains have the genetic capacity to produce all three polysaccharides (Stover et al., 2000). RSCV isolates may be an exception to this rule, since they have been shown by transcriptional analysis to express both the *Psl* and *Pel* operons simultaneously (Starkey et al., 2009). The structure of the alginate and Psl exopolysaccharides have been determined (Evans and Linker, 1973; Byrd et al., 2009). Alginate is a high molecular weight acidic polysaccharide composed of non-repeating subunits of selectively O-acetylated D-mannuronic acid and its C5′ epimer L-guluronic acid (**Figure 1A**; Linker and Jones, 1964; Chitnis and Ohman,

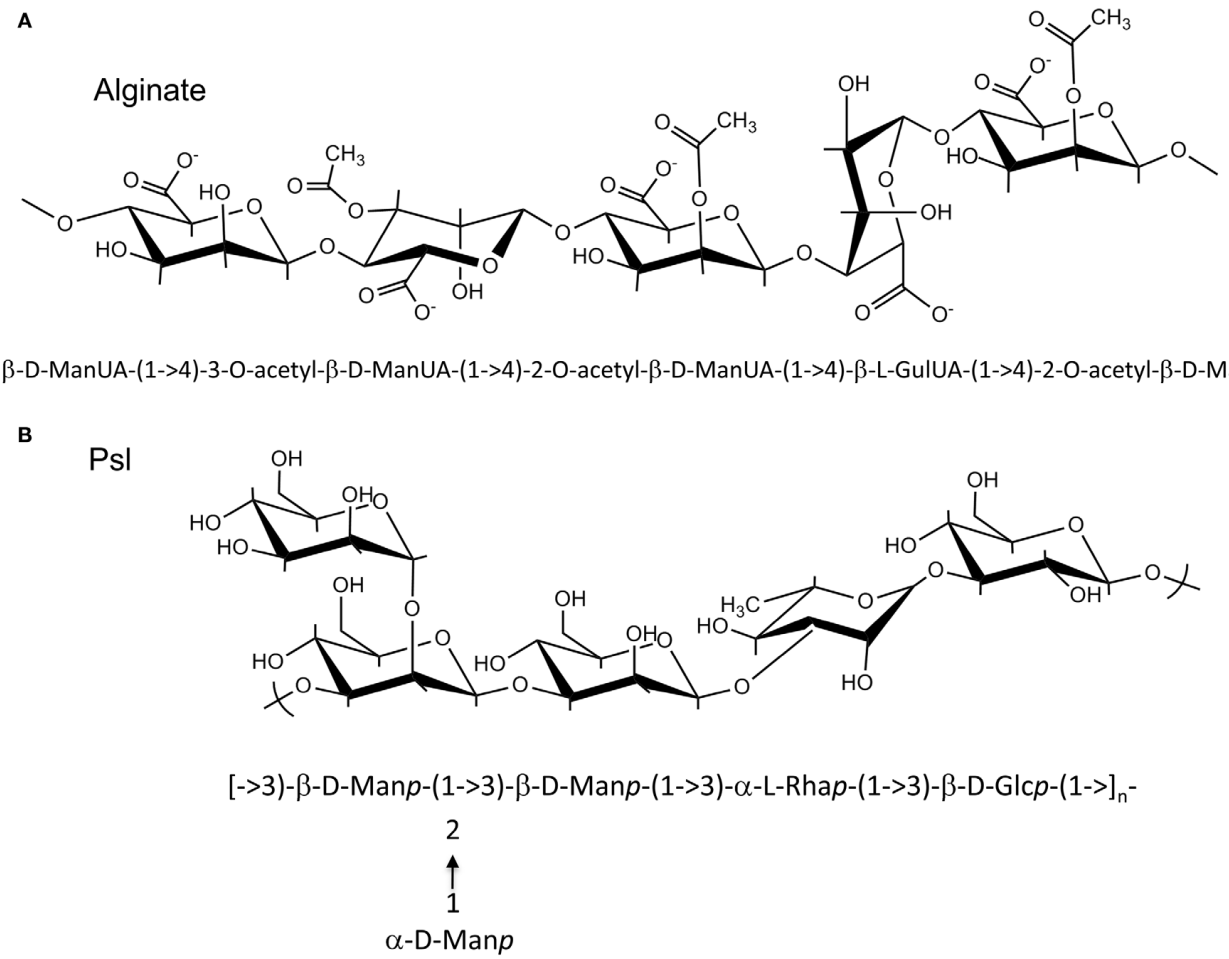

FIGURE 1 | Structures of Alginate and Psl polysaccharide. (A) *P. aeruginosa* alginate is composed of D-mannuronic acid residues interspersed with L-guluronic acid residues. The hydroxyl groups of the D-mannuronic acid residues may be O-acetylated at the C2′ and/or C3′ positions. Since modification of the D-mannuronate residues occurs at the polymer level, alginate has a random structure. Shown is an example of a possible alginate subunits arrangement, where D-mannuronate subunits are epimerized to L-guluronate or decorated with O-acetyl groups. **(B)** Psl polysaccharide is composed of a repeating pentamer consisting of D-mannose, L-rhamnose, and D-glucose residues.

1990; Franklin and Ohman, 1993). In mucoid strains, alginate is secreted into the surrounding medium and it is not covalently linked to the cell surface. Alginate produced by the mucoid CF *P. aeruginosa* FRD1 isolate, results in a highly viscous appearance of the colonies on agar medium that can be visualized by atomic force microscopy as a relatively soft but dense gelatinous structure that surrounds the cells (**Figure 2A**). The Psl polysaccharide is composed of a repeating pentamer containing D-mannose, L-rhamnose, and D-glucose (**Figure 1B**). Lectin staining of Psl indicated that it forms a helical distribution that surround the cell surface of *P. aeruginosa* PAO1, thereby facilitating its ability to play a role in cell–cell and cell–surface interactions during biofilm formation (Ma et al., 2009). Fluorescent staining and confocal laser scanning microscopy of *P. aeruginosa* PAO1 biofilms suggest that Psl forms a fabric-like matrix connecting the biofilm cells (**Figure 2B**). At present, the structure of Pel has not been determined, but it is proposed to be a glucose-rich polysaccharide that is distinct from cellulose (Friedman and Kolter, 2004b). Scanning electron microscopy of a *P. aeruginosa* PA14 pellicle shows matrix that connects the cells, allowing them to form a structured assembly at the air-liquid interface (**Figure 2C**). The connecting matrix is likely composed of Pel polysaccharide, but may contain other secreted polymers including the O-antigen of LPS, and cyclic glucans (Coulon et al., 2010). Diversification of subpopulations of bacteria that produce different polysaccharides has been observed in environmental and CF biofilms, particularly during the production of RSCV. This diversification is hypothesized to be a survival strategy providing members of the biofilm community with a means to withstand changes in environmental conditions (Boles et al., 2004).

Since Psl and Pel were discovered fairly recently, the biosynthetic mechanisms of these two exopolysaccharides are not well established and many aspects of alginate biosynthesis still remain unclear. However, the functional characterization and structures of several proteins involved in alginate production have recently been determined (Regni et al., 2002; Snook et al., 2003; Keiski et al., 2010; Whitney et al., 2011). Using this information and the predicted structures of other alginate, Psl and Pel proteins, herein we describe predictive models for the biosynthetic complexes of Psl and Pel. We predict that the biosynthetic mechanisms for the three *P. aeruginosa* exopolysaccharides fall into two general classes – an isoprenoid lipid carrier dependent mechanism and most likely a lipid carrier independent mechanism. Biosynthesis of many bacterial exopolysaccharides requires oligosaccharide subunit assembly on a lipid carrier prior to transport across the inner membrane and polymerization of full-length polymer in the periplasm (Whitfield, 2006). As described below, Psl is likely synthesized using this strategy based on the predicted structural similarities to proteins involved in group 1 capsular polysaccharide production in *Escherichia coli*, such as the K30 antigen (Whitfield, 2006). No isoprenoid lipid carrier has been observed as an intermediate for alginate biosynthesis. Rather, alginate is believed to be polymerized and directly transported across the inner membrane using its glycosyltransferase, Alg8, in conjunction with Alg44, a bis-(3′–5′)-cyclic-dimeric guanosine monophosphate (c-di-GMP) binding protein which has been shown to be required for alginate polymerization (Remminghorst and Rehm, 2006b; Merighi et al., 2007; Oglesby et al., 2008). The mechanism for alginate biosynthesis appears to be similar to that of bacterial cellulose biosynthesis, as this exopolysaccharide similarly does not require a lipid carrier (Romling, 2002). Pel biosynthesis is the least characterized *P. aeruginosa* exopolysaccharide, but our analysis suggests that it may not require a lipid carrier. Structural modeling of the Pel proteins suggest that its biosynthesis is more similar to that of alginate and cellulose, than Psl, but significant differences also exist (see below).

Each *P. aeruginosa* exopolysaccharide is encoded on distinct regions of the *P. aeruginosa* genome (Stover et al., 2000; **Figure 3**). Alginate is encoded on a 12 gene operon, from PA3540–PA3551 (on the PAO1 genome), Psl on a 12 gene operon from PA2231–PA2242, and Pel are on a seven gene operon from PA3058–PA3064.

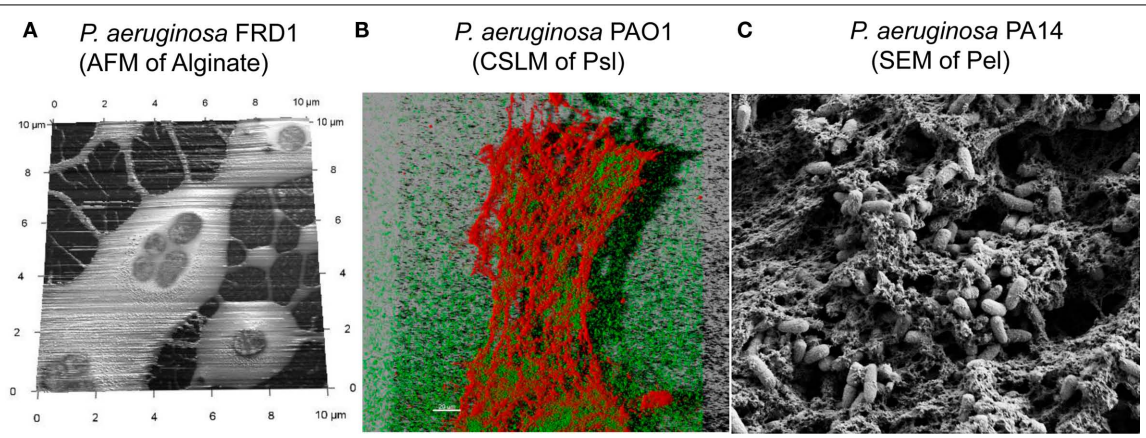

FIGURE 2 | Extracellular polysaccharides of *P. aeruginosa*, visualized using three approaches. (A) Atomic Force Microscopy image of *P. aeruginosa* FRD1, showing alginate as a soft loosely adhered polymer that surrounds the cells. **(B)** Confocal laser scanning microscopy (CLSM) image of hydrated *P. aeruginosa* PAO1, cultured as a pellicle. The CLSM image shows a three-dimensional reconstruction of the pellicle, with the *P. aeruginosa* cells expressing the green fluorescent protein and the Psl polysaccharide counterstained with CellMask Orange (Invitrogen Corp.). **(C)** Scanning Electron Microscopy image of *P. aeruginosa* PA14, cultured as a pellicle. The image shows the extracellular matrix, which includes Pel, as a fabric-like matrix that surrounds and connects the cells that form a microbial mat at the air-water interface.

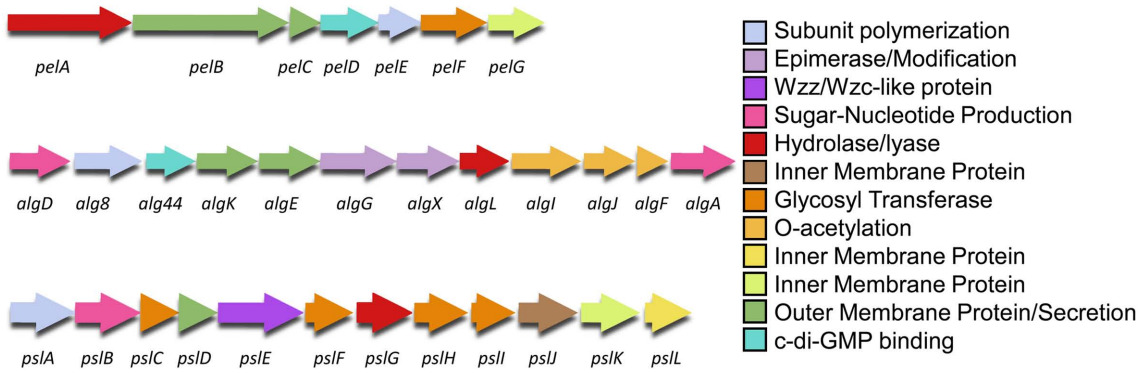

FIGURE 3 | Genetic structure of the *Pel, alg,* and *Psl* operons. The genes are color coded by proposed function as shown in **Figures 4–6**.

It should be noted that while PA2243–PA2245 have been termed *Psl* genes, the results of mutagenesis studies indicate that they are not required for Psl synthesis (Byrd et al., 2009). **Figure 3** shows the organization of the exopolysaccharide biosynthetic operons, with the genes color coded by the proposed role(s) that each protein product plays in the biosynthetic process. The biosynthesis of the polymer can be divided into the following distinct steps (Hay et al., 2009): (i) production of the activated sugar-nucleotide precursor; (ii) polymerization and transport across the inner membrane; (iii) post-polymer modification of the polymer in, and passage through the periplasm and; (iv) export from the cell. In the sections below, we combine available published data with the results of our protein modeling studies to describe the four steps in the biosynthetic pathways for each of the three *P. aeruginosa* extracellular polysaccharides.

ALGINATE BIOSYNTHESIS

Alginate is first synthesized as a linear homopolymer of D-mannuronic acid residues. The polymer is then modified in the periplasm through selective O-acetylation by the concerted action of AlgI, AlgJ, and Alg,F and epimerized by AlgG (Franklin and Ohman, 1993, 2002; Franklin et al., 1994). Since AlgG converts the D-mannuronic acid residues to L-guluronic acid at the polymer level and acetylation can occur at the hydroxyl groups of either the C2′ and/or C3′ positions, alginate has a somewhat random structure (**Figure 1A**). This distinguishes alginate from many of the *E. coli* capsular polysaccharides and from Psl as these polymers are composed of regular repeating subunits (**Figure 1B**).

Although direct protein–protein interactions of the 13 alginate proteins have not yet been demonstrated experimentally, genetic and physiological evidence suggest that most of the proteins form a complex that spans the inner membrane, periplasm, and outer membrane (**Figure 4** and **Table 1**). Evidence for a multi-protein biosynthetic apparatus comes from *in vitro* assays, which found that the cell envelope, including both inner and outer membranes, were required for alginate polymerization (Remminghorst and Rehm, 2006b). The structure of the periplasmic/outer membrane lipoprotein AlgK (PDB ID:3E4B; Keiski et al., 2010), which contains multiple copies of the protein–protein interaction tetratricopeptide-like repeat (TPR), reinforces this hypothesis, as

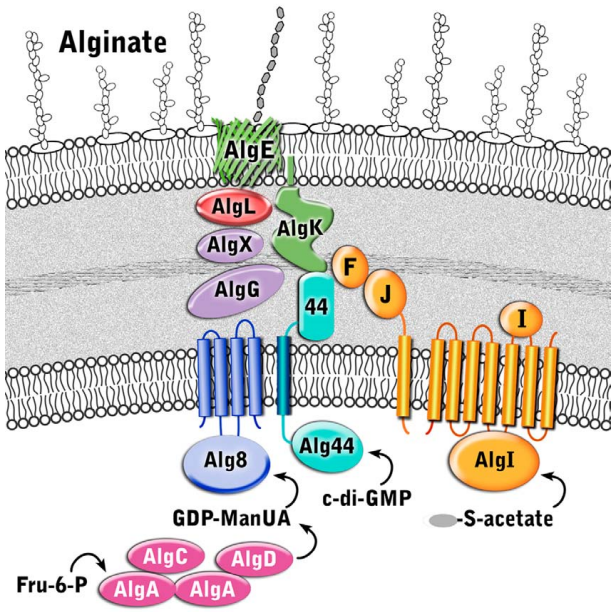

FIGURE 4 | Proposed structure of the alginate biosynthetic complex. The proteins are color coded according to proposed function as described in **Figure 3**.

TPR proteins often act as scaffolds for the assembly of multi-protein complexes (Blatch and Lassle, 1999; D'Andrea and Regan, 2003). Further evidence of a multi-protein biosynthetic complex was found when examining the phenotypes of *algK*, *algX*, and *algG* deletion mutants (Jain and Ohman, 1998; Jain et al., 2003; Robles-Price et al., 2004). Deletion of any one of these genes results in the secretion of depolymerized uronic acid subunits, presumably degraded by the periplasmic alginate lyase, AlgL (Jain and Ohman, 1998). These results suggest that each of these proteins is part of the biosynthetic complex and that when this is not formed, the synthesized polymer is exposed to AlgL and degraded. Given its structure, AlgK is likely a scaffold protein, which plays a key role in the assembly of the other periplasmic proteins that form the multi-protein biosynthetic complex (Keiski et al., 2010).

ALGINATE PRECURSOR SYNTHESIS

Exopolysaccharide biosynthesis requires sugar-nucleotide precursors. These are sugar subunits that have been activated with a high-energy bond. For alginate, the sugar subunits are activated with GDP, in a pathway where fructose-6-phosphate, derived from central carbon metabolism (Hay et al., 2009), is converted into GDP-mannuronate in a four-step process:

$$\text{Fructose - 6 - P} \xrightarrow{1} \text{Mannose - 6 - P} \xrightarrow{2} \text{Mannose - 1 - P}$$

$$\xrightarrow{3} \text{GDP - Mannose} \xrightarrow{4} \text{GDP - Mannuronate}$$

The enzymes required for GDP-mannuronate production include: (i) the bifunctional enzyme, AlgA which exhibits phosphomannose isomerase (PMI) and GDP-mannose pyrophosphorylase (GMP) activity (reactions 1 and 3); (ii) AlgC, a phosphomannomutase (reaction 2); and (iii) AlgD, which is a GDP-mannose dehydrogenase (reaction 4; Darzins et al., 1986; Deretic et al., 1987; Zielinski et al., 1991). Two of the genes required for this process are found on the alginate operon, *algA* and *algD*. However, *algC* is located elsewhere in the genome at PA5322 (Zielinski et al., 1991). AlgC appears to be crucial for general exopolysaccharide biosynthesis, not just alginate, as it is also required for precursor synthesis of Psl, as well as LPS and rhamnolipids (Goldberg et al., 1993; Olvera et al., 1999).

The crystal structures of two of these enzymes, AlgD and AlgC, have been determined (Regni et al., 2002; Snook et al., 2003). A common structural feature of enzymes involved in nucleotide binding, such as in the generation of activated sugars, is the presence of at least one β/α/β nucleotide binding domain. This domain, often referred to as a Rossmann fold, has a secondary structure consisting of alternating β-strands and α-helices arranged such that they form a central six-stranded parallel β-sheet linked to five surrounding α-helices. Many variations of the "classical" Rossmann fold or nucleotide binding domain have been observed in literature and AlgD (PDB ID:1MV8) is one such example (Snook et al., 2003). This protein forms a dimer with each individual subunit containing one complete N-terminal nucleotide binding domain and a C-terminal nucleotide-like binding domain, which lacks the third β-strand and final α-helix of this motif (Snook et al., 2003). The two nucleotide binding domains are separated by a long 33 residue α-helix, that has been termed the spacer-helix. Interestingly, the protein forms a domain-swapped dimer, whereby the N-terminal nucleotide binding domain of one subunit interacts with the C-terminal nucleotide binding domain of the second subunit. The interface of these two domains forms the active site; the location of which was verified by the structure of AlgD in complex with its substrate, NAD(H) and product GDP-mannuronate (ManUA). Two dimers of AlgD likely interact to form a tetrameric structure in the cell cytoplasm, creating the GDP-ManUA product, which is the irreversible step in alginate precursor formation.

Table 1 | Structures or structural predictions of alginate biosynthetic proteins.

Protein[a]	PDB code for structure or structurally related protein[a]	Fold or function of protein or structurally related protein	Amino acid range modeled (% coverage)[b]	% Identity	Confidence level (%)	Reference
AlgD	**1MV8**	Nucleotide binding domains/GDP-mannose dehydrogenase				Regni et al. (2002)
Alg8	1XHB	CAZy glycosyltransferase family 2	72–335 (54%)	13	99.9	Fritz et al. (2004)
Alg44	1YWU	PilZ-like domain	13–120 (27%)	16	99.1	Ramelot et al. (2007)
	3LNN	Membrane-fusion protein	192–372 (46%)	17	99.9	DeAngelis et al. (2010)
AlgK	**3E4B**	TPR-like protein				Keiski et al. (2010)
AlgE	**3RBH**	β-Barrel porin				Whitney et al. (2011)
AlgG	2PYG	CASH domain	149–507 (65%)	18	99.7	Rozeboom et al. (2008)
AlgX	1K7C	SGNH hydrolase superfamily	89–213 (26%)	16	96.1	Molgaard and Larsen (2002)
AlgL	1QAZ	CAZy polysaccharide lyase family 5	48–364 (86%)	24	100	Yoon et al. (1999)
AlgI	N.D.					
AlgJ	1K7C	SGNH hydrolase superfamily	111–231 (28%)	17	97.6	Molgaard and Larsen (2002)
AlgF	N.D.					
AlgA	2X65	Nucleotide binding domains/PMI-GMP	1–346 (71%)	38	100	Pelissier et al. (2010)
AlgC	**3CO4**	Mixed α/β topology and TATA-box binding protein-like fold/phosphomannomutase				Snook et al. (2003)

[a] *Bold letters indicate that the structure has been experimentally determined.*

[b] *Extent of the alginate protein modeled.*

N.D. not determined – Phyre2 was unable to model the protein.

The second enzyme involved in alginate precursor production whose structure has been determined is the magnesium dependent mutase, AlgC (PDB ID:3CO4), which exhibits specificity for both phosphomannose and phosphoglucose substrates (Regni et al., 2002). This protein contains four domains of approximately equal size. The first three domains have a common topological core consisting of a four-stranded β-sheet sandwiched between two α-helices, while the fourth domain is unrelated structurally to the first three, and is a member of the TATA-box binding protein-like fold superfamily. This domain consists of a four-stranded anti-parallel β-sheet, flanked by two α-helices and two short β-strands. Residues from all four domains contribute to the formation of a large active site cleft at the center of this "heart" shaped molecule. The specificity (or lack thereof) for glucose versus mannose in this class of enzymes is thought to be determined, at least in part, by a conserved sequence motif GEMS(G/A) found in domain three, which has been postulated to act as the sugar binding loop.

While the structure for AlgA has not been determined, it is predicted by structural modeling to have extensive similarity to other proteins with GDP-mannose pyrophosphorylase activity, such as the *Thermotoga maritima* guanosine-diphospho-D-mannose pyrophophorylase (PDB ID:2X65; Pelissier et al., 2010; **Table 1**) and a putative mannose-1-phosphate guanyltransferase from *Thermus thermophilus* (PDB ID:2CU2). Both of these proteins contain Rossmann-like β/α/β nucleotide binding domains characteristic of proteins that generate or bind sugar-nucleotide precursors.

POLYMANNURONATE POLYMERIZATION

Deletion mutations of the genes for the cytoplasmic membrane proteins Alg8 and Alg44 result in no alginate production (Maharaj et al., 1993; Remminghorst and Rehm, 2006a; Oglesby et al., 2008). This is in contrast to deletions of the periplasmic proteins, which result in secretion of degraded polymer and therefore suggests that Alg8 and Alg44 play a key role in alginate polymerization. Alg8 is predicted to have four transmembrane (TM) domains and a large cytoplasmic glycosyltransferase (GT) domain (Remminghorst and Rehm, 2006a; Oglesby et al., 2008). The protein has been classified as a member of the GT-2 family, a large family of inverting glycosyltransferases that include cellulose, chitin, and hyaluronan synthases (Carbohydrate Active Enzymes database, CAZy: http://www.cazy.org/; Coutinho et al., 2003; Cantarel et al., 2009). The cytoplasmic domain is predicted by Phyre^2 (Kelley and Sternberg, 2009) to be structurally homologous to UDP-GalNAc:polypeptide α-N-acetylgalactosaminyl transferase-T1 (PDB ID:1XHB; Fritz et al., 2004). As seen above for AlgD, Alg8 is predicted to contain two closely abutting β/α/β Rossmann-like nucleotide binding domains or a GT-A fold. Interestingly, Remminghorst and Rehm (2006b) have demonstrated using an *in vitro* assay for alginate polymerization with ^{14}C labeled GDP-mannuronic acid as a precursor that alginate chain extension only occurs in the cell envelope fraction. As *in vitro* polymerization does not occur in the absence of Alg8, this suggests that polymerization requires an association between both the cytoplasmic and outer membranes, and as mentioned above, is one of the primary lines of evidence that an alginate multi-protein biosynthetic complex exists. In complementation studies using a *P. aeruginosa* PAO1 strain that was engineered to produce alginate (termed *P. aeruginosa* PDO300), *alg8* overexpression *in trans* resulted in up to a 20-fold increase in alginate production compared to the wild-type strain, and an altered acetylation and epimerization profile (Remminghorst and Rehm, 2006b). This result is surprising, if it is assumed that the alginate biosynthetic proteins form a multi-protein complex; why should extra copies of one component cause an increase in polymer production? Thus, the investigators speculate that Alg8 may be the bottleneck for alginate biosynthesis (Remminghorst and Rehm, 2006b).

The second cytoplasmic membrane protein that has been shown to be absolutely required for alginate production is Alg44 (Merighi et al., 2007). Alg44 has a single TM domain located near the middle of the protein. The protein is predicted to contain a cytoplasmic N-terminal PilZ domain (Merighi et al., 2007). PilZ domains play a role in binding the secondary messenger c-di-GMP, and point mutations in this domain that result in loss of c-di-GMP binding lead to loss of alginate production. While it is clear that binding of c-di-GMP by Alg44 is required for alginate production, the exact molecular mechanism by which this interaction regulates polymerization is not known. It is interesting to note, that Pel biosynthesis also requires c-di-GMP binding to one of its biosynthetic proteins; making c-di-GMP binding a potentially common post-translational regulator of exopolysaccharide production. The C-terminal periplasmic domain of Alg44 is predicted to resemble the membrane-fusion protein MexA from the MexAB-OprM multidrug efflux pump (Remminghorst and Rehm, 2006a). This structural similarity and the requirement for a complete envelope fraction for *in vitro* polymerization suggest that this domain of Alg44 may play a role in the assembly of the multi-protein complex.

POLYMANNURONATE MODIFICATION

In the periplasm, polymannuronic acid is modified to the mature alginate polymer by a series of enzymes that include the O-acetylation complex, AlgI/AlgJ/AlgF, the polymannuronan epimerase, AlgG (Chitnis and Ohman, 1990; Franklin and Ohman, 1993, 2002; Franklin et al., 1994), and possibly, AlgX (Weadge et al., 2010). AlgX does not have a known function, but it likely associates with the multi-protein complex, since removal of AlgX by deletion mutagenesis results in the secretion of depolymerized alginate (Robles-Price et al., 2004). Interestingly, AlgX exhibits 69% sequence similarity to one of the O-acetylation enzymes, AlgJ. Homology modeling suggests that the N-terminal region, exhibits structural similarity to members of the SGNH hydrolase superfamily of enzymes, which typically remove acyl groups from carbohydrates and other compounds (PDB ID:1K7C). This finding suggests that AlgX could have a potential role in polymer modification (Weadge et al., 2010).

Deletion of any of the O-acetylation proteins, AlgI/AlgJ/AlgF, does not impair polymer synthesis or lead to the secretion of depolymerized alginate, indicating that removal of the O-acetylation proteins does not disrupt the formation of the biosynthetic complex and/or allow AlgL access to the polymer (Franklin

and Ohman, 2002). AlgI/AlgJ/AlgF O-acetylate mannuronate residues on the hydroxyl groups of the C2′ and/or C3′ positions (Franklin and Ohman, 2002; **Figure 1A**). The membrane topology of AlgI was mapped using alkaline phosphatase fusion proteins and found to contain seven TM domains, and two large cytoplasmic domains (Franklin and Ohman, 2002). AlgF and AlgJ both localize to the periplasm, where AlgJ is anchored in the cytoplasmic membrane by an uncleaved signal peptide. We have been unable with any confidence to find structural homologs for AlgI or AlgF, but AlgJ shares sequence similarity to the N-terminal region of AlgX and is also predicted to be structurally similar to members of the SGNH hydrolase superfamily (**Table 1**). The cellular location of AlgI/AlgJ/AlgF suggests a model for alginate O-acetylation, where the acetyl group contained on a donor molecule (possibly an acyl carrier protein or Coenzyme A) is transferred across the membrane by AlgI, then transferred to AlgJ or AlgF for O-acetylation of the mannuronate residues at the polymer level. This seems like an unusual strategy for polysaccharide modification, particularly when compared to N-acetylation of capsular polysaccharides where modification occurs on the sugar precursor prior to polymerization. However, it appears that the AlgI/AlgJ/AlgF esterification mechanism evolved as an ancient strategy to modify a wide variety of exopolysaccharides (Franklin et al., 2004). A similar strategy is used in O-alanylation of lipoteichoic acids in Gram-positive bacteria (via the DltB/DltD complex; Heaton and Neuhaus, 1992), and O-acetylation of cellulose in *P. fluorescens* (Spiers et al., 2002). AlgI and AlgJ homologs are also found in a wide variety of distantly related bacteria that do not produce alginate, but almost certainly produce other types of exopolysaccharides. The *algI, algJ*, and *algF* genes were likely incorporated into the alginate biosynthetic operon by an ancient lateral gene transfer event, as determined by phylogenetic analysis of AlgI and its homologs (Franklin et al., 2004).

AlgG catalyzes the epimerization of the D-mannuronate to L-guluronate at the polymer level (Franklin et al., 1994). This epimerization process alters the structural properties of alginate, including its gelling ability and its ability to bind divalent ions such as calcium (Gacesa, 1988). The structure of the C-terminal domain of AlgG has been modeled and is predicted to contain a right-handed β-helix (RHβH) fold, characteristic of proteins with carbohydrate-binding and sugar hydrolase (CASH) domains (Douthit et al., 2005). The structure of the extracellular alginate epimerase, AlgE4 (PDB ID:2PYG), from *Azotobacter vinelandii* reinforces this prediction (Rozeboom et al., 2008). CASH domain proteins include the pectate lyase of *Erwinia chrysanthemi* and several other proteins with glycosidase activity (Jenkins and Pickersgill, 2001; Jenkins et al., 2001). AlgG is the first known CASH domain protein with epimerase rather than lyase activity. The RHβH fold of AlgG includes a shallow cleft that contains the epimerase active site (Douthit et al., 2005). Point mutations of conserved residues in this cleft result in the production of alginate lacking L-guluronate (i.e., O-acetylated polymannuronic acid). Therefore, point mutations in AlgG differ from the *algG* deletion mutants, which cause secretion of depolymerized alginate (Jain et al., 2003). These results suggest that as long as AlgG maintains its proper tertiary structure it can associate with its protein partner(s) in the multi-protein complex, even if it is enzymatically inactive. It also suggests that alginate may slide through the biosynthetic complex, including the active cleft of AlgG, where it epimerizes approximately every other mannuronate residue.

Some exopolysaccharide biosynthetic operons contain a gene for an enzyme that degrades the polymer. In the case of alginate, AlgL fulfills this function (Boyd et al., 1993; Schiller et al., 1993). Initial attempts at constructing *algL* deletion mutations in a mucoid strain were unsuccessful, suggesting that AlgL is an essential protein. In a strain where alginate production was regulated by isopropyl β-D-thiogalactopyranoside (IPTG) addition, an *algL* deletion mutant could be constructed as long as the *alg* operon was down regulated by lack of IPTG (Jain and Ohman, 2005). Induction of the *algL* mutant strain with IPTG resulted in cell death and electron micrographs indicated that the periplasm of the mutant strain filled up with a polymer, likely alginate (Jain and Ohman, 2005). This result suggested a role for AlgL in periplasmic processing of alginate or secretion of the polymer, although a role as part of the secretion complex cannot be ruled out. The AlgL protein differs structurally from CASH domain lyases described above and predicted for AlgG. Instead, AlgL is a member of polysaccharide lyase family 5, which is predicted by homology modeling to be an $\alpha_6\alpha_5$ barrel and structurally similar to the alginate lyase A1–III from *Sphingomonas* spp. (PDB ID:1QAZ; Yoon et al., 1999).

ALGINATE PERIPLASMIC TRANSLOCATION AND SECRETION

The structure of the putative scaffold protein, AlgK was recently determined (PDB ID:3E4B) and shown to contain at least 9.5 TPR-like repeats (Keiski et al., 2010). The TPR repeat consists of a pair of anti-parallel α-helices, which are typically found in multiple copies where they pack together to form superhelical structures. This motif and the closely related SEL-1 motif have been implicated in complex formation in a variety of different systems (Blatch and Lassle, 1999; D'Andrea and Regan, 2003). AlgK also contains a lipid moiety that anchors the protein to the outer membrane (Keiski et al., 2010). Deletion mutants of *algK* suggest that this protein may play a role in the localization of the outer membrane porin protein, AlgE, which led to the hypothesis that AlgK/AlgE interact to form a novel type of secretin that differs structurally from other bacterial capsular polysaccharide secretion systems. AlgE is an outer membrane protein that is capable of spontaneously incorporating into planar lipid bilayers and forming a highly anion specific channel (Rehm et al., 1994). Epitope tagging suggests that it is a monomeric 18-stranded β-barrel porin (Hay et al., 2010), a prediction that the recent structure of AlgE has confirmed (PDB ID:3RBH; Whitney et al., 2009, 2011). AlgE is characterized by a highly electropositive pore constriction formed by an arginine-rich conduit that is occluded on either side by an extracellular loop and an unusually long periplasmic loop, T8. Characterization *in vitro* and *in vivo* of a AlgE-T8 loop detection mutant suggests that this loop may play a role in regulating the transport of alginate across the outer membrane.

PEL BIOSYNTHESIS

Little is known about Pel biosynthesis, as to date only two of the seven *Pel* gene products, PelC, and PelD, have been individually

examined experimentally (Lee et al., 2007; Vasseur et al., 2007; Ueda and Wood, 2009; Kowalska et al., 2010), and a model of one, PelB, proposed (Keiski et al., 2010). Using these data and our structural homology analysis of the Pel proteins we have generated a preliminary model for Pel biosynthesis and export (**Figure 5** and **Table 2**). Our model suggests that Pel biosynthesis is more closely related to the pathways for alginate and bacterial cellulose biosynthesis (Romling, 2002; Saxena and Brown, 2005), than for capsule production (Whitfield, 2006), but notable differences appear to exist in Pel that would distinguish it from the currently proposed mechanisms for these other secretion systems.

Pel PRECURSOR PRODUCTION

Unlike alginate, which contains *algA* and *algD* on the biosynthetic operon, the *Pel* operon does not appear to carry any genes for the synthesis of its sugar-nucleotide precursor. This suggests that the biosynthesis of Pel sugar-nucleotide precursors is derived from central carbon metabolism and uses enzymes from other carbohydrate synthesis pathways, similar to the observation that AlgC is required for precursor production for alginate, Psl polysaccharide, rhamnolipid, and LPS production (Goldberg et al., 1993; Coyne et al., 1994; Olvera et al., 1999; Byrd et al., 2009).

Pel POLYMERIZATION

Predictions based on the sequences of the Pel proteins suggest that only *PelF* encodes a protein that localizes to the cytoplasm. PelF is predicted to be a glycosyltransferase that resembles enzymes of the CAZy GT-4 family (Coutinho et al., 2003). Structural homology modeling suggests that PelF has a classical GT-B fold with two Rossmann-like domains and therefore it is likely that this protein is involved in polymerization. Unlike Alg8, which contains multiple TM domains that are thought to aid in polymer export across the IM (Remminghorst and Rehm, 2006b; Oglesby et al., 2008), the cytoplasmic location of PelF and lack of TM domains suggests that Pel export across the inner membrane occurs by a different mechanism. Given that the *Pel* operon contains only one glycosyltransferase, we anticipate that Pel is a linear homopolymer, and therefore, like alginate, it may not require a lipid carrier for transport across the inner membrane. In contrast to the Psl polysaccharide, no gene for an undecaprenyl-phosphate glycosylphosphotransferase is found on the *Pel* operon. While it is not clear how the polymer is transported across the inner membrane, the most likely candidates involved in this process are PelD, PelE, and PelG, all of which are located in the inner membrane and are predicted to contain multiple TM domains. The predictions that Pel is a homopolymer and that an isoprenoid lipid does not act as a carrier assumes that there are no *Pel* genes that are unlinked to *PelA-G*, that have yet to be identified.

PelD is an inner membrane protein that is essential for Pel polysaccharide production (Lee et al., 2007). PelD binds c-di-GMP and point mutants that abrogate this binding prevent polymer formation. Similarly, manipulation of the cellular levels of c-di-GMP,

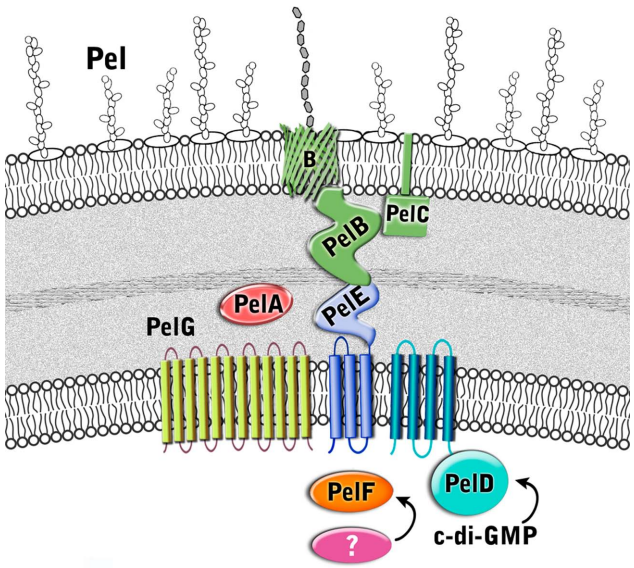

FIGURE 5 | Proposed structure of the Pel biosynthetic complex. The enzymes are color coded according to proposed function as described in **Figure 3**.

Table 2 | Structural predictions of Pel biosynthetic proteins.

Protein	PDB code of structurally related protein	Fold or function of structurally related protein	Amino acid range modeled (% coverage)[a]	% Identity	Confidence level (%)	Reference
PelA	2AAM	Glycosyl hydrolase	47–303 (27%)	18	100	N/A
	2VYO	Deacetylase/esterase	511–794 (29%)	15	98.6	Urch et al. (2009)
PelB	2XPI	TPR-like domain	254–728 (39%)	13	100	Zhang et al. (2010)
	IUYN	β-Barrel porin	966–1188 (18%)	17	96.5	Oomen et al. (2004)
PelC	2HQS	TolB-like N-terminal domain	30–169 (80%)	20	99.6	Bonsor et al. (2007)
PelD	3MF0	GAF domain	131–316 (40%)	10	98.6	Wang et al. (2010)
	3EZU	GGDEF domain	294–445 (33%)	16	100	N/A
PelE	3FP4	TPR-like protein	77–327 (55%)	11	99.7	Li et al. (2009)
PelF	2R60	CAZy glycosyltransferase family 4	13–505 (97%)	21	100	Chua et al. (2008)
PelG	3MKU	MATE transporter family	15–450 (95%)	15	98.6	He et al. (2010)

[a] *Extent of the Pel protein modeled.*

N/A – Not available.

through a deletion of the tyrosine phosphatase, TpbA, also influence the amount of Pel production and pellicle formation (Ueda and Wood, 2009). Therefore, PelD is functionally similar to Alg44 with respect to its c-di-GMP mediated regulation of polysaccharide production. However, in contrast to Alg44, which has one central TM domain that positions approximately half of Alg44 in the periplasm and the c-di-GMP binding PilZ domain in the cytoplasm (Merighi et al., 2007; Oglesby et al., 2008), the N-terminus of PelD is predicted to contain four TM domains. The presence of these TM domains results in the N-terminus of PelD being positioned in the inner membrane and the C-terminal region being in the cytoplasm where it interacts with c-di-GMP and regulates Pel production (Lee et al., 2007). Also in contrast to Alg44, structural homology predictions using Phyre^2 (Kelley and Sternberg, 2009) suggest that the C-terminal domain, encompassing residues 111–455 contains tandemly arranged GAF and GGDEF domains (PDB ID:3MFO and 3EZU), despite the fact that PelD lacks the GG(D/E)EF consensus motif (Lee et al., 2007). How PelD regulates polymer production and why this protein requires four TM domains are questions that remain to be answered.

PelG is an integral inner membrane protein predicted to contain 12 TM domains (Krogh et al., 2001). PelG has structural similarity to NorM from *Vibrio cholerae* (PDB ID:3MKU), a member of the multidrug and toxic compound extrusion (MATE) family of proteins (He et al., 2010). MATE proteins couple electrochemical gradients to extrusion of molecules across the inner membrane. The similarity of PelG to this family of proteins, suggests that PelG may play a role in export of Pel polysaccharide across the inner membrane or alternatively, like AlgI, it may transport a small molecule substrate that is subsequently used, perhaps by PelA, to modify the Pel polymer.

The third inner membrane protein, PelE, appears to be highly unique. PelE contains a type 1 export signal and two predicted TM domains. This arrangement positions most of the C-terminus of PelE, residues 90–320, in the periplasm. The C-terminal region is predicted to be all α-helical with at least four to five and possibly as many as six copies of the TPR protein–protein interaction motif. Given the role of TPR motifs in the assembly of large protein complexes (Blatch and Lassle, 1999; D'Andrea and Regan, 2003), PelE, may function as a scaffold protein in an analogous manner to AlgK. PelE, may help in the assembly of a secretion complex through interactions possibly with PelA, and PelB. A role in complex assembly at the inner membrane has also been suggested for the periplasmic domain of Alg44 (Hay et al., 2009), indicating that the periplasmic domains of Alg44 and PelE may be functionally equivalent. Multimerization of PelE and/or its association with the four TM domains of PelD could potentially provide the required portal in the inner membrane for the export of the polymer.

Pel TRANSLOCATION AND EXPORT

A second Pel protein with extensive predicted TPR structure is PelB (Keiski et al., 2010). PelB is likely localized to the periplasm and/or outer membrane. In addition to a large TPR containing domain at the N-terminus which homology modeling suggests is similar to the TPR containing anaphase-promoting complex/cyclosome subunit Cdc 16/Cut9 (PDB ID:2XPI; Zhang et al., 2010), PelB is also predicted to be rich in β-sheet structure at its C-terminus, a property that is characteristic of outer membrane porins. PelB may be functionally similar to the alginate secretin, AlgK/AlgE, but rather than two separate proteins, PelB appears to be a fusion protein containing both periplasmic and outer membrane domains in a single polypeptide. Keiski et al. (2010) suggested that this domain structure may be conserved in several other polysaccharide secretion complexes, including the bacterial cellulose and poly-β-1,6-*N*-acetylglucosamine (PAG) export proteins which contain the TPR/β-barrel fusion proteins, BcsC and PgaA (Romling, 2002; Wang et al., 2004). As in the case of AlgK, the periplasmic domain of PelB is anticipated to serve as a scaffold for the assembly of the secretion complex, and to protect the polysaccharide from degradation as it moves through the periplasm.

The *Pel* operon contains a second outer membrane protein, the lipoprotein PelC (Vasseur et al., 2007; Kowalska et al., 2010). PelC is lipidated at cysteine 19, and is predicted to contain a short stretch of ∼15 disordered residues followed by a single domain that has mixed α/β topology. Modeling results suggest that residues ∼43 to the C-terminus are structurally similar to the periplasmic N-terminal domain of *E. coli* TolB (PDB ID:2HQS; Bonsor et al., 2007), a protein that interacts with the peptidoglycan associated lipoprotein, Pal, and required for maintaining outer membrane integrity. TolB contains a three stranded β-sheet flanked by two α-helices. This model is somewhat different from the model proposed by Vasseur et al. (2007), who have suggested that the C-terminal amphipathic α-helix of PelC, which is required for Pel production, is inserted into the membrane in a Wza-like manner (Dong et al., 2006). In this model, PelC would be anchored to the OM by both its lipid anchor and C-terminal α-helix. Until the structure of PelC has been determined experimentally, it is not possible to resolve this discrepancy, but it is clear from the current functional characterization that PelC is essential for polymer production and that the C-terminal α-helix will play a key role either in protein folding and/or in the insertion of the protein into the OM.

The final protein of the *Pel* operon is PelA, a large 105 kDa protein that is predicted to contain an N-terminal TIM α/β-barrel domain with structural similarity to glycoside hydrolases (PDB ID:2AAM) and a C-terminal carbohydrate esterase domain (PDB ID:2VYO; Urch et al., 2009). Since the chemical structure of the Pel exopolysaccharide is unknown, it is difficult to predict the role of PelA in polymer production, but our model would suggest that the polymer, like alginate, may be modified after polymerization in the periplasm. Deacetylation of exopolysaccharides is not unprecedented as it is well established that N-deacetylation of the PGA polymer is required for biofilm formation in both Gram-negative and Gram-positive bacteria (Agladze et al., 2003). Similarly, if PelA also exhibits hydrolase activity, it may be functionally similar to AlgL, and thus required for proper processing and/or clearance of the Pel polysaccharide in the periplasm (Jain and Ohman, 2005).

PSL BIOSYNTHESIS

Although the Psl pathway has not been characterized, there is evidence to suggest that its biosynthetic mechanism functionally

resembles the isoprenoid lipid-based biosynthesis of *E. coli* group 1 capsular and extracellular polysaccharides (CPS and EPS; **Figure 6** and **Table 3**). First, Psl has a repeating oligosaccharide structure characteristic of CPS, as opposed to the homomeric (prior to epimerization) structure of alginate, which is polymerized a single sugar at a time. Second, the *Psl* operon does not encode a TPR protein which is characteristic of alginate and Pel polysaccharide export scaffolds, but does encode several proteins that have structural similarity to group 1 CPS/EPS proteins. These include PslA, PslE, and PslD, which are similar to WbaP, Wzc, and Wza, respectively. Therefore, the Psl biosynthetic pathway appears to be a Wzy-dependent pathway where polymers are built on an isoprenoid lipid carrier (undecaprenyl diphosphate) and then assembled and exported through the action of the group 1 CPS/EPS export process that requires polysaccharide copolymerases (PCP) and outer membrane polysaccharide export (OPX) proteins (Cuthbertson et al., 2009).

Psl PRECURSOR PRODUCTION

Since Psl is composed of D-mannose, L-rhamnose, and D-glucose (**Figure 1**), activated sugar-nucleotide precursors are required for each of these subunits. Only one enzyme from the *Psl* operon, PslB, is involved in sugar-nucleotide precursor production. The remaining enzymes for precursor activation (AlgC, RmlC, and GalU) are encoded by genes associated with other polysaccharide biosynthetic pathways (Byrd et al., 2009). Byrd et al. (2009) constructed non-polar deletion mutations of each of the *Psl* genes (*PslA-L*) and demonstrated that of these mutations only the *PslB* deletion was still capable of producing Psl polysaccharide. PslB is a bifunctional enzyme that is similar to AlgA, as it is predicted to possess an N-terminal GMP domain and C-terminal PMI domain. *P. aeruginosa* PAO1 encodes three PMI/GMPs; PslB, WbpW (PA5452 on an LPS biosynthetic operon), and AlgA. A double mutant of *PslB* and *wbpW* resulted in loss of Psl production suggesting that the different PMI/GMPs are functionally interchangeable for the production of the GDP-mannose precursor (an *algA/PslB* double mutant was not tested for Psl production, since *algA* is not expressed in *P. aeruginosa* PAO1). As described for

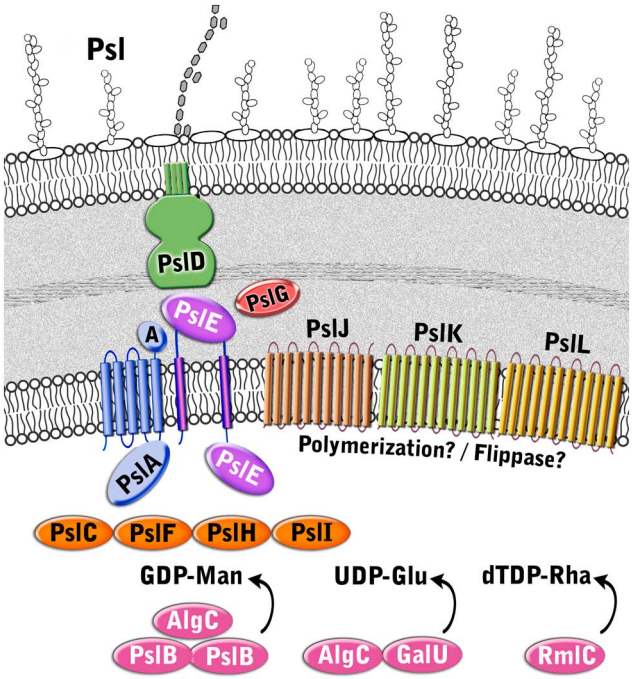

FIGURE 6 | Proposed structure of the Psl biosynthetic complex. The enzymes are color coded according to proposed function as described in **Figure 3**.

Table 3 | Structural predictions of Psl biosynthetic proteins.

Protein	PDB Code of structurally related protein or PFAM	Fold or function of structurally related protein	Amino acid range modeled (% coverage)[a]	% Identity	Confidence level (%)	Reference
PslA	3NKL	Nucleotide sugar dehydrogenase	149–268 (25%)	24	99	N/A
PslB	2X65	Nucleotide binding domains/PMI-GMP	6–361(72%)	35	98	Pelissier et al. (2010)
PslC	1XHB	CAZy glycosyltransferase family 2	1–219 (72%)	17	100	Fritz et al. (2004)
PslD	2J58	Outer membrane lipoprotein (Wza)	30–207 (69%)	28	100	Dong et al. (2006)
PslE	2EFR	Coiled-coil protein	212–406 (29%)	15	96	N/A
	3LA6	Tyrosine kinase (Wzc)	474–646(26%)	21	100	Bechet et al. (2010)
PslF	2GEK	CAZy glycosyltransferase family 4	1–395 (100%)	14	100	Guerin et al. (2007)
PslG	1UHV	CAZy glycosyl hydrolase family 39	37–395 (80%)	19	100	Yang et al. (2004)
PslH	2GEK	CAZy glycosyltransferase family 4	1–399 (99%)	19	100	Guerin et al. (2007)
PslI	2GEK	CAZy glycosyltransferase family 4	1–367 (100%)	16	100	Guerin et al. (2007)
PslJ	PF04932	PFAM for Wzy	253–382 (26%)			
PslK	3MKU	MATE transporter family	1–416 (89%)	17	100	He et al. (2010)
PslL	PF01757	Acyl transferase domain	5–324(89%)			

[a] *Extent of the Psl protein modeled.*

N/A – Not available.

AlgA in the alginate pathway shown above, PslB provides the first and third steps (PMI/GMP) for converting fructose-6-phosphate from central metabolism into GDP mannose. AlgC provides the phosphomannomutase activity for the second step (Byrd et al., 2009). Since AlgC also has phosphoglucomutase activity, it is also proposed to be involved in the production of glucose-1-phosphate, which is the substrate for GalU to convert into the UDP-glucose precursor. Byrd et al. (2009) also demonstrated that the L-rhamnose precursor is a product of the Rml pathway, and that a deletion of *rmlC* results in a defect in Psl production. Taken together, the results indicate that one enzyme for Psl precursor production (PslB) is encoded on the *Psl* operon, while the other enzymes overlap with other polysaccharide biosynthetic pathways.

Four enzymes with GT domains are encoded on the *Psl* operon (PslF, PslH, PslI, and PslC). Three of these, PslF, PslH, and PslI, are predicted to belong to the CAZy GT-4 family of enzymes that transfer glycosyl residues by a retaining mechanism and adopt a GT-B fold (Coutinho and Henrissat, 1999). Structural homology predictions using Phyre^2 (Kelley and Sternberg, 2009) suggest that PslF, PslH, and PslI all have structural relatedness to the GT-4 enzyme, PimA, a phosphatidylinositol mannosyltransferase from Mycobacteria (PDB ID:2GEK; Guerin et al., 2007). Therefore, these three enzymes may be responsible for the incorporation of the activated sugar subunits into the Psl repeating structure. The remaining soluble GT, PslC, is predicted to belong to the CAZy GT-2 family, which adopt a GT-A fold and catalyze glycosyl transfer by an inverting mechanism (Coutinho et al., 2003). GT-A folded enzymes have a single Rossmann-like fold for nucleotide binding and also have a characteristic Asp-Xxx-Asp (DXD) or equivalent motif (EAD or TDD) that is responsible for coordination of the phosphates of the nucleotide donor via a divalent cation (Coutinho and Henrissat, 1999). The Phyre^2 (Kelley and Sternberg, 2009) structural homology predictions for PslC suggest that it is similar to the murine polypeptide α-*N*-acetylgalactosaminyltransferase-T1 (PDB ID:1XHB; Fritz et al., 2004) and to a putative *Bacteriodes fragilis* glycosyltransferase (PDB ID:3BCV). However, unlike these proteins, PslC lacks a clear DXD motif, but does possess an EXD sequence that may act as a substitute. Enzymes in the GT-2 family transfer a wide variety of nucleotide activated sugars (including glucose, rhamnose, and mannose), so it is difficult to speculate which of these substrates PslC specifically recognizes. Based on the predicted structures of PslF, PslH, PslI, and PslC, these enzymes likely participate in the transfer of the precursor sugars from activated nucleotide donors to form the repeating unit of the Psl polysaccharide, which is then recognized by the membrane-associated Psl biosynthetic complex.

Psl POLYMERIZATION

Five Psl proteins (PslA/PslE/PslJ/PslK/PslL) have inner membrane spanning domains and therefore likely make up the Psl polymerization complex. Two of these proteins, PslA and PslE, have structural similarities to the Wzy-dependent capsule synthesis pathway (Whitfield, 2006). PslA is predicted to contain four TM domains at its N-terminus and one at the C-terminus. Between these transmembrane segments is a cytoplasmically located region that is predicted to have an EPS biosynthesis polyprenyl glycosylphosphotransferase activity and a Rossmann fold. PslA likely plays a similar role to WbaP in providing a site for the assembly of the oligosaccharide repeating unit onto the isoprenoid lipid at the cytoplasmic face of the inner membrane (Whitfield, 2006). PslE has characteristic domains of a Wzz (or Wzc) homolog and is therefore predicted to act as the PCP component in this system. PslE, like other PCPs, possesses two TM domains that flank a large periplasmic domain and a cytoplasmically located C-terminal tail. The large periplasmic domain is predicted to adopt a coiled-coil structure, similar to that seen for Wzz where monomers assemble into a hexameric (possibly octameric) structure with a central pore (Larue et al., 2009). This periplasmic domain is proposed to affect polysaccharide chain length (Tocilj et al., 2008) and is thought to form critical interactions with the CPS/EPS export component (PslD) thereby completing a complex that facilitates transfer of the polymer through the periplasm (Cuthbertson et al., 2009). The C-terminal cytoplasmic domain of PslE has structural similarity to Wzc, another subclass of PCP proteins. Wzc homologs are related to Wzz, but are distinguished by the presence of a C-terminally located tyrosine autokinase domain (Grangeasse et al., 2007). Interestingly, Wzc contains 6 tyrosines in the final 17 amino acids of its C-terminus, while PslE contains only one (Y658). In the case of Wzc, the phosphorylation of all six tyrosines contributes to the export of CPS and no single tyrosine was found to have a dominant phenotype. Thus, it will be interesting to see if the phophorylation of the single tyrosine in PslE affects this process.

The function of the other three inner membrane proteins (PslJ/PslK/PslL) is more difficult to predict. Each of these has extensive membrane topology throughout the protein, with PslJ predicted to have 11 TM domains, PslK, 12 TM domains, and PslL, 11 TM domains. No structural homologs are apparent for PslJ, but sequence similarity suggests that it may have an O-antigen ligase motif (Pfam 04932) that is also found in Wzy.

Similar to PelG, PslK resembles NorM from *V. cholerae* (PDB ID:3MKU), which is a member of the MATE family (He et al., 2010). Given that group 1 CPS/EPS biosynthesis typically couples Wzy and Wzz proteins to a flippase (Wzx), it is possible that PslJ fills the role of Wzy in the Psl system, and PslK acts as the flippase. However, this remains to be determined. Sequence similarity of PslL suggests that it has an Acyltransferase_3 (Pfam 1757) domain. Since the Psl polysaccharide is not known to be modified by small functional groups it is difficult to speculate what the role of PslL is in the Psl biosynthetic system. However, it is not unreasonable to hypothesize that a modification could be lost during the extraction and analysis of the structure of Psl.

Psl TRANSLOCATION AND EXPORT

PslD is a periplasmic (and possibly outer membrane) protein with structural similarity to the *E. coli* K30 capsule translocase, Wza (PDB ID:2J58; Dong et al., 2006). Wza belongs to the OPX family of proteins that are responsible for the final stage of CPS/EPS export (Cuthbertson et al., 2009). Based on the structure of Wza, the OPX family of proteins are predicted to be lipoproteins that adopt an octameric configuration with a large central cavity that

facilitates CPS export through the periplasm and across the outer membrane. The large periplasmic domain of Wza forms three discrete rings layered on top of each other, which extend the protein toward the inner membrane and thereby facilitate interaction with Wzc to form a molecular scaffold that spans the periplasm. The extreme C-terminus of Wza folds into a final domain that has a novel α-helical barrel structure that forms a pore in the outer membrane (Dong et al., 2006). While most of PslD can be structurally modeled onto Wza, there are some clear differences. PslD possess only two of the three discrete ring domains of Wza and appears to lack the α-helical barrel domain located in the outer membrane. Therefore, it is difficult to understand how the Psl polymer would pass through the outer membrane if the pore is lacking and if there is enough protein present to form a continuous structure with PslE (the Wzc homolog) in order to span the periplasm. One hypothesis is that this may indicate a requirement for additional periplasmic components. Alternatively, the answer may lie in the inherent sequence differences between Wza/Wzc and PslD/PslE. For example, the C-terminal 49 amino acids of PslD that can not be modeled using Wza may still come together in a manner that facilitates the formation of an outer membrane pore. As for the shorter periplasmic portion of PslD, it is interesting to note that the periplasmic domain of PslE is 49 amino acids larger than the same domain of Wzc, so perhaps this provides the additional periplasmic coverage that is needed. These hypotheses will require further experimental evidence.

Structural homology modeling with Phyre^2 (Kelley and Sternberg, 2009) suggests that the periplasmic protein PslG, most closely resembles β-D-xylosidases (e.g., PDB ID:1UHV) from the CAZy glycosyl hydrolase family 39 (Cantarel et al., 2009). These enzymes have a $(\beta/\alpha)_8$ TIM barrel fold that typically consists of an inner ring of eight β-strands surrounded by an outer ring of eight α-helices and a deep active site cleft along the top of the barrel. Since these enzymes hydrolyze glycosidic linkages, it is likely that PslG performs a similar function to AlgL and PelA from the alginate and Pel systems, respectively. Thus, PslG may be responsible for processing the growing polysaccharide for export and/or act as a failsafe enzyme that rids the periplasm of aberrant polymer when the export process goes awry. Indirect evidence for the second function can be implied from *wza* or *wzc* mutants that produce short oligosaccharides of CPS instead of the large molecules formed during proper CPS export (Drummelsmith and Whitfield, 1999).

SUMMARY AND FUTURE PERSPECTIVES

Pseudomonas aeruginosa has the genetic capacity to produce three different secreted extracellular polysaccharides. The biosynthetic mechanism of alginate has been well studied. However, little has been determined experimentally for Psl and Pel biosynthesis. With the ability to predict domain structures of the Psl and Pel biosynthetic proteins, it was possible to construct models for the synthesis of these secreted extracellular polysaccharides (**Figures 4–6**). The analyses suggest that *P. aeruginosa* extracellular polysaccharides are synthesized using two general biosynthetic strategies, an isoprenoid lipid carrier dependent mechanism (for Psl) and a lipid carrier independent mechanism (for alginate and likely for Pel). The evidence for a lipid carrier dependent mechanism for Psl include the presence of several proteins with structural similarity to the EPS/CPS Wzy-dependent pathway of *E. coli* capsule biosynthesis, and the presence of a repeating oligosaccharide structure, that is characteristic of polysaccharides that are first assembled in association with the isoprenoid lipid. The lipid independent mechanism for alginate and possibly for Pel is based on structural similarities of proteins for these two biosynthetic complexes to enzymes involved in bacterial cellulose and Pga biosynthesis (Romling, 2002; Wang et al., 2004) thereby forming a new class of exopolysaccharide biosynthesis/export systems. Hallmarks of this new class are found in both the Pel and alginate biosynthetic pathways. First, both pathways contain a c-di-GMP binding protein that is essential for polysaccharide polymerization. Second, they both appear to utilize TPR-like proteins to form the biosynthetic scaffolds. Finally, the presence of only one glycosyltransferase on the *Pel* operon, like alginate, suggests that these polysaccharides are first synthesized as a homopolymer. While the models shown for Pel and Psl (**Figures 5** and **6**) are based primarily on protein structural predictions, they provide a framework and starting point for testing experimentally the structure and function of these biosynthetic proteins and pathways.

Pseudomonas aeruginosa encoding three different polysaccharide biosynthetic operons raises the following questions: (1) Why maintain the genetic potential to synthesize several different polysaccharides? (2) Do environmental signals induce production of one polysaccharide compared to another? and (3) What survival advantages are imparted by the different polysaccharides under different conditions? The structure of a polysaccharide is important for its function, and production of several structurally different polysaccharides may allow cells to adapt to certain environments. Alginate, Pel, and Psl have all been shown to provide cohesive and adhesive properties that allow cells to form pellicles, microcolonies, or biofilms (Nivens et al., 2001; Ma et al., 2009; Colvin et al., 2011). However, the proper structure of the polysaccharide is likely required for formation of these structured communities. For example, a mutant strain that is impaired in the O-acetylation of alginate was unable to form biofilms (Nivens et al., 2001), the predominant phenotype exhibited by strains isolated from chronic disease in the CF lung.

Switching between production of different *P. aeruginosa* polysaccharides may be induced by specific stress responses (Wood et al., 2006). *P. aeruginosa* strains in the CF lung are initially derived from environmental reservoirs (Burns et al., 2001). Therefore, these strains likely produce Pel or Psl. Perhaps due to the selective pressures of the CF lung, *P. aeruginosa* develops alternate phenotypes, including mucoidy associated with alginate production (Lyczak et al., 2002). Alginate production by pulmonary isolates helps protect the cells from host defensive processes, including opsonic phagocytosis (Pier et al., 2001). Interestingly, regulation of both alginate and Psl require alternative sigma factors that are generally associated with stress responses – σ^E for alginate and σ^S for Psl (Flynn and Ohman, 1988; Martin et al., 1994; Ramsey and Wozniak, 2005; Irie et al., 2010).

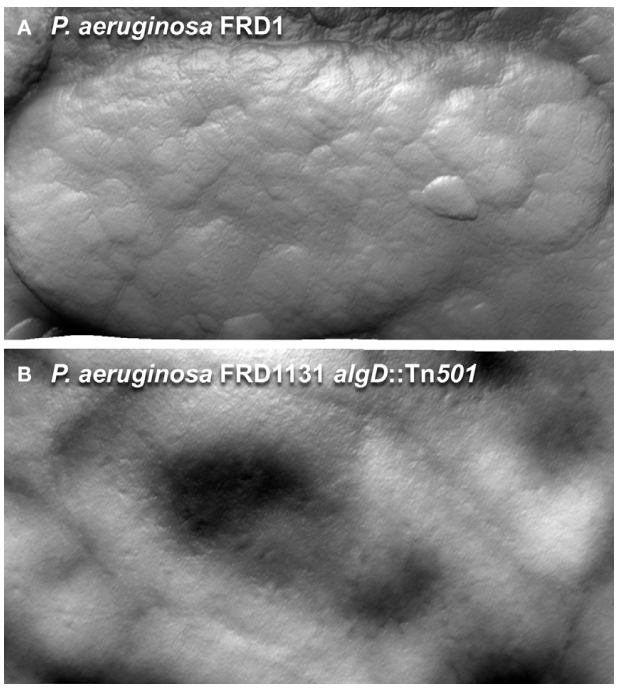

FIGURE 7 | Atomic force microscopy images of hydrated *P. aeruginosa* FRD1 and FRD11131 *algD*::Tn501 biofilm cells, collected at 90% humidity. (A) When living at an air/water interface, FRD1 is able to maintain its shape during dehydration. **(B)** FRD1131 biofilm cells dry out quickly and collapses under the same conditions. Once dry, the extracellular polymer is removed from FRD1 by imaging, but the surface of the FRD1 cells is distinct from FRD1131.

The production of extracellular polymers may temper conditions found in inhospitable environments. Alginate has been shown to play a role in protecting cells from desiccation (Berry et al., 1989; DeVault et al., 1989). Characterization of *P. aeruginosa* by AFM shows that when an alginate producing strain of *P. aeruginosa* is transferred from an aqueous environment to a high humidity condition, the strain maintains its shape (**Figure 7A**). In contrast, when an *algD* null mutant strain is transferred to equivalent dehydrating conditions, the cells show a collapsed structure (**Figure 7B**), suggesting that the extracellular polysaccharide may allow cell survival under certain inhospitable conditions. Having the potential to secrete three different polymers with varying hydrophobicity, viscoelastic properties, and charge may provide *P. aeruginosa* with the flexibility to adjust the physicochemical nature of its microenvironment, and survive in different niches.

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REFERENCES

Agladze, K., Jackson, D., and Romeo, T. (2003). Periodicity of cell attachment patterns during *Escherichia coli* biofilm development. *J. Bacteriol.* 185, 5632–5638.

Bechet, E., Gruszczyk, J., Terreux, R., Gueguen-Chaignon, V., Vigouroux, A., Obadia, B., Cozzone, A. J., Nessler, S., and Grangeasse, C. (2010). Identification of structural and molecular determinants of the tyrosine-kinase Wzc and implications in capsular polysaccharide export. *Mol. Microbiol.* 77, 1315–1325.

Berry, A., Devault, J. D., and Chakrabarty, A. M. (1989). High osmolarity is a signal for enhanced algD transcription in mucoid and non-mucoid *Pseudomonas aeruginosa* strains. *J. Bacteriol.* 171, 2312–2317.

Blatch, G. L., and Lassle, M. (1999). The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *Bioessays* 21, 932–939.

Boles, B. R., Thoendel, M., and Singh, P. K. (2004). Self-generated diversity produces "insurance effects" in biofilm communities. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16630–16635.

Bonsor, D. A., Grishkovskaya, I., Dodson, E. J., and Kleanthous, C. (2007). Molecular mimicry enables competitive recruitment by a natively disordered protein. *J. Am. Chem. Soc.* 129, 4800–4807.

Boyd, A., Ghosh, M., May, T. B., Shinabarger, D., Keogh, R., and Chakrabarty, A. M. (1993). Sequence of the algL gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. *Gene* 131, 1–8.

Burns, J. L., Gibson, R. L., Mcnamara, S., Yim, D., Emerson, J., Rosenfeld, M., Hiatt, P., Mccoy, K., Castile, R., Smith, A. L., and Ramsey, B. W. (2001). Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. *J. Infect. Dis.* 183, 444–452.

Byrd, M. S., Sadovskaya, I., Vinogradov, E., Lu, H., Sprinkle, A. B., Richardson, S. H., Ma, L., Ralston, B., Parsek, M. R., Anderson, E. M., Lam, J. S., and Wozniak, D. J. (2009). Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Mol. Microbiol.* 73, 622–638.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, D233–D238.

Chitnis, C. E., and Ohman, D. E. (1990). Cloning of *Pseudomonas aeruginosa* algG, which controls alginate structure. *J. Bacteriol.* 172, 2894–2900.

Chua, T. K., Bujnicki, J. M., Tan, T. C., Huynh, F., Patel, B. K., and Sivaraman, J. (2008). The structure of sucrose phosphate synthase from *Halothermothrix orenii* reveals its mechanism of action and binding mode. *Plant Cell* 20, 1059–1072.

Colvin, K. M., Gordon, V. D., Murakami, K., Borlee, B. R., Wozniak, D. J., Wong, G. C., and Parsek, M. R. (2011). The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog.* 7, e1001264. doi: 10.1371/journal.ppat.1001264

Coulon, C., Vinogradov, E., Filloux, A., and Sadovskaya, I. (2010). Chemical analysis of cellular and extracellular carbohydrates of a biofilm-forming strain *Pseudomonas aeruginosa* PA14. *PLoS ONE* 5, e14220. doi: 10.1371/journal.pone.0014220

Coutinho, P. M., Deleury, E., Davies, G. J., and Henrissat, B. (2003). An evolving hierarchical family classification for glycosyltransferases. *J. Mol. Biol.* 328, 307–317.

Coutinho, P. M., and Henrissat, B. (1999). Carbohydrate-Active Enzymes. Avialable at: http://afmb.cnrs-mrs.fr/(cazy/CAZY/index.html

Coyne, M. J. Jr., Russell, K. S., Coyle, C. L., and Goldberg, J. B. (1994). The *Pseudomonas aeruginosa* algC gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. *J. Bacteriol.* 176, 3500–3507.

Cuthbertson, L., Mainprize, I. L., Naismith, J. H., and Whitfield, C. (2009).

Pivotal roles of the outer membrane polysaccharide export and polysaccharide copolymerase protein families in export of extracellular polysaccharides in gram-negative bacteria. *Microbiol. Mol. Biol. Rev.* 73, 155–177.

D'Andrea, L. D., and Regan, L. (2003). TPR proteins: the versatile helix. *Trends Biochem. Sci.* 28, 655–662.

Darzins, A., Frantz, B., Vanags, R. I., and Chakrabarty, A. M. (1986). Nucleotide sequence analysis of the phosphomannose isomerase gene (pmi) of *Pseudomonas aeruginosa* and comparison with the corresponding *Escherichia coli* gene manA. *Gene* 42, 293–302.

De Angelis, F., Lee, J. K., O'connell, J. D. III, Miercke, L. J., Verschueren, K. H., Srinivasan, V., Bauvois, C., Govaerts, C., Robbins, R. A., Ruysschaert, J. M., Stroud, R. M., and Vandenbussche, G. (2010). Metal-induced conformational changes in ZneB suggest an active role of membrane fusion proteins in efflux resistance systems. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11038–11043.

Deretic, V., Gill, J. F., and Chakrabarty, A. M. (1987). Gene algD coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa. J. Bacteriol.* 169, 351–358.

DeVault, J. D., Berry, A., Misra, T. K., Darzins, A., and Chakrabarty, A. M. (1989). Environmental sensory signals and microbial pathogenesis: *Pseudomonas aeruginosa* infection in cystic fibrosis. *Biotechnology (N.Y.)* 7, 352–357.

DeVault, J. D., Kimbara, K., and Chakrabarty, A. M. (1990). Pulmonary dehydration and infection in cystic fibrosis: evidence that ethanol activates gene expression and induction of mucoidy in *Pseudomonas aeruginosa. Mol. Microbiol.* 4, 737–745.

Dong, C., Beis, K., Nesper, J., Brunkan-Lamontagne, A. L., Clarke, B. R., Whitfield, C., and Naismith, J. H. (2006). Wza the translocon for *E. coli* capsular polysaccharides defines a new class of membrane protein. *Nature* 444, 226–229.

Douthit, S. A., Dlakic, M., Ohman, D. E., and Franklin, M. J. (2005). Epimerase active domain of *Pseudomonas aeruginosa* AlgG, a protein that contains a right-handed beta-helix. *J. Bacteriol.* 187, 4573–4583.

Drummelsmith, J., and Whitfield, C. (1999). Gene products required for surface expression of the capsular form of the group 1 K antigen in *Escherichia coli* (O9a:K30). *Mol. Microbiol.* 31, 1321–1332.

Evans, L. R., and Linker, A. (1973). Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa. J. Bacteriol.* 116, 915–924.

Flynn, J. L., and Ohman, D. E. (1988). Cloning of genes from mucoid *Pseudomonas aeruginosa* which control spontaneous conversion to the alginate production phenotype. *J. Bacteriol.* 170, 1452–1460.

Franklin, M. J., Chitnis, C. E., Gacesa, P., Sonesson, A., White, D. C., and Ohman, D. E. (1994). *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. *J. Bacteriol.* 176, 1821–1830.

Franklin, M. J., Douthit, S. A., and Mcclure, M. A. (2004). Evidence that the algI/algJ gene cassette, required for O acetylation of *Pseudomonas aeruginosa* alginate, evolved by lateral gene transfer. *J. Bacteriol.* 186, 4759–4773.

Franklin, M. J., and Ohman, D. E. (1993). Identification of algF in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. *J. Bacteriol.* 175, 5057–5065.

Franklin, M. J., and Ohman, D. E. (2002). Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa. J. Bacteriol.* 184, 3000–3007.

Friedman, L., and Kolter, R. (2004a). Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol. Microbiol.* 51, 675–690.

Friedman, L., and Kolter, R. (2004b). Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J. Bacteriol.* 186, 4457–4465.

Fritz, T. A., Hurley, J. H., Trinh, L. B., Shiloach, J., and Tabak, L. A. (2004). The beginnings of mucin biosynthesis: the crystal structure of UDP-GalNAc:polypeptide alpha-N-acetylgalactosaminyltransferase-T1. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15307–15312.

Gacesa, P. (1988). Alginates. *Carbohydr. Polym.* 8, 161–182.

Goldberg, J. B., Hatano, K., and Pier, G. B. (1993). Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. *J. Bacteriol.* 175, 1605–1611.

Govan, J. R., and Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and Burkholderia cepacia. *Microbiol. Rev.* 60, 539–574.

Grangeasse, C., Cozzone, A. J., Deutscher, J., and Mijakovic, I. (2007). Tyrosine phosphorylation: an emerging regulatory device of bacterial physiology. *Trends Biochem. Sci.* 32, 86–94.

Guerin, M. E., Kordulakova, J., Schaeffer, F., Svetlikova, Z., Buschiazzo, A., Giganti, D., Gicquel, B., Mikusova, K., Jackson, M., and Alzari, P. M. (2007). Molecular recognition and interfacial catalysis by the essential phosphatidylinositol mannosyltransferase PimA from mycobacteria. *J. Biol. Chem.* 282, 20705–20714.

Hay, I. D., Rehman, Z. U., Ghafoor, A., and Rehm, B. H. (2009). Bacterial biosynthesis of alginates. *J. Chem. Technol. Biotechnol.* 85, 752–759.

Hay, I. D., Rehman, Z. U., and Rehm, B. H. (2010). Membrane topology of outer membrane protein AlgE, which is required for alginate production in *Pseudomonas aeruginosa. Appl. Environ. Microbiol.* 76, 1806–1812.

He, X., Szewczyk, P., Karyakin, A., Evin, M., Hong, W. X., Zhang, Q., and Chang, G. (2010). Structure of a cation-bound multidrug and toxic compound extrusion transporter. *Nature* 467, 991–994.

Heaton, M. P., and Neuhaus, F. C. (1992). Biosynthesis of D-alanyl-lipoteichoic acid: cloning, nucleotide sequence, and expression of the Lactobacillus casei gene for the D-alanine-activating enzyme. *J. Bacteriol.* 174, 4707–4717.

Hendrickson, E. L., Plotnikova, J., Mahajan-Miklos, S., Rahme, L. G., and Ausubel, F. M. (2001). Differential roles of the *Pseudomonas aeruginosa* PA14 rpoN gene in pathogenicity in plants, nematodes, insects, and mice. *J. Bacteriol.* 183, 7126–7134.

Irie, Y., Starkey, M., Edwards, A. N., Wozniak, D. J., Romeo, T., and Parsek, M. R. (2010). *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol. Microbiol.* 78, 158–172.

Jackson, K. D., Starkey, M., Kremer, S., Parsek, M. R., and Wozniak, D. J. (2004). Identification of psl, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J. Bacteriol.* 186, 4466–4475.

Jain, S., Franklin, M. J., Ertesvag, H., Valla, S., and Ohman, D. E. (2003). The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa. Mol. Microbiol.* 47, 1123–1133.

Jain, S., and Ohman, D. E. (1998). Deletion of algK in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. *J. Bacteriol.* 180, 634–641.

Jain, S., and Ohman, D. E. (2005). Role of an alginate lyase for alginate transport in mucoid *Pseudomonas aeruginosa. Infect. Immun.* 73, 6429–6436.

Jenkins, J., Mayans, O., Smith, D., Worboys, K., and Pickersgill, R. W. (2001). Three-dimensional structure of *Erwinia chrysanthemi* pectin methylesterase reveals a novel esterase active site. *J. Mol. Biol.* 305, 951–960.

Jenkins, J., and Pickersgill, R. (2001). The architecture of parallel beta-helices and related folds. *Prog. Biophys. Mol. Biol.* 77, 111–175.

Keiski, C. L., Harwich, M., Jain, S., Neculai, A. M., Yip, P., Robinson, H., Whitney, J. C., Riley, L., Burrows, L. L., Ohman, D. E., and Howell, P. L. (2010). AlgK is a TPR-containing protein and the periplasmic component of a novel exopolysaccharide secretin. *Structure* 18, 265–273.

Kelley, L. A., and Sternberg, M. J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.* 4, 363–371.

King, J. D., Kocincova, D., Westman, E. L., and Lam, J. S. (2009). Review: lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa. Innate Immun.* 15, 261–312.

Kintz, E., and Goldberg, J. B. (2008). Regulation of lipopolysaccharide O antigen expression in *Pseudomonas aeruginosa. Future Microbiol.* 3, 191–203.

Kowalska, K., Soscia, C., Combe, H., Vasseur, P., Voulhoux, R., and Filloux, A. (2010). The C-terminal amphipathic alpha-helix of *Pseudomonas aeruginosa* PelC outer membrane protein is required for its function. *Biochimie* 92, 33–40.

Krogh, A., Larsson, B., Von Heijne, G., and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–580.

Larue, K., Kimber, M. S., Ford, R., and Whitfield, C. (2009). Biochemical and structural analysis of bacterial O-antigen chain length regulator proteins reveals a conserved quaternary structure. *J. Biol. Chem.* 284, 7395–7403.

Lee, V. T., Matewish, J. M., Kessler, J. L., Hyodo, M., Hayakawa, Y., and Lory, S. (2007). A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Mol. Microbiol.* 65, 1474–1484.

Li, J., Qian, X., Hu, J., and Sha, B. (2009). Molecular chaperone Hsp70/Hsp90 prepares the mitochondrial outer membrane translocon receptor Tom71 for preprotein loading. *J. Biol. Chem.* 284, 23852–23859.

Linker, A., and Jones, R. S. (1964). A polysaccharide resembling alginic acid from a *Pseudomonas* microorganism. *Nature* 204, 187–188.

Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2000). Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect.* 2, 1051–1060.

Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* 15, 194–222.

Ma, L., Conover, M., Lu, H., Parsek, M. R., Bayles, K., and Wozniak, D. J. (2009). Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog.* 5, e1000354. doi: 10.1371/journal.ppat.1000354

Ma, L., Jackson, K. D., Landry, R. M., Parsek, M. R., and Wozniak, D. J. (2006). Analysis of *Pseudomonas aeruginosa* conditional Psl variants reveals roles for the Psl polysaccharide in adhesion and maintaining biofilm structure postattachment. *J. Bacteriol.* 188, 8213–8221.

Maharaj, R., May, T. B., Wang, S. K., and Chakrabarty, A. M. (1993). Sequence of the alg8 and alg44 genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. *Gene* 136, 267–269.

Martin, D. W., Schurr, M. J., Yu, H., and Deretic, V. (1994). Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to sigma E and stress response. *J. Bacteriol.* 176, 6688–6696.

Merighi, M., Lee, V. T., Hyodo, M., Hayakawa, Y., and Lory, S. (2007). The second messenger bis-(3′-5′)-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 65, 876–895.

Molgaard, A., and Larsen, S. (2002). A branched N-linked glycan at atomic resolution in the 1.12 A structure of rhamnogalacturonan acetylesterase. *Acta Crystallogr. D Biol. Crystallogr.* 58, 111–119.

Nivens, D. E., Ohman, D. E., Williams, J., and Franklin, M. J. (2001). Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J. Bacteriol.* 183, 1047–1057.

Oglesby, L. L., Jain, S., and Ohman, D. E. (2008). Membrane topology and roles of *Pseudomonas aeruginosa* Alg8 and Alg44 in alginate polymerization. *Microbiology* 154, 1605–1615.

Ohman, D. E. (1986). Molecular genetics of exopolysaccharide production by mucoid *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 5, 6–10.

Olvera, C., Goldberg, J. B., Sanchez, R., and Soberon-Chavez, G. (1999). The *Pseudomonas aeruginosa* algC gene product participates in rhamnolipid biosynthesis. *FEMS Microbiol. Lett.* 179, 85–90.

Oomen, C. J., Van Ulsen, P., Van Gelder, P., Feijen, M., Tommassen, J., and Gros, P. (2004). Structure of the translocator domain of a bacterial autotransporter. *EMBO J.* 23, 1257–1266.

Pelissier, M. C., Lesley, S. A., Kuhn, P., and Bourne, Y. (2010). Structural insights into the catalytic mechanism of bacterial guanosine-diphospho-D-mannose pyrophosphorylase and its regulation by divalent ions. *J. Biol. Chem.* 285, 27468–27476.

Pier, G. B., Coleman, F., Grout, M., Franklin, M., and Ohman, D. E. (2001). Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. *Infect. Immun.* 69, 1895–1901.

Ramelot, T. A., Yee, A., Cort, J. R., Semesi, A., Arrowsmith, C. H., and Kennedy, M. A. (2007). NMR structure and binding studies confirm that PA4608 from *Pseudomonas aeruginosa* is a PilZ domain and a c-di-GMP binding protein. *Proteins* 66, 266–271.

Ramsey, D. M., and Wozniak, D. J. (2005). Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Mol. Microbiol.* 56, 309–322.

Regni, C., Tipton, P. A., and Beamer, L. J. (2002). Crystal structure of PMM/PGM: an enzyme in the biosynthetic pathway of *P. aeruginosa virulence factors. Structure* 10, 269–279.

Rehm, B. H., Boheim, G., Tommassen, J., and Winkler, U. K. (1994). Overexpression of algE in *Escherichia coli*: subcellular localization, purification, and ion channel properties. *J. Bacteriol.* 176, 5639–5647.

Remminghorst, U., and Rehm, B. H. (2006a). Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. *FEBS Lett.* 580, 3883–3888.

Remminghorst, U., and Rehm, B. H. (2006b). In vitro alginate polymerization and the functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 72, 298–305.

Robles-Price, A., Wong, T. Y., Sletta, H., Valla, S., and Schiller, N. L. (2004). AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 7369–7377.

Romling, U. (2002). Molecular biology of cellulose production in bacteria. *Res. Microbiol.* 153, 205–212.

Rozeboom, H. J., Bjerkan, T. M., Kalk, K. H., Ertesvag, H., Holtan, S., Aachmann, F. L., Valla, S., and Dijkstra, B. W. (2008). Structural and mutational characterization of the catalytic A-module of the mannuronan C-5-epimerase AlgE4 from *Azotobacter vinelandii*. *J. Biol. Chem.* 283, 23819–23828.

Saxena, I. M., and Brown, R. M. Jr. (2005). Cellulose biosynthesis: current views and evolving concepts. *Ann. Bot.* 96, 9–21.

Schiller, N. L., Monday, S. R., Boyd, C. M., Keen, N. T., and Ohman, D. E. (1993). Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (algL): cloning, sequencing, and expression in *Escherichia coli*. *J. Bacteriol.* 175, 4780–4789.

Sibley, C. D., Duan, K., Fischer, C., Parkins, M. D., Storey, D. G., Rabin, H. R., and Surette, M. G. (2008). Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. *PLoS Pathog.* 4, e1000184. doi: 10.1371/journal.ppat.1000184

Silo-Suh, L., Suh, S. J., Sokol, P. A., and Ohman, D. E. (2002). A simple alfalfa seedling infection model for *Pseudomonas aeruginosa* strains associated with cystic fibrosis shows AlgT (sigma-22) and RhlR contribute to pathogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15699–15704.

Snook, C. F., Tipton, P. A., and Beamer, L. J. (2003). Crystal structure of GDP-mannose dehydrogenase: a key enzyme of alginate biosynthesis in *P. aeruginosa*. *Biochemistry* 42, 4658–4668.

Spiers, A., Kahn, S., Bohannon, J., Travisano, M., and Rainey, P. (2002). Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. *Genetics* 16, 33–46.

Starkey, M., Hickman, J. H., Ma, L., Zhang, N., De Long, S., Hinz, A., Palacios, S., Manoil, C., Kirisits, M. J., Starner, T. D., Wozniak, D. J., Harwood, C. S., and Parsek, M. R. (2009). *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *J. Bacteriol.* 191, 3492–3503.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., and Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 406, 959–964.

Tan, M. W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G., and Ausubel, F. M. (1999). *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proc. Natl. Acad. Sci. U.S.A.* 96, 2408–2413.

Tocilj, A., Munger, C., Proteau, A., Morona, R., Purins, L., Ajamian, E., Wagner, J., Papadopoulos, M., Van Den Bosch, L., Rubinstein, J. L., Fethiere, J., Matte, A., and Cygler, M. (2008). Bacterial polysaccharide co-polymerases share a common framework for control of polymer length. *Nat. Struct. Mol. Biol.* 15, 130–138.

Ueda, A., and Wood, T. K. (2009). Connecting quorum sensing, c-di-GMP, Pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog.* 5, e1000483. doi: 10.1371/journal.ppat.1000483

Urch, J. E., Hurtado-Guerrero, R., Brosson, D., Liu, Z., Eijsink, V. G., Texier, C., and Van Aalten, D. M. (2009). Structural and functional characterization of a putative polysaccharide deacetylase of the human parasite *Encephalitozoon cuniculi*. *Protein Sci.* 18, 1197–1209.

Vasseur, P., Soscia, C., Voulhoux, R., and Filloux, A. (2007). PelC is a *Pseudomonas aeruginosa* outer membrane lipoprotein of the OMA family of proteins involved in exopolysaccharide transport. *Biochimie* 89, 903–915.

Wang, H., Robinson, H., and Ke, H. (2010). Conformation changes, N-terminal involvement, and cGMP signal relay in the phosphodiesterase-5 GAF domain. *J. Biol. Chem.* 285, 38149–38156.

Wang, X., Preston, J. F. III, and Romeo, T. (2004). The pgaABCD locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J. Bacteriol.* 186, 2724–2734.

Weadge, J. T., Yip, P. P., Robinson, H., Arnett, K., Tipton, P. A., and Howell, P. L. (2010). Expression, purification, crystallization and preliminary X-ray analysis of *Pseudomonas aeruginosa* AlgX. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 66, 588–591.

Whitfield, C. (2006). Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Annu. Rev. Biochem.* 75, 39–68.

Whitney, J. C., Hay, I. D., Li, C., Eckford, P. D. W., Robinson, H., Amaya, M. F., Wood, L. F., Ohman, D. E., Bear, C. E., Rehm, B. H., and Howell, P. L. (2011). Structural basis for alginate secretion across the bacterial outer membrane. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13083–13088.

Whitney, J. C., Neculai, A. M., Ohman, D. E., and Howell, P. L. (2009). Expression, refolding, crystallization and preliminary X-ray analysis of *Pseudomonas aeruginosa* AlgE. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 65, 463–466.

Wood, L. F., Leech, A. J., and Ohman, D. E. (2006). Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. *Mol. Microbiol.* 62, 412–426.

Yang, J. K., Yoon, H. J., Ahn, H. J., Lee, B. I., Pedelacq, J. D., Liong, E. C., Berendzen, J., Laivenieks, M., Vieille, C., Zeikus, G. J., Vocadlo, D. J., Withers, S. G., and Suh, S. W. (2004). Crystal structure of beta-D-xylosidase from *Thermoanaerobacterium saccharolyticum*, a family 39 glycoside hydrolase. *J. Mol. Biol.* 335, 155–165.

Yoon, H. J., Mikami, B., Hashimoto, W., and Murata, K. (1999). Crystal structure of alginate lyase A1-III from *Sphingomonas* species A1 at 1.78 A resolution. *J. Mol. Biol.* 290, 505–514.

Zhang, Z., Kulkarni, K., Hanrahan, S. J., Thompson, A. J., and Barford, D. (2010). The APC/C subunit Cdc16/Cut9 is a contiguous tetratricopeptide repeat superhelix with a homo-dimer interface similar to Cdc27. *EMBO J.* 29, 3733–3744.

Zielinski, N. A., Chakrabarty, A. M., and Berry, A. (1991). Characterization and regulation of the *Pseudomonas aeruginosa* algC gene encoding phosphomannomutase. *J. Biol. Chem.* 266, 9754–9763.

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Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide

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Lipopolysccharide (LPS) is an integral component of the *Pseudomonas aeruginosa* cell envelope, occupying the outer leaflet of the outer membrane in this Gram-negative opportunistic pathogen. It is important for bacterium–host interactions and has been shown to be a major virulence factor for this organism. Structurally, *P. aeruginosa* LPS is composed of three domains, namely, lipid A, core oligosaccharide, and the distal O antigen (O-Ag). Most *P. aeruginosa* strains produce two distinct forms of O-Ag, one a homopolymer of D-rhamnose that is a common polysaccharide antigen (CPA, formerly termed A band), and the other a heteropolymer of three to five distinct (and often unique dideoxy) sugars in its repeat units, known as O-specific antigen (OSA, formerly termed B band). Compositional differences in the O units among the OSA from different strains form the basis of the International Antigenic Typing Scheme for classification via serotyping of different strains of *P. aeruginosa*. The focus of this review is to provide state-of-the-art knowledge on the genetic and resultant functional diversity of LPS produced by *P. aeruginosa*. The underlying factors contributing to this diversity will be thoroughly discussed and presented in the context of its contributions to host–pathogen interactions and the control/prevention of infection.

Keywords: lipopolysaccharide, serotyping, biosynthesis, motility, virulence, seroconversion, bacteriophage, nucleotide sugars

INTRODUCTION AND HISTORICAL PERSPECTIVES

To classify the differences among *Pseudomonas aeruginosa* strains isolated from environmental or clinical settings, serological methods have been employed whereby polyclonal antisera were raised against representative strains, then cross-adsorbed with other *P. aeruginosa* strains in order to yield a collection of strain-specific antisera. In order to exploit the specificity of the antibodies within these antisera, a number of immunochemical techniques were employed including slide agglutination tests and gel-diffusion precipitation reactions. The former constitutes a rather simple method of visualizing clumping of bacteria on a microscope slide by a small aliquot of specific antiserum in a matter of seconds, while the latter allows the diffusion of antibodies and antigen, usually bacterial cell lysates, from adjacent sample wells in agarose gels until equivalent amounts of these reagents meet to form visible precipitins (Mutharia and Lam, 2007). Interestingly, the precipitin reactions also provide the resolution to distinguish between cross-reacting groups, thereby giving rise to subgrouping among a specific serotype, for instance, serogroups 2a2b, 2a2c, etc. (Stanislavsky and Lam, 1997). Though tedious, these serotyping methods were the gold standards approximately 30 years ago, and are still being used by certain public health laboratories due to the low cost and rapidity in obtaining results. Since various laboratories from different parts of the world prepare their own antisera, many *P. aeruginosa* serotyping schemes have been established, such as the Homma, Habs, Lanyi and Bergan, and Fisher Immunotyping systems (reviewed in Stanislavsky and Lam, 1997). In order to standardize results stemming from the different serotyping systems, Liu et al. (1983) coordinated a special meeting between the *P. aeruginosa* serotyping experts, with the initiative giving rise to a standardized serotype classification termed the International Antigenic Typing Scheme (IATS), which has 17 serotypes based on Habs serotypes 1–12 plus 5 more serotypes from other serotyping systems (Stanislavsky and Lam, 1997). A subsequent study added 3 more serotypes to bring the total to 20 (Liu and Wang, 1990). Note that from this point onward in this review, the serotypes of *P. aeruginosa* strains mentioned are based on the IATS classification. Although it has been known that variations in the cell-surface lipopolysaccharide (LPS) of *P. aeruginosa* are responsible for serotyping, knowledge of the chemical structures of LPS and the underlying genetics of the biosynthetic process was lacking and prompted new research interests in this area.

The IATS serotyping scheme has been generally effective for classifying *P. aeruginosa* strains that are wildtype organisms producing smooth LPS possessing three distinct domains, namely, lipid A, core oligosaccharide (OS), and *O* polysaccharides or O antigens (O-Ag). This is not often the case in clinical settings as many of these isolates are found to be either partially lacking or completely devoid of O-Ag. Serotyping of chronic bacterial isolates from cystic fibrosis (CF) patients for epidemiological studies was particularly problematic because a very high proportion of these bacteria were found to be either polytypeable by more than one serotyping antisera or non-typable (NT). This has prompted the generation of new typing reagents based on monoclonal antibodies (mAbs) specific against each of the original 17 IATS serotypes (Lam et al., 1987a,b). In ensuing studies, comparison of the *P. aeruginosa* typing efficiencies with the complete set of anti-O1 to anti-O17 mAbs versus other methods, such as phage-susceptibility typing, pyocin typing, and restriction fragment length polymorphism (RFLP) DNA fingerprinting was performed (Ojeniyi et al., 1989, 1990; Speert et al., 1994); these methods differed substantially in their capacity to indentify unique typing patterns. Although RFLP was deemed to have the greatest discrimination power, LPS-based serotyping appears

preferable for other indications because it is the simplest to perform. The use of mAb typing reagents was found to be more specific and accurate than the use of polyclonal antibody typing kits in assigning a specific serotype designation to many of the polytypeable *P. aeruginosa* isolates (Dasgupta et al., 1994). However, to classify strains that have been determined as NT by serotyping, other methods are required, for instance, the use of molecular techniques (discussed below as a consequence of sequencing results of all 20 IATS O-Ag biosynthesis gene clusters). This provided the rationale for investigating LPS biosynthesis and its assembly using genetic and biochemical approaches.

GENETICS OF O-SPECIFIC ANTIGEN BIOSYNTHESIS

Most *P. aeruginosa* strains produce two different forms of O-Ag, one of which contains a homopolymer of D-rhamnose, arranged in $\alpha1 \rightarrow 3$, $\alpha1 \rightarrow 2, \alpha1 \rightarrow 3$ trisaccharide repeats. This homopolymeric D-rhamnan is the common polysaccharide antigen (CPA; formerly termed A band) among *P. aeruginosa* strains. More details of the properties of CPA will be discussed in later sections. The other form of O-Ag produced by *P. aeruginosa* is a heteropolymer with three to five distinct sugars in its repeat units, and is named O-specific antigen (OSA; formerly termed B band). The differences of the composition of the O units form the basis of the IATS serotyping scheme. Knirel et al. (2006) have made a significant contribution to our understanding of all the serotypes by systematically elucidating the chemical structures of the OSA of all 20 IATS serotypes (**Table 1**). To appreciate the complexity and diversity of LPS produced by *P. aeruginosa*, it is noteworthy that heterogeneity exists in the chain length of either form of O-Ag present on the cell surface used to "cap" the core OS of *P. aeruginosa* (**Figure 1**). This accounts for the "ladder-banding" pattern when LPS from *P. aeruginosa* is analyzed by silver-stained SDS-PAGE gels and Western immunoblotting (Rocchetta et al., 1998a).

The importance of the OSA toward virulence and its use in serotyping has made it an attractive target for genetic studies to help understand both the plasticity of this region of the LPS and the complex steps of OSA biosynthesis. OSA biosynthesis follows the Wzy-dependent pathway model, originally proposed by Whitfield (1995); it involves the sequential activities of a series of integral inner membrane (IM) proteins, for which we have recently obtained comprehensive topological data helping to explain their respective functions (Islam et al., 2010). In this model, OSA sugar repeats are sequentially built on the lipid carrier undecaprenyl pyrophosphate (UndPP) on the cytoplasmic face of the IM. The UndPP-linked OSA repeats are then translocated to the periplasmic face of the IM by the flippase Wzx (Burrows and Lam, 1999), where they are polymerized by Wzy (De Kievit et al., 1995) through a putative catch-and-release mechanism (Islam et al., 2011), to modal lengths regulated by Wzz_1 (Burrows et al., 1997) and

Table 1 | O-Antigen repeating units which compose the 20 IATS reference strains.

IATS serotype	O-specific antigen repeat*
O1	→4)-D-GalNAc-(1 → 4)-D-GlcNAc3NAcA-D-(β1→3)-D-FucNAc-(1→3)-D-QuiNAc-(1→
O2	→4)-D-ManNAc3NAmA-(β1→4)-L-GulNAc3NAcA-(1→3)-D-FucNAc-(β1→
O5	→4)-D-ManNAc3NAmA-(β1→4)-D-ManNAc3NAcA-(β1→3)-D-FucNAc-(1→
O16	→4)-D-ManNAc3NAmA-(β1→4)-D-ManNAc3NAcA-(β1→3)-D-FucNAc-(β1→
O18	→4)-L-GulNAc3NAmA-(1→4)-D-ManNAc3NAcA-(β1→3)-D-FucNAc-(1→
O20	→4)-L-GulNAc3NAmA-(1→4)-D-ManNAc3NAcA-(β1→3)-D-FucNAc4OAc-(1→
O3	→2)-L-Rha3OAc-(1→6)-D-GlcNAc-(1→4)-L-GalNAc4OAcA-(1→3)-D-QuiNAc4N*S*Hb (β1→
O4	→2)-L-Rha-(1→3)-L-FucNAc-(1→3)-L-FucNAc-(1→3)-D-QuiNAc-(1→
O6	→3)-L-Rha-(1→4)-D-GalNAc3OAcAN-(1→4)-D-GalNFoA-(1→3)-D-QuiNAc-(1→
O7	→4)-Pse4OAc5N*R*Hb7NFo-(2→4)-D-Xyl-(β1→3)-D-FucNAc-(1→
O8	→4)-Pse4OAc5NAc7NFo-(2→4)-D-Xyl-(β1→3)-D-FucNAc-(1→
O9**	→3′)-Pse4OAc5NAc7N*R*Hb-(β2→4)-D-FucNAc-(1→3)-D-QuiNAc-(β1→
O10	→3)-L-Rha2OAc-(1→4)-L-GalNAcA-(1→3)-D-QuiNAc-(1→
O19	→3)-L-Rha-(1→4)-L-GalNAcA-(1→3)-D-QuiNAc-(1→
O11	→2)-D-Glc-(β1→3)-L-FucNAc-(1→3)-D-FucNAc-(β1→
O12	→8)-8eLeg5NAc7NAc-(2→3)-L-FucNAm-(1→3)-D-QuiNAc-(1→
O13	→)-L-Rha-(1→3)-L-Rha-(1→4)-D-GalNAc3OAcA-(1→3)-D-QuiNAc-(β1→
O14	→)[D-Glc-1→3)]-L-Rha-(1→3)-L-Rha-(1→4)-D-GalNAc3OAcA-(1→3)-D-QuiNAc-(β1→
O15	→2)-D-Rib*f*-(β1→4)-D-GalNAc-(1→
O17	→4)-L-Rha-(1→3)-D-ManNAc-(β1→

**Adapted from Knirel et al. (2006), O-Ag repeats are clustered according to structural similarity. Conserved sugars, colored by backbone structure, are adorned with various side groups depicting the diverse phenotypes generated by the OSA gene cluster throughout all 20 serotypes. Anomeric conformations are α unless marked β. Sugars have the pyranose form except ribose in O15. Ac, acetyl; 8eLeg, 5,7-diamino-3,5,7,9-tetradeoxy-L-glycero-D-galacto-non-2-ulosonic (8-epilegionaminic) acid; Fo, formyl; FucN, 2-amino-2,6-dideoxy-galactose; GalN, 2-amino-2-deoxy-galactose; GlcNA, 2-amino-2-deoxy-glucuronic acid; GulNA, 2-amino-2-deoxy-guluronic acid; ManN 2-amino-2-deoxy-mannose; ManNA, 2-amino-2-deoxy-mannuronic acid; N, amino; NAc, acetamido; NAμ, acetamidino; OAc, O-acetyl; Pse, 5,7-diamino-3,5,7,9-tetradeoxy-L-glycero-L-manno-non-2-ulosonic (pseudaminic) acid; QuiN, 2-amino-2,6-dideoxyglucose; Rha, rhamnose; RHb (R)-3-hydroxybutanoyl; Rib, ribose; SHb, (S)-3-hydroxybutanoyl.*

In the polymer, QuiNAc is linked to the pseudaminic acid via the 3-hydroxybutanoyl group.

***QuiNAc is linked to the pseudaminic acid via the 3-hydroxybutanoyl group.*

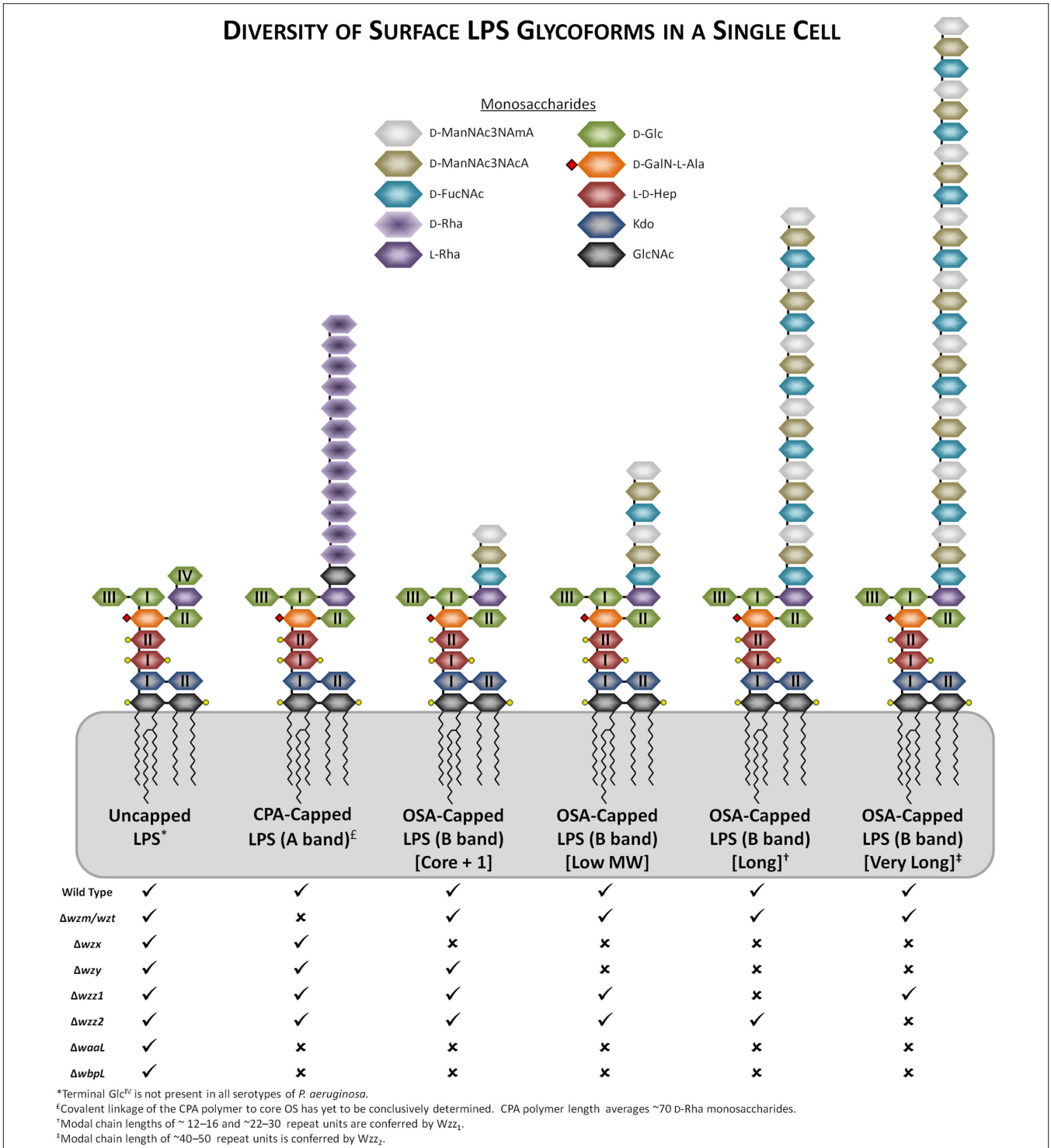

FIGURE 1 | Representations of the heterogeneity/diversity of the LPS glycoforms present on the surface of a single *P. aeruginosa* cell. Genetic defects in members of various assembly pathway genes and their resultant changes to the variety of LPS glycoforms present have been indicated, with "✓" or "×" representing their presence or absence, respectively. Substitutions of various lipid A and core OS sugars with phosphate groups and L-Ala have been indicated with yellow circles and red diamonds, respectively. The OSA polymer from serotype O5 has been displayed as a representative polymer.

Wzz_2 (Daniels et al., 2002). The modal length of OSA imparted by Wzz_2 (40–50 repeat length) is longer than that imparted by Wzz_1 (12–16 repeats and 22–30 repeats; Daniels et al., 2002). However, Wzz_1 is apparently more important for virulence than Wzz_2 (Kintz et al., 2008). Finally, the complete OSA chain is ligated to lipid A-core by the O-Ag ligase WaaL (Abeyrathne et al., 2005; Abeyrathne and Lam, 2007). Preliminary investigations mapped the OSA cluster to 37 min of the *P. aeruginosa* PAO1 (serotype O5) genome (Lightfoot

and Lam, 1993), corresponding to *pa3141* to *pa3160* in the annotated genome of strain PAO1 (Stover et al., 2000). This first reported LPS OSA cluster was isolated from a cosmid-based genomic library. Clone pFV100 from the library was able to complement mutant ge6, a Tn*5–751* insertional mutant of PAO1, defective in B band (OSA) biosynthesis (Lightfoot and Lam, 1993). Subsequently, Burrows et al. (1996) obtained the sequence of the entire OSA cluster. To characterize the function of the genes encoded in this cluster, knockout mutant constructs were generated for each of the genes and the mutants were examined for their effect on LPS production in *P. aeruginosa*. The list of OSA biosynthesis genes in this serotype O5 cluster and their functions were determined based on genetic studies as well as biochemical and chemical evidence (**Figure 2A**; **Table 2,** Burrows et al., 1996). Following the success in characterizing the O5 OSA biosynthesis locus, the sequences of the O6 (Belanger et al., 1999) and O11 (Dean et al., 1999) OSA loci were also determined. Comparisons among the newly sequenced loci revealed that all three OSA loci were flanked by *himD/ihfB* (*pa3161*) on the 5′ end and terminated with *wbpM* (*pa3141*) on the 3′ end. These observations are essential for establishing the conserved chromosomal locus for the OSA cluster, though the genes within the locus are the most varied in the *P. aeruginosa* genomes regardless of serotype. This information eventually allowed Raymond et al. (2002) to clone and sequence the OSA loci from all 20 IATS serotypes. Based on their sequencing data, the general genetic structures of the OSA loci of all the serotypes could be divided into 11 distinct groups based on the protein families that the genes in these loci encode, as well as the presence of insertion sequences (IS) and deletions (**Figure 2B**; Raymond et al., 2002). This group has also presented the sequences of a set of primers for PCR amplification of each of the IATS serotypes, meaning that a PCR-based approach can be used to correctly type clinical isolates that have previously been evaluated as NT by any typing antisera. However, thus far, there has been no systematic study conducted by any group to test the capability of using these primers for consistently typing clinical strains of *P. aeruginosa* even though the potential to do so exists. These initial genetic investigations helped to reveal differences among the OSA clusters; worth noting is the anomaly discovered regarding the entire loss of the OSA cluster in the O15 serotype. Strains that belong to this serotype were previously identified using both polyclonal antibody typing kits and mAb-based serotyping (Lam et al., 1987b). It was proposed by Raymond et al. (2002) that in serotype O15, the genes involved in its OSA biosynthesis may not necessarily be residing in the usual OSA locus as in other serotypes. Additionally, in serotype O6, the *wzy* gene does not reside in the OSA cluster (Belanger et al., 1999). Further, in serotype O5, the transcriptional start site for *wzx* exists within the *wzy* gene, and there is a large IS at the 3′ end of the O5 cluster upstream of *wbpM* (Burrows et al., 1996). This variation helps to explain the diversity of the LPS in *P. aeruginosa* as a property of the genetic differences among the IATS serotypes. Other factors that influence the OSA diversity are outlined in the following sections.

EVIDENCE OF HORIZONTAL GENE TRANSFER REVEALS THE PRESENCE OF CONSERVED AND DUPLICATED CLUSTERS IN CERTAIN SEROTYPES

The high conservation of the *wbpM* gene and its localization at the 3′ end of the OSA loci among all 20 serotypes is not the only commonality among all of the OSA loci. A major factor in genetic reassortment

Table 2 | *P. aeruginosa* PAO1 serotype O5 OSA biosynthesis cluster*.

Gene	Proposed/demonstrated function	Mol% G + C	References
wzz /pa3160	OSA chain length regulator	49.5	Burrows et al. (1997), Daniels et al. (2002)
wbpA/pa3159	UDP-*N*-acetyl-D-glucosamine 6-dehydrogenase	54.5	Miller et al. (2004)
wbpB/pa3158	UDP-2-acetamido-2-deoxy-D-glucuronic acid 3-dehydrogenase	52.8	Westman et al. (2009)
wbpC/pa3157	Possible *O*-acetyltransferase	53.1	
wbpD/pa3156	UDP-2-acetamido-3-amino-2,3-dideoxy-D-glucuronic acid *N*-acetyltransferase	53.9	Wenzel et al. (2005), Westman et al. (2009)
wbpE/pa3155	UDP-2-acetamido-2-dideoxy-D-ribo-hex-3-uluronic acid transaminase	52.8	Westman et al. (2009)
wzy/pa3154	OSA α-polymerase	44.6	De Kievit et al. (1995), Islam et al. (2010), Islam et al. (2011)
wzx/pa3153	OSA unit flippase	49.3	Burrows and Lam (1999), Islam et al. (2010)
hisH2/pa3152	Imidazole glycerol phosphate synthase subunit	49.3	King et al. (2009)
hisF2/pa3151	Imidazole glycerol phosphate synthase subunit	50.0	King et al. (2009)
wbpG/pa3150	Amidotransferase	44.5	
wbpH/pa3149	Glycosyltransferase	45.6	
wbpI/pa3148	UDP-*N*-acetylglucosamine 2-epimerase	50.2	Westman et al. (2009)
wbpJ/pa3147	Glycosyltransferase (GT-4)	54.5	
wbpK/pa3146	NAD-dependent epimerase/dehydratase	56.8	
wbpL/pa3145	Glycosyltransferase	55.5	Rocchetta et al. (1998a)
wbpM/pa3141	Nucleotide sugar epimerase/dehydratase	61.9	Creuzenet and Lam (2001)

**Modified from Burrows et al. (1996), the information in this table has been updated with references which indicate that either genetic or biochemical evidence have been collected to demonstrate the function of each of these genes within the OSA biosynthesis cluster of PAO1 serotype O5. Note that in contrast to the genome of P. aeruginosa, which has a relatively high mol% G + C content (67%), the genes within this cluster showed significantly lower mol% G + C, ranging from 45.2 to 61.9%, evidence of horizontal gene transfer.*

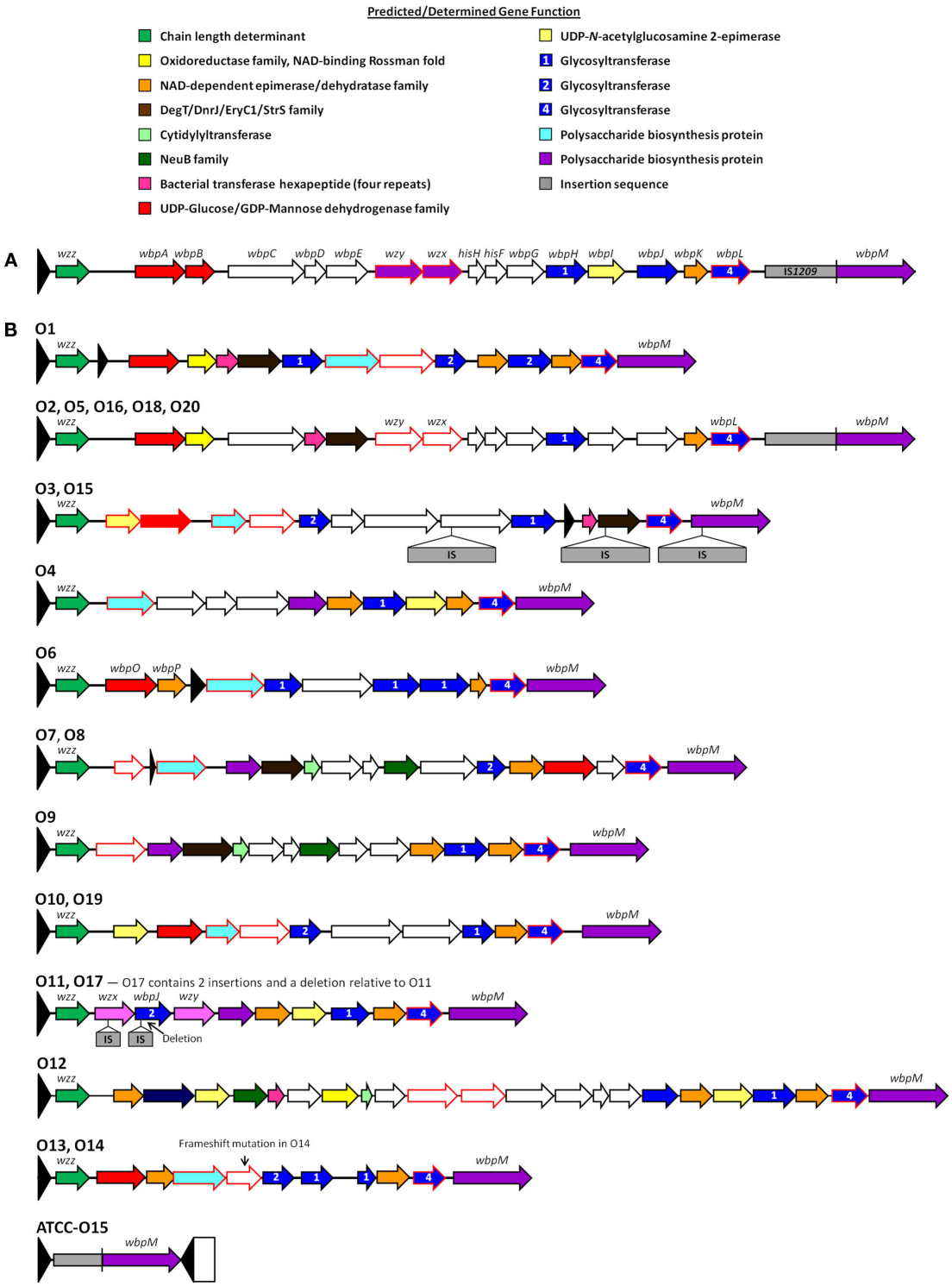

FIGURE 2 | Organization of the genes within OSA biosynthesis clusters. **(A)** OSA biosynthesis cluster of serotype O5 adapted from Burrows et al. (1996). The gene cluster is located on the complementary strand; genes which match the PFAM designation are colored accordingly. Genes not involved in OSA biosynthesis are depicted in gray including a large insertion sequence (IS). **(B)** Adapted from Raymond et al. (2002), the OSA biosynthesis gene clusters were organized into 11 groups based on sequence conservation. Genes were designated using the PFAM database; specific protein families which occur a minimum of three times throughout all 20 OSA biosynthesis clusters are represented by a specific color. A red outline depicts an ORF with potential transmembrane-spanning domains. Previously identified genes are labeled above the respected cluster if present within the serotype. Insertion sequences (IS) present within genes are depicted by a secondary gray box.

is horizontal gene transfer (HGT), i.e., the direct transmission of genetic information across the species barrier, which often results in similar/identical gene products. Interestingly, Dean and Goldberg (2000) probed all 20 serotypes for the presence of genes that are conserved in the O11 OSA biosynthesis cluster, and discovered that the IATS O17 strain possesses a cryptic copy of the O11 locus within its genome. The O11 cluster was apparently rendered non-functional due to the presence of IS*PA11B* disrupting the 5′ end of the *wzy* gene. However, when a complete O11 OSA cluster was supplied *in trans*, LPS produced by the complemented strain reacted with anti-O11 antiserum in Western immunoblotting. This suggested that the cryptic O11 serotype genes found within the O17 strain were at one time functional. The presence of a secondary locus within one genome lends support to HGT. Apparently, the OSA LPS genes for the O17 serotype that were acquired or have evolved may have caused a gradual loss of function or suppression to the O11 OSA biosynthesis gene cluster through the incorporation of an IS element. Interestingly, the O17 OSA serotype is very similar to the *Burkholderia cepacia* serogroup O5 antigen, confirming that this may be an ancestral locus which was acquired by HGT (Dean and Goldberg, 2000). Similarly, Raymond et al. (2002) discovered the presence of an additional cryptic locus corresponding to the O3 cluster within the genome of serotype O15, with further investigation revealing that the O3 cluster in the O15 strain was rendered non-functional due to IS elements at two different sites within the cluster.

Evidence that HGT contributes to the diversity of *P. aeruginosa* LPS is not limited to transfer of the entire OSA biosynthesis locus. Varying degrees of genetic information exchange between bacteria are apparent since bacterial pathogens of different genera share conserved metabolic pathways for the biosynthesis of nucleotide sugars. A number of rare sugars are found in the OSA of *P. aeruginosa* LPS, e.g., L-FucNAc (a constituent of the O11 OSA), di-*N*-acetylated mannuronic acid (D-ManNAc3NAcA, a constituent of the O5 OSA), and a uronamide sugar GalNAcAN (a constituent of O6 OSA). The metabolic pathways for the biosynthesis of the nucleotide-activated precursors for these rare sugars are only found in a few bacterial pathogens but not in humans (Kneidinger et al., 2003; Westman et al., 2008, 2009; King et al., 2010). The biosynthesis of UDP-L-FucNAc from the substrate UDP-D-GlcNAc, requires the products of three genes from the serotype O11 OSA cluster, namely, *wbjB*, *wbjC*, and *wbjD*. Two sets of functional homologs of these three genes are found in *Staphylococcus aureus*, namely *cap5E-cap5F-cap5G* and *cap8E-cap8F-cap8G* for the biosynthesis of serogroup-5 and serogroup-8 capsular polysaccharides, respectively (Kneidinger et al., 2003). The complexity of the biosynthesis of the UDP-activated precursor of D-ManNAc3NAcA requires five steps. Indeed, the activity of the encoded products of *wbpA*, *wbpB*, *wbpE*, *wbpD*, and *wbpI*, acting in this particular order, and also starting with UDP-GlcNAc as the starting substrate, have been fully characterized (Westman et al., 2008, 2009). The same pathway is shared by *Bordetella pertussis* as it also possesses D-ManNAc3NAcA as a sugar in its LPS. However, only four of the five genes, *wlbA*, *wlbB*, *wlbC*, *wlbD*, which are functional homologs of *wbpB*, *wbpE*, *wbpD,* and *wbpI*, respectively, are localized within the O-Ag biosynthesis gene clusters. Two copies of the homolog of *wbpA* were found elsewhere in the *B. pertussis* genome (Westman et al., 2008). In a separate investigation, our group has determined that *wbpO, wbpP,* and *wbpS* encode the enzymes for the three metabolic steps that convert UDP-GlcNAc by oxidation (WbpO), epimerization (WbpP), and amidotransfer (WbpS) to form the uronamide sugar precursor UDP-GalNAcAN for O6 OSA. Interestingly, functional homologs of these three genes were found in *Escherichia coli* O121. As well, homologs of these three genes were also found in *Francisella tularensis* (King et al., 2010); however, they have not been tested to determine whether they could complement knockout mutants of the *P. aeruginosa* genes to restore production of O6 LPS that contains GalNAcAN in its O-Ag unit. Therefore, *P. aeruginosa* and other bacterial pathogens likely acquired genes for the biosynthesis of the abovementioned UDP-sugars from common ancestors. However, investigation of the evolutionary derivations and lineages of these pathways is beyond the scope of this review.

EVIDENCE OF DIVERSITY IN LPS EXPRESSION IN *P. AERUGINOSA* DUE TO CHROMOSOMAL INSERTIONS

Large chromosomal inversions (LCIs), which can be induced by ISs, persist throughout Gram-negative bacteria and have altered the balance between chromosomal stability and variability. Clonal isolates taken from CF patients were found to contain additional, non-conserved LCIs (Schmidt et al., 1996). *P. aeruginosa* "clone C," found in various geographic locations and in non-CF patients (Kidd et al., 2011), possesses a particular additional sequence, IS*6100*, which was first discovered in *Mycobacterium tuberculosis* and known to cause genetic rearrangements in heterologous hosts. The LCIs of the "clone C" strains have been associated with *wbpM* (*pa3141*) disruption causing loss of the OSA (Kresse et al., 2003). The genomes of two CF isolates were compared to that of the wildtype PAO1, with regions of high variability localized to the OSA biosynthesis locus. Targeted sequencing of the OSA cluster provided a high-resolution view of the genetic changes that arise during clonal infection of CF patients. Genomes of all late-stage clinical isolates contained ISs, as judged by gel electrophoresis (Spencer et al., 2003). The OSA region of a clonal isolate (strain 1–60) was nearly identical to serotype O1 except for the presence of a large 1.5-kb IS, which disrupted a reading frame responsible for the biosynthesis of an unspecified nucleotide sugar. Additionally, a second strain (2–164) was found to contain a 2-bp deletion in *wbpQ*, which has not yet been characterized. Both of these observed mutations caused defects in LPS production and conferred a NT phenotype. Interestingly, some of the earlier NT strains did not contain the IS elements (Spencer et al., 2003). Strain-specific islands were discovered with high similarity to previously identified sequences (e.g., PAGI-1 in O-Ag biosynthesis) as well as some phage-related sequences; however, these contained a low mol% G + C content possibly indicating acquisition from other bacterial species (Spencer et al., 2003), since the *P. aeruginosa* genome has a high mol% G + C content. Whole-genome sequencing has begun to provide snapshots of these genetic changes, which occur during clonal infection; future work using this approach may provide a roadmap for navigating the infection stages of *P. aeruginosa.*

LYSOGENIC CONVERSION BY BACTERIOPHAGE ALTERS THE OSA SEROTYPE TO PROMOTE RESISTANCE

A natural part of bacteriophage biology is engagement of a lysogenic cycle wherein a temperate phage invades a bacterial cell and incorporates its genetic material into the host genome, leading to the

propagation of phage DNA during host cell replication. This part of the phage life cycle could also lead to the incorporation of exogenous bacterial genes into the host genome (Miller et al., 1974). The D3 phage is a temperate phage that readily infects *P. aeruginosa* (Cavenagh and Miller, 1986). LPS-specific phage-dependent conversion of the OSA occurs throughout Gram-negative bacteria (Robbins et al., 1965; Bagdian et al., 1966). Holloway and Cooper (1962) observed that after strain PAO1 (serotype O5) was infected with D3 phage, at least 20% of the new colonies from subcultures tested could not be agglutinated by O5-specific typing antiserum. The mechanism of the changes occurring at the bacterial cell surface was unknown at the time. In a later study, a similar observation was made by the Hancock group who showed that lysogenized PAO1 cells had an altered LPS phenotype (Hancock et al., 1983), and this change was later determined to be due to the addition of an acetyl group at position 4 of the fucosamine residue and an alteration in the linkage of the trisaccharide repeating unit bond from $\alpha 1 \rightarrow 4$ to $\beta 1 \rightarrow 4$ (Kuzio and Kropinski, 1983). In a more recent study by our group, a region of the D3 genome was found to contain a gene that hybridized in Southern blots to an O-acetylation gene found in the PAO1 genome (Newton et al., 2001). This region spans 3.6 kb of the D3 genome, and contains three genes: (i) *oac* (encodes an *O*-acetylase), which adds an *O*-acetyl group to the OSA FucNAc residue, (ii) wzy_{β} (encodes a β-polymerase) which polymerizes the OSA repeats to form $\beta 1 \rightarrow 4$ linkages, and (iii) *iap* (encodes an α-polymerase inhibitor, a small 31 amino acid peptide with a single transmembrane domain). Iap possesses the activity to render Wzy_{α} non-functional thereby preventing the formation of the usual $\alpha 1 \rightarrow 4$ linkage among O-Ag subunits of strain PAO1 (Newton et al., 2001). Expression of this operon *in trans* produced an LPS banding pattern similar to that of LPS from O16, and these bands reacted strongly with the anti-O16 mAb MF47-4 in Western immunoblotting but not with the anti-O5 mAb MF15-4 (Newton et al., 2001). O-Ag subunits of serotypes O2 and O16 are linked by $\beta 1 \rightarrow 4$ bonds, and both serotype strains contain a functional wzy_{α} that is actively being repressed by a previously undetected *iap*. Interestingly, the wzy_{β} gene in O2 and O16 was not localized to the OSA biosynthesis cluster, further supporting the idea that this could be a D3 phage-associated gene. The authors initially suggested that wzy_{β} and *iap* identified in *P. aerugionsa* O2 and O16 strains are xenologs of the D3 phage. However the mol% G + C of wzy_{β} and *iap* respectively are inconsistent when compared to the mol% G + C values of both the PAO1 and the D3 phage genomes; therefore, the authors attributed the origin of these genes to an exogenous source (Kaluzny et al., 2007).

CHROMOSOMAL INSTABILITY PROMOTES MUTATIONS THAT DECREASE VIRULENCE AND INCREASE SURVIVAL

It has become dogma that once *P. aeruginosa* has colonized the lungs of CF patients, it adapts to a biofilm lifestyle to protect the bacterial community from hostile host defense and promote survival. The emergence of mucoid colony morphology is a hallmark of such adaptation and provides selective pressures that favor the occurrence of mutator phenotypes. These mutators in the bacterial community accumulate genetic changes resulting in altered phenotypes and gene expression profiles. The cause of the mutator phenotype is the inactivation/suppression of the methyl-directed mismatch repair (MMR) mechanism used to repair DNA replication errors and decrease the likelihood of homologous recombination events (Kunkel and Erie, 2005). Mutations in this region are known to cause loss of DNA proof reading thereby increasing the likelihood of mutant phenotypes which may convey survival advantages (Leclerc et al., 1996). This "hypermutable" phenotype was observed in 20% of *P. aeruginosa* isolates collected from CF airways and absent in isolates collected from blood and from the airway of non-CF patients (Oliver et al., 2000). One of the major genes responsible for the MMR mechanism is *mutS*; inactivation of this gene effectively shuts off the MMR cascade. Genetic analysis of the *mutS* gene from the previously characterized hypermutable *P. aeruginosa* strain JMSMA7 revealed a 3.3-kb IS and an 8-nucleotide repeat which flanked a 54-nucleotide deletion (Oliver et al., 2000). Interestingly, contained within the 3.3-kb IS lies another 1.2-kb IS element that is 90% homologous to IS*222* from the *P. aeruginosa* D3 phage (Kropinski et al., 1994; Oliver et al., 2000). This region was interpreted as a veritable "hot-spot" of recombination within the *mutS* gene promoting its loss of function and increasing the number of mutations (Oliver et al., 2002). An in-depth longitudinal study was undertaken by Smith et al. (2006) to examine the presence of the mutator phenotype in CF patients. The information gained from this study was used to further characterize the hypermutable isolates and determine the locations of these genetic errors. Sequencing of genes in two CF isolates taken at 6 months and a comparable isolate taken at 96 months during time course experiments revealed the accumulation of 68 mutations, wherein 13 were associated with virulence factors including genes associated with O-Ag biosynthesis (Smith et al., 2006). A continuing in-depth study using the genomes of these two isolates revealed an apparent increase in mutations within genes responsible for antibiotic resistance, as well as in *lasR*, a gene responsible for the regulation of quorum sensing in *P. aeruginosa* (Mena et al., 2008). The inactivation of *lasR* and subsequently *rhlI* in the hypermutable phenotype may result in a decrease of *migA* expression as indicated earlier by Yang et al. (2000). MigA is a glycosyltransferase responsible for the uncapped core OS phenotype, under the control of the RhlI–RhlR system (described below); when inactivated, the levels of MigA decrease causing an accumulation of "core-plus-one," a LPS glycoform with a single OSA repeat (Yang et al., 2000; **Figure 1**), a less virulent form of LPS. This degree of genotype plasticity is proposed to aid in the survival of bacteria within stressful environments during the transition to a chronic infection (Smith et al., 2006).

QUORUM-SENSING ALTERS CORE OS STRUCTURE THAT AFFECTS O-AG LIGATION

Cell density-dependent gene regulation plays an integral role in cell-surface virulence factor expression, altering gene expression based on population size (Whiteley et al., 1999). As previously mentioned, most *P. aeruginosa* CF isolates from chronically infected patients are devoid of OSA and begin to favor the typical "rough" phenotype, i.e., producing LPS that lacks the full-length O antigen side chain, being non-motile and non-piliated (Hancock et al., 1983). Although previous studies have shown the genetic relationship of the alginate-based mucoid phenotype (Ma et al., 1998), the regulation of changes governing the transition from smooth to rough LPS is not well defined. The "mucus inducible gene" *migA* (described above) is a putative glycosyltransferase and was discovered when *P. aeruginosa* was grown in the presence of CF mucus (Wang et al., 1996). Expression of the *migA* gene was found to be a result of

quorum sensing (Whiteley et al., 1999), dependent on the release of autoinducer molecules by *P. aeruginosa* into the local environment. When the bacterial density increases to an appropriate level, the local concentration of autoinducer molecules reaches a critical threshold past which they can consistently bind to transcription regulators across the entire cell population, resulting in alternative gene expression across the entire community (Fuqua et al., 1994). The *migA* gene was found to be under the control of RhlI–RhlR regulatory system, one of the two principal quorum-sensing systems, due to its severe decrease in expression in a *rhlI–rhlR* double knockout as compared to wildtype. Furthermore, it possesses an upstream *las*-box-like sequence (CT-N_{11})-AG), known to be a quorum-sensing recognition site for quorum-sensing regulators (Yang et al., 2000). Interestingly, overexpression of *migA* resulted in a loss of "core-plus-one" but not the higher molecular weight LPS, leading the authors to speculate the existence of a secondary glycosyltransferase (Yang et al., 2000). Recently that second glycosyltransferase was identified by our group as *wapR*, located 158-bp downstream of the core biosynthesis locus (Poon et al., 2008). Genetic and biophysical analysis of these two genes and their products revealed that both are responsible for core modifications in LPS biosynthesis (**Figure 3**). The expression of these genes results in an altered core structure: *migA* is responsible for the addition of the L-rhamnose^{A} (L-Rha^{A}) in the $\alpha 1 \rightarrow 6$ linkage, preventing the addition of O-Ag to lipid A-core by the O-Ag ligase WaaL (Yang et al., 2000; Poon et al., 2008). The *wapR* gene is responsible for the addition of the core sugar L-Rha^{B}, creating the favored linkage site for the addition of the O-Ag by WaaL (Poon et al., 2008). A genomic knockout of *wapR* resulted in the loss of higher molecular weight LPS bands, corresponding with the addition of O-Ag to lipid A-core. Work is currently underway in our laboratory to establish the relationship between *migA* and *wapR* gene expression. The cell density-dependent effects on gene regulation are of particular importance as CF isolates are often found in mature biofilms, a direct byproduct of quorum sensing.

THE AGE OF TECHNOLOGY BROADENS GENE EXPRESSION INVESTIGATIONS

In silico-based algorithms and bioinformatic methods have moved to the forefront of genomic research, performing a variety of predictions to provide a global view of genome expression profiles. When combined with microarray data, *in silico* modeling can be applied to examine an entire genome, particularly for investigating up and down regulations of genes throughout a longitudinal experiment. Using this approach, Oberhardt et al. (2010) examined the gene expression in a number of CF isolates throughout a progressive CF infection by a clonal strain of *P. aeruginosa*. They observed that the genes relating to LPS and capsule biosynthesis were downregulated during chronic CF infection. The development of new computer-based programs and ever-improving novel DNA sequencing techniques will help to broaden our understanding of the polymorphic nature of OSA of *P. aeruginosa* during CF infection.

STRUCTURE, GENETICS, AND FUNCTION OF CPA (A BAND)

DISCOVERY OF CPA

In the 1980s, CPA was detected to be a *P. aeruginosa* antigen that reacted with a human monoclonal antibody produced by Sawada's group (Sawada et al., 1985). Further analysis found the antigen to be a neutral polysaccharide composed of D-rhamnose (D-Rha) that is separable as well as structurally and immunologically distinct from the predominant OSA (Yokota et al., 1987; Kocharova et al., 1988; Rivera et al., 1988). In a study by McGroarty's group, the authors used gel filtration chromatography and a buffer containing deoxycholate, which helped to disperse LPS from micelles and aggregates, to obtain two separate fractions of surface polysaccharide from *P. aeruginosa* (Rivera et al., 1988). The fast-migrating fraction contained OSA (named B band) that was reactive to serotype-specific antibodies, while the slower-migrating fraction (CPA, i.e., A band) did not react with any of the mAbs that the Lam laboratory had raised against the IATS serotypes. Upon receiving the CPA from McGroarty, our group raised a number of hybridoma cell lines (e.g., N1C9, N1F10) that secreted mAbs that are specific against

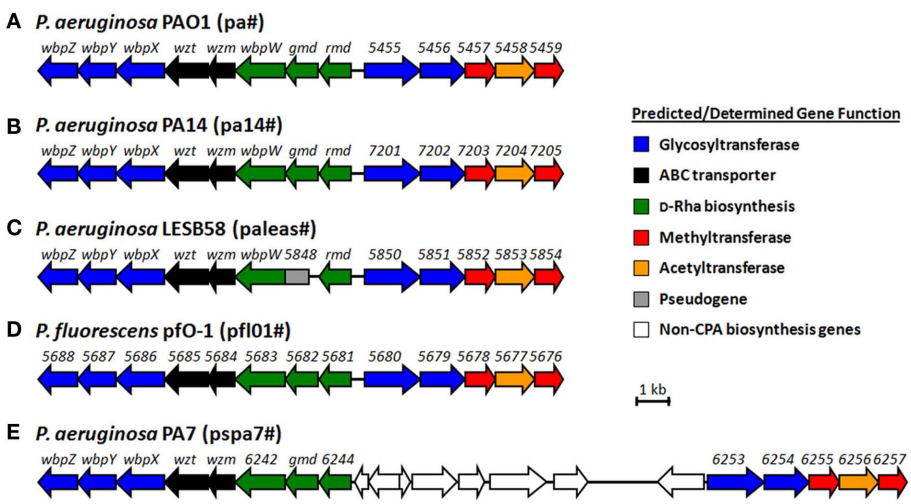

FIGURE 3 | Proposed CPA gene clusters of (A) *P. aeruginosa* PAO1, (B) PA14, (C) LESB58, (E) PA7, and (D) *P. fluorescens* pfO-1. The organism-specific identifier for numbered genes of unknown function is indicated in brackets beside each organism.

CPA. Using mAb N1F10 in dot-blot and Western-immunoblotting analyses, the presence of CPA was detected in 14 of the 20 *P. aeruginosa* IATS serotypes. The strains that did not react to the mAb belong to serotypes O7, O12, O13, O14, O15, and O16 (Lam et al., 1989; Currie et al., 1995). However, a majority of clinical *P. aeruginosa* isolates examined since then were found to be devoid of OSA but produce CPA as the predominant surface polysaccharide antigen (Lam et al., 1989; Weisner et al., 2007). In a separate study, CPA was found to be a receptor for bacteriophage A7, indicating its significance as a cell-surface polysaccharide component of *P. aeruginosa* and its role in the co-evolution with temperate bacteriophages (Rivera et al., 1992).

STRUCTURAL STUDY OF CPA

Structural elucidation of CPA from different *P. aeruginosa* strains has been reported. Yokota's group was the first to discover that the predominant structure of the common polysaccharide from *P. aeruginosa* strain IID 1008 (ATCC 27584; Yokota et al., 1987) reactive to a human mAb is composed of a trisaccharide repeating unit: →3) D-Rha(α1 → 3)D-Rha(α1 → 2)D-Rha(α1→. Our group prepared CPA from PAO1 (serotype O5; Arsenault et al., 1991) and by using high-field nuclear magnetic resonance (NMR) and mass spectrometry (MS) methods, we collected data to show that this neutral polysaccharide contains a D-rhamnan with the same structural repeat as demonstrated by Yokota et al. (1987). Two other groups who examined CPA from a serotype O7 strain (Lanyi–Bergan classification; Kocharova et al., 1988) and strain PA103 (serotype O11; Choudhury et al., 2005) also observed the same trisaccharide repeat structure. However, aside from the predominant D-Rha trisaccharide repeat units, small amounts of other components have also been reported with less consistency among the different studies. For example, the presence of 3-*O*-methylrhamnose, ribose, mannose, glucose, and a 3-*O*-methylhexose have been detected from CPA prepared from strain PAO1 (Arsenault et al., 1991); 3-*O*-methyl-6-deoxyhexose, glucose, xylose, alanine, galactosamine, and phosphorus have all been reported as components of CPA fractions for strain IID 1008 (ATCC 27584; Yokota et al., 1987). Glucose, mannose, and phosphate have been found in the study of CPA from the O7 strain (Kocharova et al., 1988), while mannose and GlcNAc, as well as small amounts of *O*- and *N*-acetyl substitutions, were found in the CPA from strain PA103 (Choudhury et al., 2005). These differences reported in the minor components of CPA could be due to several factors including genuine strain differences, the details in the preparation of CPA, and the sensitivity and accuracy of the detection methods used. In conclusion, the precise structure of CPA has not been defined to date. Further study is currently underway in our lab to determine the identity of the initiating sugar, the terminal sugar, the position and amount of other sugar components, as well as the degree and position of the methylation and acetylation modifications of CPA from strain PAO1.

GENETICS OF CPA BIOSYNTHESIS

A cosmid clone pFV3 that is able to restore CPA biosynthesis in a CPA-deficient mutant (strain AK1401) was first obtained from a cosmid library of *P. aeruginosa* prepared from strain PAO1. Mutant strain AK1401 was a phage-resistant mutant derived from the PAO1 background (Lightfoot and Lam, 1991). When expressed *in trans*, cosmid pFV3 was also found to be able to restore CPA biosynthesis in five (O7, O13, O14, O15, and O16) out of the six IATS serotypes that lacked CPA LPS (Lightfoot and Lam, 1991). Sequencing and genetic analysis of this clone revealed a cluster of eight genes (**Figure 3**, *rmd-wbpZ*) that are required for CPA biosynthesis. Our group went on to construct a non-polar knockout mutant of each of these genes (Rocchetta and Lam, 1997; Rocchetta et al., 1998a,b). Three of the genes *rmd*, *gmd*, *wbpW* (*pa5454*, *pa5453*, *pa5452*) plus another gene located in the alginate biosynthesis cluster, *algC* (*pa5322*), encode enzymes responsible for the biosynthesis of GDP-D-Rha, the nucleotide sugar precursor for CPA. The glycosyltransferase gene *wbpL* located in the OSA gene cluster described earlier was found to be required for the initiation of both CPA and OSA assembly (Rocchetta et al., 1998a). Another three genes in the cluster, *wbpX*, *wbpY*, and *wbpZ* (*pa5449*, *pa5448*, and *pa5447*), encode putative rhamnosyltransferases responsible for the specific linkages of the α1 → 3, α1 → 3, α1 → 2 bonds that form the D-Rha trisaccharide repeating unit (Rocchetta et al., 1998a). The last two genes, *wzm* and *wzt* (*pa5451* and *pa5450*), encode proteins for an ABC transport system that transfers the UndPP-linked CPA polymer precursor across the IM to the periplasmic surface where CPA is ligated to lipid A-core to complete its synthesis (Rocchetta and Lam, 1997). Southern-blotting analysis revealed that this cluster of eight genes is present in all of the 20 IATS reference strains, although some strains that do not produce CPA (O12, O13, O15, and O16) showed variation in the blotting profiles suggesting DNA rearrangement within the cluster (Currie et al., 1995).

In recent years, the genomes of several *P. aeruginosa* strains have been sequenced including PAO1 (serotype O5) (Stover et al., 2000), PA14 (serotype O10) (Lee et al., 2006), PA7 (a multidrug resistance serotype O12 strain; Roy et al., 2010), and the NT Liverpool Epidemic strain LESB58 (Winstanley et al., 2009). LESB58 has a rough LPS phenotype devoid of OSA; however, its genome sequence consists of the OSA biosynthesis locus that shares a high degree of homology and gene organization with the serotype O6 OSA LPS cluster sequenced by Raymond et al. (2002). Analysis of these genomes revealed that all four strains contain the *rmd-wbpZ* eight-gene cluster in a conserved manner. However, the clinical isolate LESB58 contains a non-functional *gmd* that can be deemed a pseudogene. In three of the sequenced genomes (PAO1, PA14, and LESB58), immediately adjacent to the *rmd-wbpZ* eight-gene cluster and located in the opposite strand, a five-gene cluster (*pa5455–pa5459* in PAO1, *pa14_7201-pa14_7205* in PA14, and *paleas_5850-paleas_5854* in LESB58) was also present in a conserved manner (**Figures 3A–C**). This cluster of genes encoding putative glycosyltransferases (the first two genes in the cluster, blue), methyltransferases (the third and the fifth genes in the cluster, red) and an acetyltransferase (the fourth gene in the cluster, orange), was also proposed to be involved in CPA biosynthesis and modification (King et al., 2009). Mutation and complementation analyses of these five genes clearly showed that all five are required for CPA biosynthesis (Hao and Lam, unpublished data). This five-gene cluster is also found in the genome of strain PA7 (serotype O12; **Figure 3E**, *pspa7_6253-pspa7_6257*). However, unlike in the aforementioned three strains, this five-gene cluster is located on the same strand and is separated from the *rmd-wbpZ* eight-gene cluster by a 10-kb DNA fragment in PA7 (**Figure 3**). This could have resulted from a chromosomal rearrangement event

and might have disrupted the promoter region of this gene cluster, which would explain the lack of CPA expression in this strain. We also found that this genomic region from strain PA7 showed a much lower sequence identity to that of the other three strains. The strains PAO1, PA14, and LESB58 share more than 95% sequence identity in most of the proteins encoded by genes of the CPA biosynthesis cluster, while PA7 showed less than 50% identity to these three strains. This may explain why the cosmid pFV3 did not complement CPA biosynthesis in this serotype while still being able to do so for the other five serotypes that lack CPA LPS. Interestingly, we found that homologous CPA biosynthesis genes are also present in the genome of *P. fluorescens* pfO-1 (Silby et al., 2009), in a conserved manner similar to *P. aeruginosa* PAO1 and PA14 (**Figure 3D**), showing 54–75% amino acid sequence identity to these two strains. To date there is no report about the structure of LPS of *P. fluorescens* pfO-1. It would be interesting to study whether this strain produces the same CPA LPS, and whether it also produces two types of LPS as in *P. aeruginosa*.

The same structure of D-Rha repeating units [→3) D-Rha(α1 → 3)D-Rha(α1 → 2)D-Rha(α1→)] has been reported for the LPS of several other bacterial species including the plant pathogen *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* (Senchenkova et al., 2002), the opportunistic human pathogen *Stenotrophomonas maltophilia* serotype O7 (Winn and Wilkinson, 1998), *P. syringae* strains (Ovod et al., 1996), and a *Burkholderia cepecia* strain (Cerantola and Montrozier, 1997). However, to our knowledge, the OSA biosynthesis genes have not been identified and investigated in any of these bacterial strains.

FUNCTIONAL STUDY OF CPA

Most *P. aeruginosa* strains simultaneously produce both OSA-containing LPS and CPA-containing LPS, with the former being the more proportionally predominant LPS species. However, it was reported that due to long-term selective pressure against the OSA (which is the more immunogenic of the two O-Ag glycoforms), CPA is apparently selectively maintained as the preferred O-Ag on the cell surface of *P. aeruginosa* during chronic lung infection in CF patients, and becomes the major LPS antigen over time (Hancock et al., 1983; Lam et al., 1989).

Thus far, there is only limited information about the biological function of CPA besides being part of the *P. aeruginosa* cell wall. Our lab has demonstrated in a cell adhesion assay study that a *rmd* mutant of *P. aeruginosa*, defective in D-Rha biosynthesis and thereby CPA production, is significantly less efficient in adhering to cultured human bronchial epithelial cells, suggesting that CPA may play an important role in adherence of *P. aeruginosa* to tissues (Matewish, 2004). More work is currently underway to investigate the significance of CPA for *P. aeruginosa* to attach to different surfaces using biophysical techniques based on atomic force microscopy (AFM).

CONTRIBUTION OF VARIATIONS IN CORE OLIGOSACCHARIDE TO LPS DIVERSITY

For the sake of full coverage of each region of the LPS molecule that contributes to diversity in the LPS produced by *P. aeruginosa*, in this section, we will briefly discuss the variability in core OS structures. Our group has recently published a review on the structural diversity of the complete and the truncated core OS (Kocíncová and Lam, 2011), hence more details on structural diversity of core OS can be found there. In general, core OS, which is the linker region between O-Ag and lipid A can be divided into two regions, namely, the inner and outer core.

STRUCTURE OF INNER CORE OS

Composition of the sugar constituents in inner core OS is identical among *P. aeruginosa* strains and it consists of two residues of 3-deoxy-D-manno-octulosonic acid (Kdo^{I} and Kdo^{II}) and two residues of L-*glycero*-D-*manno*-heptose (Hep^{I} and Hep^{II}; **Figure 4**). The inner core is highly conserved when compared to other Gram-negative bacteria. A distinguishing feature of the *P. aeruginosa* inner core is a high degree of phosphorylation that is essential for viability of *P. aeruginosa*, since mutation of either of the two genes (*wapP* and *waaP*) encoding kinases that are responsible for adding phosphate groups to the inner core heptoses is lethal (Walsh et al., 2000). In most *P. aeruginosa* strains, three phosphorylation sites have been identified; positions 2 and 4 on Hep^{I} and position 6 on Hep^{II}. In a CF isolate, Hep^{II} has been shown to possess an additional phosphorylation site on position 4 (Knirel et al., 2006). In theory, monophosphate, diphosphate, or triphosphate groups might occupy each of the phosphorylation sites (Kooistra et al., 2003; Choudhury et al., 2005; Bystrova et al., 2006). Some of the analyzed *P. aeruginosa* strains have shown the non-stoichiometric substitution of a phosphate group on position 2 of Hep^{I} by ethanolamine-phosphate or ethanolamine-diphosphate (Bystrova et al., 2006). However, Knirel et al. (2006) suggested that the actual content of phosphates and ethanolamine-phosphates in the inner core might be higher, since these phosphate groups can be released from the core during preparation of LPS prior to structural analyses. In addition to phosphorylation, Hep^{II} is modified with another non-carbohydrate substituent, namely an *O*-carbamoyl group on position 7 (Beckmann et al., 1995).

STRUCTURE OF OUTER CORE OS

The outer core of *P. aeruginosa* LPS is composed of one D-galactosamine (GalN), one L-Rha, and three or four D-glucose (Glc^{I}–Glc^{IV}) residues. GalN is further substituted on position 2 with an alanyl (Ala) group or in some truncated core structures with an acetyl group (Sanchez Carballo et al., 1999; Choudhury et al., 2008). The outer core OS of *P. aeruginosa* has a unique feature; it exists in two structurally distinct glycoforms, called "uncapped" and "capped" (King et al., 2009). These two glycoforms basically differ in position and linkage of an L-Rha residue in each structure. The capped glycoform is covalently attached to O-Ag on $L\text{-}Rha^{B}$ that is 1,3-linked to Glc^{I}, whereas the uncapped glycoform cannot be substituted with O-Ag and it contains an $L\text{-}Rha^{A}$ that is 1,6-linked to Glc^{II} (**Figure 4**). The presence of this $L\text{-}Rha^{A}$ likely causes steric hindrance and prevents attachment of O-Ag to the uncapped core OS; it is also possible that this 1,6-linked L-Rha cannot be recognized by WaaL (O-Ag ligase) as part of the core OS receptor during the O-Ag ligation process.

Another variability in sugar composition of core OS is the presence or absence of a fourth Glc residue (Glc^{IV}) in the uncapped core OS; among the core OSs of the 20 IATS serotypes, Glc^{IV} is present in only nine (O2, O5, O7, O8, O10, O16, O18, O19, and O20; De Kievit and

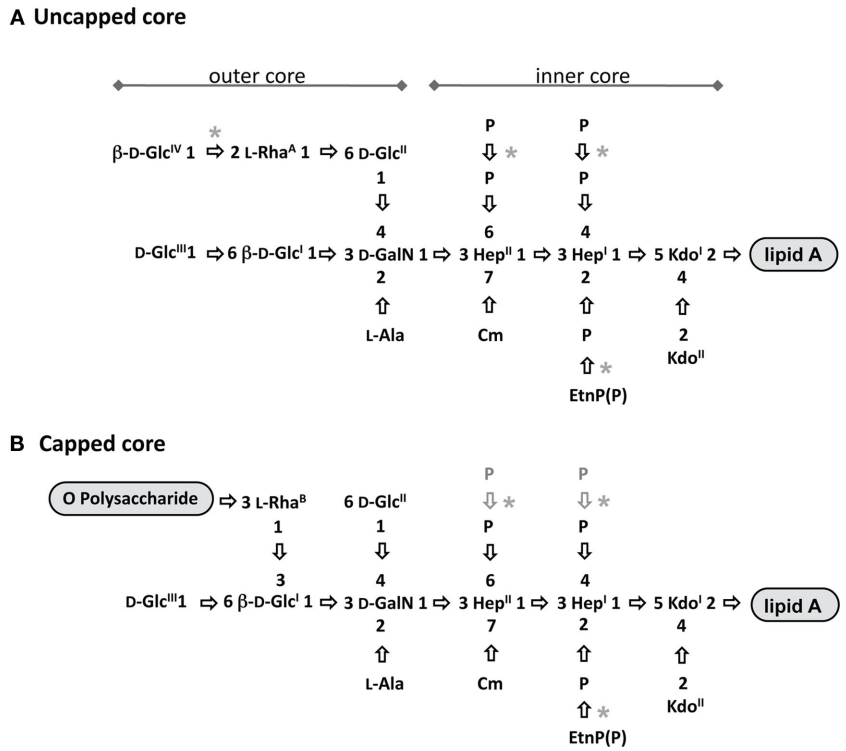

FIGURE 4 | Structures of uncapped and capped glycoforms. The structures of the uncapped core oligosaccharide **(A)**, and capped core oligosaccharide **(B)** are depicted. All the sugars shown have α configuration unless otherwise indicated. Asterisks depict variable substitutions, acetylation sites are shown since they have not been precisely identified. Ala, alanine; Cm, carbamoyl; Etn, ethanolamine; GalN, 2-amino-2-deoxy-galactose (galactosamine); Glc, glucose; Hep, L-glycero-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Rha, rhamnose. Adapted from King et al. (2009).

Lam, 1994; Bystrova et al., 2006). Additionally, a variable non-sugar substitution found in outer core OS is O-acetylation. *P. aeruginosa* strains display diversity in the degree of O-acetylation ranging from none to five acetyl groups (Bystrova et al., 2006). Since O-acetylation is non-stoichiometric, attempts to precisely identify and quantify all acetylated residues have been difficult. Thus far, only two specific residues have been found to be O-acetylated: Rha^A, particularly when this residue is not further linked to Glc^{IV}, and Glc^{II}, as observed in a number of *P. aeruginosa* mutants with defects in core OS biosynthesis producing truncated core OS (Bystrova et al., 2006; Poon et al., 2008).

SPONTANEOUS CORE TRUNCATIONS

As mentioned above, phosphorylation of inner core heptoses is essential for *P. aeruginosa* cells. Each constituent of the entire inner core seems to be required for viability of *P. aeruginosa*; thus far all of the truncated OS structures that have been examined exhibited a complete inner core, with defects only found in the outer core residues (Sadovskaya et al., 1998; Sanchez Carballo et al., 1999; Kooistra et al., 2003; Choudhury et al., 2005). Preliminary observations also suggested that GalN, the first residue of the outer core, might be essential for viability. This assumption was based on the inability to construct a mutant of *wapG*, which encodes a glycosyltransferase presumably responsible for transferring a GalN residue to the Hep residue in the core (Matewish, 2004). A spontaneous mutation causing truncation of core OS under a selective pressure could be a significant source of core diversity. Core OS has been reported to be a receptor for a number of bacteriophages and pyocins (Jarrell and Kropinski, 1981a,b; Temple et al., 1986; Yokota et al., 1994; Michel-Briand and Baysse, 2002). For more details, see the section in this review on the role of LPS in host–pathogen interactions. In earlier studies of *P. aeruginosa* LPS biosynthesis, bacteriophages E79 and 2 Lindberg were used to select for spontaneous rough mutants that also had truncation within the core OS (Jarrell and Kropinski, 1981a,b; Dasgupta et al., 1994). Two varieties of outer core truncations providing resistance to bacteriophages have been described, more specifically, the outer core of these structures in the mutant bacterial strains were composed of either (i) the first residue of outer core, GalN (representing the deepest truncation of core OS structure ever found in *P. aeruginosa*) or (ii) a disaccharide GalN–Glc^{II} (Masoud et al., 1994; Sadovskaya et al., 1998; Sanchez Carballo et al., 1999). Similarly, pyocins (i.e., "defective phages") produced by *P. aeruginosa*, that are able to kill susceptible cells of the same species but are unable to replicate within cells (Michel-Briand and Baysse, 2002), have been used to select for spontaneous rough mutants aiming to generate ones with truncations within the core region (Koval and Meadow, 1977). Another strategy to search for strains with core OS truncations has been to select for spontaneous mutants that would be resistant to a cationic antibiotic; our group used increasing concentrations of gentamicin to select for drug-resistant mutants (Dasgupta et al., 1994). However, the core OS structures from these gentamicin and pyocin-resistant strains have yet to be elucidated, and as such their chemical compositions remain unknown.

GENETICS OF CORE DIVERSITY DUE TO GLYCOSYLTRANSFERASES

Pseudomonas aeruginosa genomes possess a gene cluster (*pa4996–pa5012*) encoding proteins that are known to be involved in core OS biosynthesis: HldE, MsbA, PA4998, WaaL, WapR, PA5001, PA5002, PA5003, WapH, WapO, PA5006, WapQ, WapP, WaaP, WapG, WaaC, and WaaF. The respective function of each of these proteins (either demonstrated or proposed based on homology comparisons) has been summarized (**Table 3**; for more detailed description, see a recent review by our group (King et al., 2009). Apart from the presence or absence of the Glc^{IV} residue as a terminal sugar in uncapped core OS, the structures of core OS among *P. aeruginosa* strains are relatively conserved with no strain-to-strain variability in sugar composition. This observation is consistent with the discovery that the genes in the locus for core biosynthesis are well conserved among *P. aeruginosa* strains whose genomes have been sequenced. The amino acid identity of the gene products in this locus from one strain to the next is relatively high ranging from 77 to 100%. In addition to genes that encode kinases and heptosyltransferases, this cluster contains genes that are involved in the transportation of lipid A and ligation of O-Ag to the core (*msbA* and *waaL*, respectively). Two other genes, *pa5002* and *pa5003* have not been characterized; therefore, their functions are at present unknown.

Our group has reported the characterization of two transferase genes, *migA* (described above) and *waaA*, that are important for core OS biosynthesis, but are localized outside the usual core LPS gene locus. The *waaA* gene encodes a putative Kdo transferase (King et al., 2009). More recently, we have characterized a homolog of *migA* that is designated as *wapR*. The *wapR* gene turned out to be localized within the core OS gene cluster. Characterization of WapR showed that it is responsible for the transfer of L-Rha^{B}, the other outer core rhamnose residue that is 1,6-linked to the Glc^{I} of the capped core OS (Poon et al., 2008). We hypothesize that the ratio between expression levels of *migA* and *wapR* would influence the amount of capped and uncapped core OS being produced. Studies to understand the regulation of *wapR* are currently underway in our laboratory. Both putative rhamnosyltransferase enzymes, MigA and WapR, presumably utilize the same substrates, TDP-L-Rha as a sugar donor and core OS lacking L-Rha residues as an acceptor molecule. Therefore, they compete for the enzyme substrates and should impact on amounts of capped and uncapped glycoforms being produced. For instance, overproduction of MigA favors synthesis of uncapped core, whereas an elevated level of WapR activity leads to higher amounts of capped core substituted with O-Ag. It is plausible that under certain conditions *migA* is upregulated while *wapR* is downregulated (or *vice versa*), which may consequently influence the amount of O-Ag on the cell surface of *P. aeruginosa*.

We recently discovered yet another transferase gene called *wapB*, which shares sequence homologies with both *migA* and *wapR*. The *wapB* gene encodes a putative 1,2-glucosyltransferase that is required for transfer of terminal Glc^{IV} to uncapped core OS (Kocíncová et al., 2011). As not all of the *P. aeruginosa* strains possess Glc^{IV} as a terminal sugar residue in their uncapped core OS, not all of the *P. aeruginosa* strains have the *wapB* gene. For instance, this gene is absent from the sequenced genome of *P. aeruginosa* PA7.

Other variability in the structure of the *P. aeruginosa* core OS arises from the different degrees of phosphorylation (including ethanolamine-phosphate) and acetylation patterns. However, the variability of these phosphatidyl or acetyl substitutions is non-stoichiometric and the genetic elements that account for these minor substitutions are unknown at present. Additionally, it has yet to be determined what role (if any) genetic events such as mutations or mobile element insertions in core OS genes have played in the truncation of core OS, when rough LPS mutants were selected for resistance to bacteriophages/pyocins or antibiotics. Therefore, there are obvious areas concerning core biosynthesis and regulation that require further investigation.

Table 3 | Genes involved or potentially involved in the core OS biosynthesis of *P. aeruginosa*.

Gene	Proposed/demonstrated function	References
CORE BIOSYNTHESIS GENE CLUSTER *(PA4996-PA5012)*		
hldE/pa4996	Heptose biosynthesis	King et al. (2009)
msbA#/pa4997	Transport lipid A-core	Ghanei et al. (2007)
pa4998	Kinase	King et al. (2009)
waaL#/pa4999	O antigen ligase	Abeyrathne et al. (2005), Abeyrathne and Lam (2007)
wapR/pa5000	Glycosyltransferase (Rha^{B})	Poon et al. (2008)
pa5001	Glycosyltransferase	King et al. (2009)
pa5002	Unknown	
pa5003	Unknown	
wapH/pa5004	Glycosyltransferase (Glc^{II})	Matewish (2004)
wapO/pa5005	Carbamoyltransferase	(King et al. (2009)
pa5006	Kinase	King et al. (2009)
wapQ/pa5007	Heptose kinase	Walsh et al. (2000)
wapP/Pa5008	Heptose kinase	Walsh et al. (2000), To (2006)
waaP/pa5009	Heptose kinase: position 4 of HepI	Walsh et al. (2000), Zhao and Lam (2002), Zhao et al. (2002)
wapG/pa5010	Glycosyltransferase (GalN)	
waaC/pa5011	Glycosyltransferase (Hep^{I})	De Kievit and Lam (1997)
waaF/pa5012	Glycosyltransferase (Hep^{II})	De Kievit and Lam (1997)
GENES LOCATED OUTSIDE OF THE CORE BIOSYNTHESIS CLUSTER		
wapB/pa1014	glycosyltransferase (Glc^{IV})	Kocíncová et al. (2011)
migA/pa0705	glycosyltransferase (Rha^{A})	Poon et al. (2008)
waaA/pa4988	glycosyltransferase: (Kdo^{I}, Kdo^{II})	King et al. (2009)

 # Indicates genes involved in cellular processes other than core biosynthesis. Bold font indicates that biochemical or genetic evidence of involvement in P. aeruginosa core biosynthesis exists.

STRUCTURE AND FUNCTION DIVERSITY OF LIPID A

STRUCTURAL DIVERSITY OF *P. AERUGINOSA* LIPID A

The lipid A domain of LPS is a glucosamine-based lipid that anchors the LPS in the bacterial outer membrane (OM). The structure of *P. aeruginosa* lipid A has been studied in great detail using both MS and NMR techniques, and it has been reviewed by several groups (Knirel et al., 2006; King et al., 2009; Moskowitz and Ernst, 2010). Similar to lipid A of other Gram-negative bacteria, *P. aeruginosa*

lipid A is composed of a diglucosamine biphosphate backbone [4-P-β-D-GlcN^{II}-(1→6)-α-D-GlcN^{I}-(1→P], and *O*- and *N*-linked primary and secondary fatty acids. The major structural differences of lipid A are observed in the number, the position, the nature of the linked acyl groups, and the modification of the phosphate groups (**Figure 5**). For example, the chain lengths of the fatty acids attached to lipid A of *P. aeruginosa* (C_{10}/C_{12}) are shorter than those of *E. coli* and *Salmonella enterica* sv. Typhimurium (C_{12}/C_{14}; Trent, 2004). Heterogeneity of lipid A structure has also been observed within different *P. aeruginosa* strains, which arises from different growth conditions and isolation sources.

The lipid A of laboratory-adapted *P. aeruginosa* strains (including PAO1, PAK, and PA14) grown in rich medium was found to exhibit a hexa-acylated form (~25%; **Figure 5** lipid A form A) or a penta-acylated form (~75%; **Figure 5** lipid A form B; Bhat et al., 1990; Kulshin et al., 1991; Ernst et al., 1999; Moskowitz et al., 2004). The hexa-acylated form is a symmetrically acylated structure, with each sugar containing an *N*-linked 12:0(3-OH) [I-3-hydroxylauroyl] group at the 2 position, an *O*-linked 10:0(3-OH) [I-3-hydroxydecanoyl] group at the 3 position, and a secondary 12:0 (lauroyl) group linked to the 3-OH group of the primary *N*-linked 12:0(3-OH) at position 2 (lipid A form A). Either or both of the secondary acyl groups could be non-stoichiometrically 2-hydroxylated (**Figure 5** red labeled), converting 12:0 to 12:0(2-OH) (Kulshin et al., 1991). The penta-acylated lipid A form, which is the predominant (~75%) lipid A form of laboratory *P. aeruginosa* strains, lacks the primary *O*-linked acyl substitution at position 3 of the GlcN^{I} (**Figure 5**, lipid A form B; Kulshin et al., 1991).

As has been demonstrated in enteric bacteria such as *S. enterica* (Guo et al., 1997, 1998), *P. aeruginosa* lipid A adopts different modifications in response to different environmental conditions encountered such as different growth medium and during human infection. For example, clinically isolated *P. aeruginosa* strains produce lipid A with modified structures. Lipid A from *P. aeruginosa* isolated from patients with acute clinical infections (such as blood, ear, eye, and urinary tract) or chronic non-CF infections such as bronchiectasis has a penta-acylated form that lacks the secondary acyl group at position 2 of the sugar GlcN^{I} (**Figure 5** lipid A form C; Ernst et al., 1999, 2003). Three different types of modifications have been observed for the lipid A structure of *P. aeruginosa* isolates from CF patient airways. The first type of modification is observed in the majority of CF *P. aeruginosa* isolates: the addition of an *O*-linked secondary palmitate (16:0) to the 3-OH group of 10:0(3-OH) at the 3′ position in sugar GlcN^{II} (**Figure 5** lipid A form D, blue; Guo et al., 1997, 1998). Less than half of CF isolates examined by the Miller group showed a second type of modification to lipid A, the non-stoichiometric addition of aminoarabinose (4-amino-4-deoxy-L-arabinose, Ara4N) to either or both of the

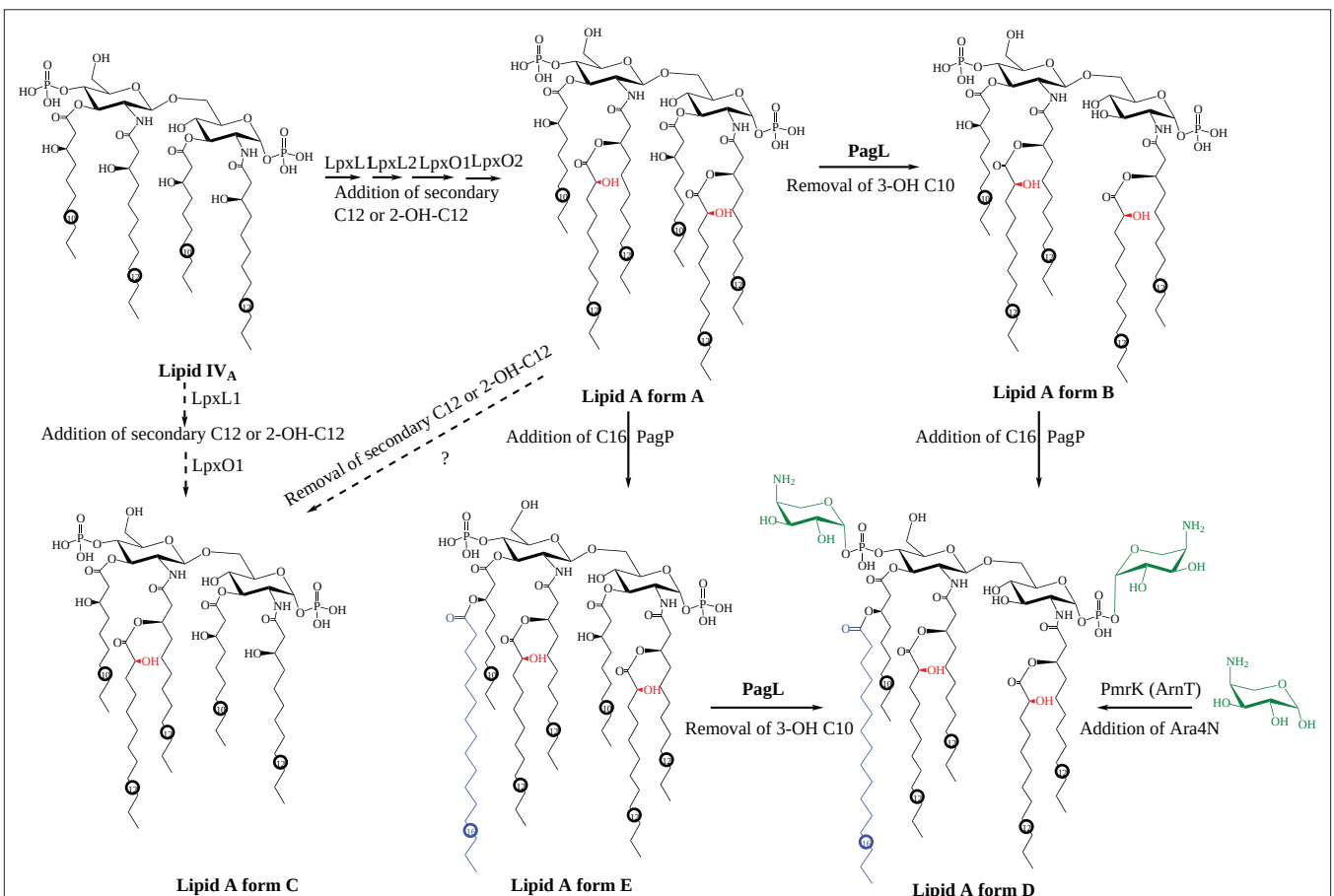

FIGURE 5 | Different lipid A forms of *P. aeruginosa* and the proposed biosynthesis and modification pathway.

terminal phosphates (**Figure 5** lipid A form D, green; Ernst et al., 2007). A hepta-acylated lipid A (**Figure 1** lipid A form E) found only in *P. aeruginosa* isolates from CF patients with very severe lung disease resulted from a third type of modification: the retention of the primary *O*-linked 10:0(3-OH) group at the 3 position of $GlcN^{I}$ (Ernst et al., 2003, 2007). Interestingly, the lipid A modifications observed in CF airway isolates (i.e., addition of palmitate and aminoarabinose) could be induced in laboratory strains, strains isolated from the environment, or non-CF clinical isolates under certain growth conditions including low Mg^{2+} concentration or the presence of cationic antimicrobial peptides (CAMPs) such as polymyxin B, indicating the enzymatic pathways for these modifications were intact and inducible in these strains (Bhat et al., 1990; Ernst et al., 1999, 2003; Bedoux et al., 2004).

PROPOSED BIOSYNTHESIS AND MODIFICATION PATHWAY OF LIPID A

Over the past 20 years, the biosynthesis pathway of lipid A has first been experimentally determined in *E. coli* as well as other enteric bacteria and reviewed by Raetz and Whitfield (2002) and Trent (2004). Bioinformatics analysis indicates that the general scheme of lipid A biosynthesis pathways are highly conserved among Gram-negative bacteria, although various modifications are present in different bacteria (Trent, 2004). In *P. aeruginosa*, in contrast to the extensive and detailed structural elucidation of lipid A, most aspects of the biosynthesis pathway have not been investigated experimentally, perhaps due to the fact that the genetics of lipid A biosynthesis were so thoroughly studied in enteric bacteria and that the pathway is relatively conserved. The current assumption about the biosynthesis pathways is mainly based on the identification of homologs to *E. coli* genes. Since all of the corresponding homologous genes encoding enzymes required for the biosynthesis of the important lipid A structure precursor lipid IV_A (**Figure 5**; including the primary acyltransferases LpxA and LpxD, the nucleotidase LpxH, the disaccharide synthase LpxB, and the kinase LpxK) were identified in the genome of *P. aeruginosa*, the biosynthesis of the lipid IV_A analog of *P. aeruginosa* is thought to be executed in a conserved manner as in *E. coli* and *S. enterica*. A detailed description of the proposed biosynthesis pathway of lipid IV_A was recently reviewed by our group (King et al., 2009). Not surprisingly, the lipid A primary acyl chain length of different bacteria is determined by the chain length preference of the acyltransferase LpxA and LpxD (Dotson et al., 1998; Wyckoff et al., 1998).

Different forms of lipid A could then be synthesized and modified from lipid IV_A by different enzymes depending on the environmental conditions (a proposed pathway is depicted in **Figure 5**). Two secondary acyltransferases (LpxL1 and LpxL2, encoded by *pa3243* and *pa0011*, respectively) are proposed to transfer the secondary lauroyl groups to lipid IV_A to form the hexa-acyl lipid A (**Figure 1**. lipid A form A; Mohan et al., 1994; King et al., 2009). However, unlike in *E. coli* and *S. enterica* sv. Typhimurium, the addition of the first core sugar 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) to the lipid IV_A is not strictly necessary for the attachment of the secondary acyl groups in *P. aeruginosa* (Goldman et al., 1988). The non-stoichiometric 2-hydroxylation of the secondary lauroyl group is proposed to be catalyzed by corresponding homologs of *S. enterica* sv. Typhimurium LpxO (Gibbons et al., 2000, 2008). In *P. aeruginosa*, two putative LpxO homologs (LpxO1 and LpxO2, encoded by *pa4512* and *pa1936*, respectively) were present; presumably each would hydroxylate one of the two secondary acyl chains (King et al., 2009). A newly identified deacylase enzyme PagL is believed to be involved in the removal of the primary 10:0(3-OH) group at the 3 position of $GlcN^{I}$ of the hexa-acylated lipid A (**Figure 5**, lipid A form A) to form the penta-acylated lipid (**Figure 5**, lipid A form B), and to convert the hepta-acylated lipid A (**Figure 5**, form E) to hexa-acylated lipid A (**Figure 5**, form D) in CF isolates (Ernst et al., 2006). As stated earlier, the lipid A from isolates of patients with acute infection or non-CF chronic infection lacks the secondary C_{12} acyl chain at $GlcN^{I}$ (**Figure 5**, lipid A form C; Ernst et al., 1999, 2003). This could be due to the lack of expression of one of the secondary acyltransferases *lpxL*, or the later removal of the secondary C_{12} acyl group from lipid A (**Figure 5**, form A) by a novel deacylase enzyme. The OM palmitoyl transferase protein PagP was originally identified in *Salmonella* and is responsible for the addition of palmitate to lipid A (Guo et al., 1998). A PagP homolog in *P. aeruginosa* is proposed to catalyze the addition of palmitate to lipid A forms A and B to synthesize lipid A forms E and D that are found in CF patient airway isolates, respectively (Trent, 2004). The hexa-acylated lipid A (form D) could also result from the removal of the primary 10:0(3-OH) group from the hepta-acyl lipid A (form E) by the deacylase enzyme PagL. It is not known which of the two steps, i.e., the addition of palmitate or the removal of the primary 10:0(3-OH), occurs first. However, it has been confirmed that the occurrence of the hepta-acylated lipid A (form E) found only in isolates from CF patients with severe infection was due to the loss of PagL function (Ernst et al., 2006). Although an *in vitro* assay indicated that a PagP-like enzyme was present in the membranes (Trent, 2004) of *P. aeruginosa*, to date, no *pagP* homolog has been identified in the genome (Stover et al., 2000). This could be due to low sequence similarity between these homologs. Similar to *Salmonella*, the addition of palmitate is regulated by the PhoP–PhoQ two-component regulatory system, as a *phoP* null mutant of *P. aeruginosa* is not able to synthesize palmitate-modified lipid A under inducing conditions for wildtype strains (such as limited Mg^{2+}; Ernst et al., 1999). The biosynthesis pathway of aminoarabinose and its addition to lipid A has been well characterized in *E. coli* and *S. enterica* (reviewed by Trent, 2004 and Bishop, 2005). The gene *ugd* (also called *pmrE*) and the *pmrHFIJKLM* (also referred to as the *arnBCADTEF*) gene cluster have been identified and characterized to encode enzymes required for the biosynthesis of aminoarabinose as well as its transport across the IM and its ligation to lipid A (Trent, 2004; Bishop, 2005). These homologous genes were identified in a single cluster in the genome of *P. aeruginosa* PAO1 (*pa3552–pa3559*; Stover et al., 2000). As in *E. coli* and *S. enterica*, it was found that both PhoP–PhoQ and another two-component regulatory system PmrA–PmrB are involved in the regulation of the expression of these genes and the addition of aminoarabinose to lipid A (Moskowitz et al., 2004; Moskowitz and Ernst, 2010). Both systems can respond to Mg^{2+}-limiting conditions (Mcphee et al., 2003, 2006); moreover, the PmrA–PmrB system can also respond to the presence of CAMPs including polymyxin B causing activation of the transcription of the *pmr* gene cluster (Mcphee et al., 2003).

BIOLOGICAL IMPLICATIONS OF LPS DIVERSITY IN *P. AERUGINOSA*

COLONIZATION AND PERSISTENCE

Physiological adaptability and metabolic plasticity have long been hallmarks of *P. aeruginosa* survival, traits that were consistent with the annotated genome sequence for strain PAO1, which revealed a wealth of predicted genes involved in various synthesis, assembly, metabolic, regulatory, and pathogenesis paradigms (Stover et al., 2000). This genomic abundance reflects the remarkable versatility of this bacterial species to colonize and persist in diverse niches ranging from different environmental settings to host animal tissues (Wolfgang et al., 2003). While several characteristics of *P. aeruginosa* facilitate initial substratum colonization and later-stage persistence, each is either directly or indirectly affected by variations in the phenotype of the LPS present on the cell surface.

In the aqueous environment, cells of *P. aeruginosa* swim with the aid of a polar flagellum (Feldman et al., 1998), but this mode of motility was impaired in mutants that lacked wildtype smooth LPS due to defects in either core OS biosynthesis, yielding truncated core OS (Lindhout et al., 2009), or ligation of OSA and CPA to the core OS, in the case of a *waaL* mutant (Abeyrathne et al., 2005). Swarming motility of *P. aeruginosa* cells, which is the process of translocation on semi-solid surfaces dependent on flagella, type IV pili, and rhamnolipid (a wetting agent produced by *P. aeruginosa*; Köhler et al., 2000), was similarly compromised in the aforementioned mutants with truncated core OS. The motility defects in the mutants were found to be a result of changes in cell-surface properties such as cell-to-cell and cell-to-substratum adhesion forces. Both types of adhesive forces became significantly increased in the mutants, as measured by AFM. This is in contrast to the initial thoughts that LPS defects might have somehow affected flagella-driven motility. In fact, when the mutant cells were examined by standard light microscopy, and the swimming speed of individual cells was quantified, no difference could be discerned between the mutant and the wildtype parent strain. The increased adhesive properties retarded all outward motility from the population of *P. aeruginosa* cells (Lindhout et al., 2009).

Biofilm formation is the preferred mode of growth for *P. aeruginosa* cells clinging to rocks in fluvial streams or surviving in the lungs of CF patients in a chronic infection situation (Hall-Stoodley et al., 2004). Following planktonic growth of *P. aeruginosa*, attachment to a substratum is a prerequisite for establishing long-term colonization at a particular site to eventually adapt to a biofilm mode of growth. Biofilms are intricate surface-associated bacterial communities that confer survival advantages to the cells residing within and for which flagellar-mediated motility is important for their maturation in *P. aeruginosa* (O'toole and Kolter, 1998; Klausen et al., 2003). Consistent with the motility defects described above, the same mutants of *P. aeruginosa* lacking complete core OS and the distal CPA and OSA moieties were found to form biofilms with significant differences in mechanical and structural properties when compared to those of wildtype bacteria. This evidence was collected from a variety of quantitative measurement studies to determine changes in ultrastructures, biophysical properties, cell–cell adhesion forces, and viscoelasticity of mutant and wildtype strains using a technique called microbead force spectroscopy (Lau et al., 2009a). Several bacterial strains including knockout mutants disrupted in *migA*, *wapR*, and *rmlC*, respectively, and with defined core OS truncation characteristics were compared to their wildtype PAO1 parent strain in these studies. Significant changes were observed in cell mechanical properties among the mutant strains compared to the wildtype PAO1. The functions of *migA* and *wapR* have been described earlier; the *rmlC* gene is responsible for TDP-L-Rha biosynthesis and hence a *rmlC* mutant produces a defective core OS truncated at the Rha^A and Rha^B residues in the two glycoforms of the core OS (**Figure 4**). The data from these studies revealed that truncation of core OS enhanced both adhesive and cohesive forces by up to 10-fold, whereas changes in instantaneous elasticity were correlated with the presence of O-Ag. Using AFM to raster-scan bacterial cells in air in contact mode for each of the aforementioned four strains showed differences in the texture of the surface "smoothness." Interestingly, LPS-"smooth" strain wildtype PAO1 with O-Ag exhibits rougher surface topography than rough strains, i.e., the mutant bacterial strains of *migA*, *wapR*, and *rmlC* (**Figure 6**). Using confocal laser scanning microscopy to quantify biofilm structural changes in these mutants, we observed that textural parameters varied with adhesion or the inverse of cohesion, while areal and volumetric parameters were linked to adhesion, cohesion, or the balance between them. Microcolonies formed by cells of the wildtype PAO1 had round perimeters, while the microcolonies formed by the mutant strains had more irregular edges (**Figure 7**; Lau et al., 2009b). These studies support the importance of O-Ag in the formation of cellular structures and the physiology of *P. aeruginosa* in a biofilm mode of growth. In a study by Ivanov et al. (2011), they showed that changes in the relative proportion of OSA modalities as well as the outright loss of OSA result in reduced virulence of *P. aeruginosa* consistent with diminished surface adhesive forces, further supporting the role of LPS-mediated adhesion in *P. aeruginosa* persistence. The observations made in these recent studies substantiated an earlier report in which rough mutants of *P. aeruginosa* lacking OSA had an LD_{50} that was 1000X higher than that of a wildtype strain in a mouse infection model (Cryz et al., 1984).

PROPERTIES OF *P. AERUGINOSA* LPS THAT INFLUENCE ITS INTERACTION WITH THE DEFENSE SYSTEMS OF THE HOST

The genomic stability of *P. aeruginosa* is such that environmental isolates have been found to maintain the potential for pathogenicity through the conservation of numerous virulence-associated genes required for host infection (Wolfgang et al., 2003). Undoubtedly, theses genetic traits contribute to the success of the bacterium as an opportunistic pathogen. In humans, opportunistic infection by *P. aeruginosa* is often seen in patients with varying degrees of compromised host defenses such as those suffering from damaged corneal lenses, cancer, AIDS, severe burn wounds, and CF (Lyczak et al., 2000). As with the more environmental physiological aspects described above, LPS of *P. aeruginosa* plays multiple key roles in the interaction between the pathogen and the infected host through direct engagement or evasion of innate and adaptive immune system responses.

During the course of infection, it is a general understanding that the humoral adaptive immune response has a lag phase of between 5 days to a week from initial infection detection; in that time period, the innate immune system plays an essential role in engaging and

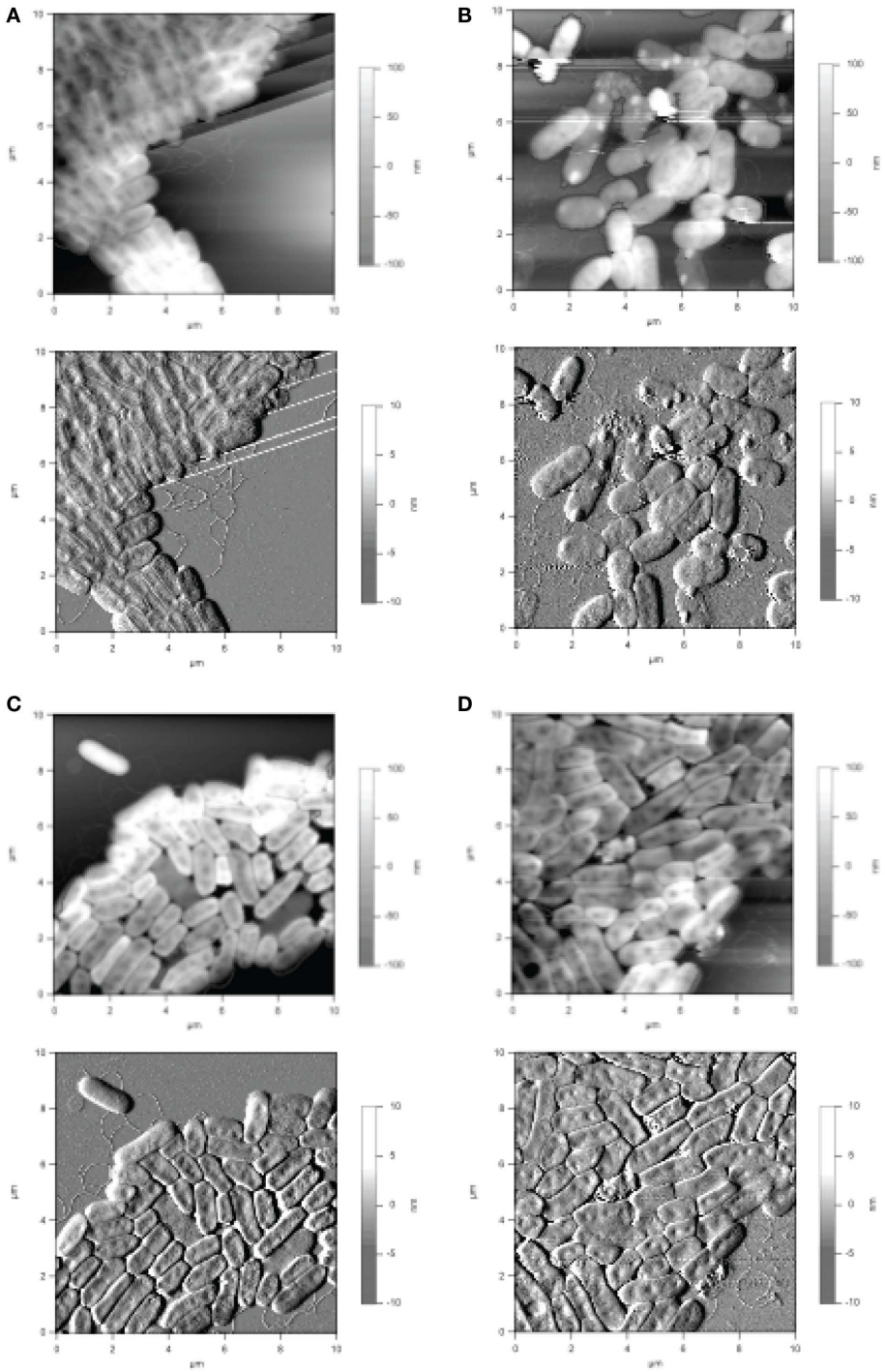

FIGURE 6 | Images obtained from Atomic Force Microscopy analyses of *P. aeruginosa* cells collected in contact mode in air. (A) Strain PAO1; **(B)** *migA* mutant; **(C)** *vapR* mutant; **(D)** *rmlC* mutant. Height images (top panels) are shown with enclosed areas for roughness calculations. Deflection images (bottom panels) reveal more details in cell morphology. The straight lines in **(A)** are steps in the mica substratum. Reproduced from Lau et al. (2009b), with permission from Copyright Clearance Centre.

managing the infection through a variety of mechanisms. One such mode of action involves the recognition of pathogen-associated molecular patterns (PAMPs) by various pattern recognition receptors in the host (Janeway and Medzhitov, 2002). Arguably, the most widely described reaction of the innate immune system to LPS is that of endotoxicity resulting from recognition of the distal lipid A moiety by the host Toll-like receptor 4 (TLR4) complex, which consists of the TLR4 receptor, the co-receptor component MD-2, and the GPI-linked LPS binding protein CD14 (Da Silva Correia et al., 2001; Hajjar et al., 2002; Palsson-Mcdermott and O'neill,

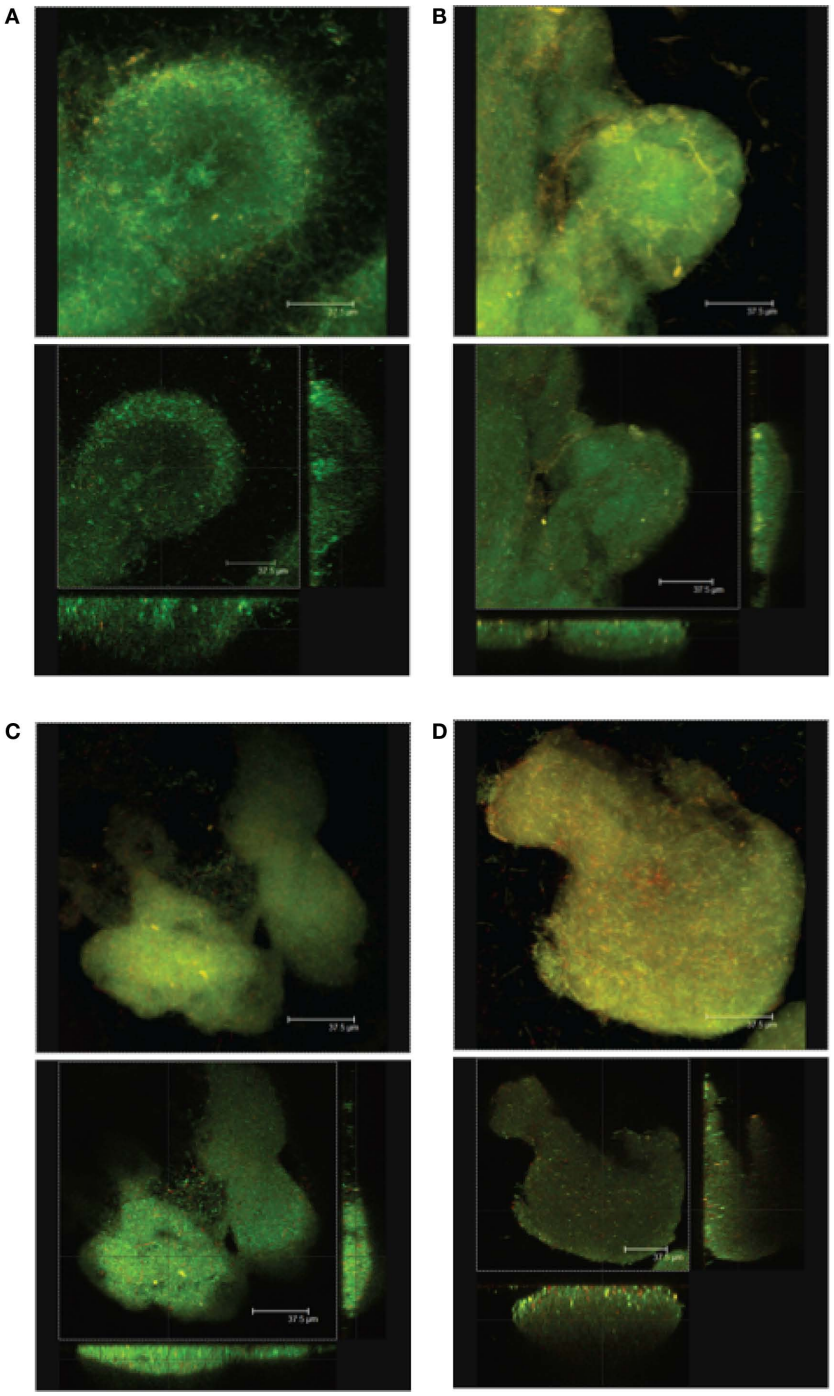

FIGURE 7 | Images from confocal laser scanning microscopy analyses of *P. aeruginosa* cells that illustrate the changes in biofilm structure resulting from truncation in the LPS core in mutant strains as comparing to the wildtype bacteria. Average projections (top panels) and midpoint cross sections (bottom panels) of representative microcolonies of **(A)** wildtype strain PAO1, **(B)** *migA* mutant, **(C)** *wapR* mutant, and **(D)** *rmlC* mutant are shown. Reproduced from Lau et al. (2009b), with permission from Copyright Clearance Centre.

2004; Miller et al., 2005). As discussed earlier, during adaptation of *P. aeruginosa* to the CF airway, the normally penta-acylated lipid A of the bacterium (**Figure 5**, lipid A form B) was modified to a hexa-acylated form (**Figure 5**, lipid A form D; Ernst et al., 2003), becoming a more robust proinflammatory stimulus mediated by the TLR4 complex (Hajjar et al., 2002). In contrast, LPS with penta-acylated lipid A is less potent. Compared to enterobacterial lipid A which has the hexa-acyl form with longer chain fatty acids (C14 versus C10–C12), *P. aeruginosa* lipid A showed a significantly lower toxicity in an experimental animal model infection (Takada and

Kotani, 1992; Backhed et al., 2003). On the surface, direct stimulation of the innate immune system by *P. aeruginosa* would appear to be a curious survival strategy for a pathogen that establishes chronic infections in CF patients; however, this strongly points to an as-yet-to-be-determined survival advantage for *P. aeruginosa* conferred by constant inflammation of the airway epithelium. Conversely, this sequence of proinflammatory responses has been recognized as a direct contributing factor to the gradual deterioration of lung capacity in CF patients with *P. aeruginosa* infection (Hoiby et al., 2010).

Modifications to the lipid A domain of LPS may also confer other survival advantages. The host innate immune system produces a wide range of CAMPs, and some of them (such as cecropin and human defensin) directly interact with bacterial membranes (Hancock and Diamond, 2000). The addition of positively charged aminoarabinose to the phosphate groups of lipid A reduces the permeability of the OM or the affinity of lipid A for those CAMPs, and has clearly been shown to promote *P. aeruginosa* resistance to CAMPs; thus, such modifications may increase bacterial survival rates during host colonization (Ernst et al., 1999, 2007; Macfarlane et al., 2000; Moskowitz et al., 2004; Trent, 2004).

The principal genetic defect resulting in altered physiology of patients with CF is a loss of function of the CF transmembrane conductance regulator (CFTR), a chloride ion channel important for maintaining the optimal viscosity of mucus membranes. Impaired CFTR function results in the production of mucus that is highly viscous, creating an ecological niche in the respiratory tract similar to a biofilm matrix, ideal for colonization by *P. aeruginosa* (Gibson et al., 2003). However, in addition to the increased viscosity of the lung environment, a deficiency in binding of *P. aeruginosa* by CFTR also contributes to pathogenesis. Uptake of bacteria by airway epithelial cells has been implicated as a key mechanism for clearance of *P. aeruginosa* and CFTR was shown to play a role as a receptor and contribute to epithelial internalization (Bajmoczi et al., 2009). Murine cells lacking recombinant human CFTR, as well as those expressing the predominant ΔF508 CFTR mutant, displayed significant impairment in the binding and endocytosis of *P. aeruginosa*, compared to expression of the wildtype human protein (Pier et al., 1997), with the core OS portion of the LPS molecule of the bacterium implicated as the ligand responsible for uptake (Pier et al., 1996). As such, in individuals with wildtype CFTR, clearance of *P. aeruginosa* is mediated by uptake of the infecting bacteria via binding to CFTR and subsequent internalization. However, studies of experimental eye infections demonstrated that CFTR-mediated internalization of *P. aeruginosa* by corneal epithelial cells is essential to the development of keratitis (Zaidi et al., 1999), a potentially severe infection of the ocular lens often developed by individuals that frequently wear contact lenses (Robertson et al., 2007). In this scenario, the core OS-mediated interaction of CFTR with *P. aeruginosa* is detrimental to the human host rather than beneficial.

Activation of the complement cascade, which is comprised of more than 30 serum and cell-surface proteins, is another response of the innate immune system essential to combating infection. Three known mechanisms lead to activation of the complement system, namely, the classical (CCP), alternative (ACP), and mannan-binding lectin (MBL) pathways, each of which results in activation of C3 to yield C3b; in turn, this can lead to opsonization of pathogens and engulfment by phagocytes or surface deposition of complement components C5b, C6, C7, C8, and C9 to form the lytic membrane-attack complex (Dunkelberger and Song, 2010). In a murine model of *P. aeruginosa*-induced pneumonia, C3-deficient mice displayed higher mortality rates than C3-expressing mice. The C3-deficient mortality rates were similar to those observed for mice deficient in ACP-specific factor B, but not for mice deficient in CCP- and MBL-specific C4, indicating a critical role for the ACP in controlling initial *P. aeruginosa* pulmonary infection. Phagocytic cells from both the C3-expressing and C3-deficient mice displayed a reduced capacity *in vitro* to bind and take up *P. aeruginosa* in the presence of C3-deficient serum compared to C3-containing serum, suggesting that phagocytic clearance of the bacterium via C3-mediated opsonization constitutes a part of the protection afforded by the ACP against *P. aeruginosa* infection(Mueller-Ortiz et al., 2004).

Clinical isolates of *P. aeruginosa* from CF patients, deficient in long-chain O-Ag production, were found to be sensitive to pooled normal human serum (PNHS) when compared to subcultured derivatives of the parental strains with restored O-Ag chain biosynthesis, which were found to be serum resistant (Schiller et al., 1984). The same derivative strains with restored O-Ag biosynthesis were subsequently shown to activate (i.e., consume) more complement from PNHS per bacterial organism than their O-Ag-deficient parental counterparts. Serum-sensitive strains were observed to accumulate more C3 than serum-resistant strains, with the C3 accumulation also occurring more rapidly in the former; both of these phenotypes were reduced upon prior treatment of the PNHS with ethylene glycol tetraacetic acid (EGTA, which blocks the CCP), suggesting that the CCP is important for maximal deposition of C3. Analysis of the form of C3 deposited on the surface of the various serum-sensitive and serum-resistant strains indicated equivalent amounts of C3b and iC3b (proteolytically inactivated C3b) in the former, while the latter contained mostly iC3b (Schiller et al., 1989). Taken together, these results indicate that complement-mediated responses to *P. aeruginosa* are an important part of the infection resolution mechanism, which is directly affected by the presence of O-Ag on the bacterial surface.

Sugar-binding proteins known as lectins, of which there are several classes, are also known to play a role in the innate immune response to *P. aeruginosa* infection. Surfactant proteins A (SP-A) and D (SP-D) are part of the calcium-dependent, collagenous C-type lectin family, containing carbohydrate-recognition domains. In the lung, they are secreted into the mucus lining the epithelium wherein they facilitate clearance of microbial pathogens via agglutination, neutralization, and opsonization (Holmskov et al., 2003). SP-A and SP-D have been shown to bind both rough and smooth strains of *P. aeruginosa* (Bufler et al., 2003), with SP-D interacting specifically with LPS (Kishore et al., 1996); incidentally, both surfactant proteins have been shown to be important for clearance of *P. aeruginosa* (Levine et al., 1998; Mariencheck et al., 1999; Restrepo et al., 1999; Ni et al., 2005) but are present at reduced levels in the lungs of CF patients (Postle et al., 1999). In a separate investigation, the signal from biotin-labeled LPS from

P. aeruginosa was shown to overlap with that obtained with antibodies against the S-type lectin galectin-3 via Western blotting of human corneal epithelial proteins, suggesting binding of the LPS by galectin-3. Additionally, pretreatment of scarified corneas in whole-eye culture with mAbs against either the outer core OS from *P. aeruginosa* or galectin-3 was found to inhibit binding of the bacterium to the cornea (Gupta et al., 1997). Coupled with the importance of SP-A and SP-D described above, the capacity of galectin-3 in binding of *P. aeruginosa* indicates a significant role for LPS-dependent lectin-mediated clearance of *P. aeruginosa* infection via the innate immune response.

It has been demonstrated that the type III secretion system (T3SS) of the bacterium mediates direct virulence-promoting primary interactions between *P. aeruginosa* and the host. This pathogenesis mechanism involves the highly regulated contact-dependent insertion of a needle-like injector assembly into a host cell by the surface-associated bacterium, followed by the extrusion of four principal effector proteins (ExoS, T, U, Y) from the bacterium directly into the cytosol of the host cell. These type III effector proteins display a wide range of substrate specificities and in turn perturb a diverse range of host cell functions (Hauser, 2009). Intriguingly, upon comparison of isogenic LPS mutants to wildtype *P. aeruginosa*, the relative levels of expression of *exoS* and *exoT* (encoding effectors) *exsA* (encoding the positive T3SS regulator), and *pcrV* (encoding the injection-facilitation component) displayed marked increases in the absence of OSA. Even more pronounced increases in gene expression were observed in the simultaneous absence of OSA and CPA, suggesting a strong regulatory association between the presence/absence of capped core OS and T3SS effector expression (Augustin et al., 2007). Intracellularly, the production of ExoS and PcrV were found to be reflective of upregulated gene expression. *P. aeruginosa* mutants defective in OSA and CPA were also found to confer increased cytotoxicity *in vitro* and *in vivo* in lung epithelial cells and a murine model of infection, respectively (Augustin et al., 2007), indicating that the presence of O-Ag is an important factor contributing to the regulation and expression of T3SS effectors.

Notwithstanding either innate immune system interplay or T3SS activity, the host–pathogen interaction dynamic is also applicable with *P. aeruginosa* as the host and bacteriophage as the infectious agents. To date, multiple motifs on the LPS molecule of *P. aeruginosa* have been identified as receptors for various bacteriophages. As discussed earlier, bacteriophage A7 was found to bind and hydrolyze the D-Rha of CPA in a *P. aeruginosa* mutant devoid of OSA (Rivera et al., 1992). In addition, specificity for OSA has also been identified through examination of bacteriophage D3; lysogenization with the phage resulted in a loss of adsorption with the same phage during subsequent infection cycles (Holloway and Cooper, 1962), a phenotypic alteration later identified to be a result of acetyl-group addition at the D-Fuc residue as well as alteration of the linkage stereochemistry between OSA repeat units from $\alpha 1 \rightarrow 4$ to $\beta 1 \rightarrow 4$, resulting in serotype conversion. Lysogenization by D3 also impeded the binding ability of the unrelated LPS-specific phage E79 (Kuzio and Kropinski, 1983). The core OS of the bacterium is not only an important ligand for interaction with eukaryotic cells, but it is also a receptor for bacteriophages. Phages φCTX, φPLS27, E79, and H22 have all been shown to recognize the core OS domain of *P. aeruginosa* LPS (Meadow and Wells, 1978; Jarrell and Kropinski, 1981b; Temple et al., 1986; Yokota et al., 1994).

Analogous to the binding of *P. aeruginosa* by bacteriophage is the interaction of pyocins with the surface of the bacterium. Pyocins are chromosomally encoded by over 90% of *P. aeruginosa* strains and are inducible upon treatment with certain mutagenic agents. Depending on the type of pyocins, which might behave like lytic proteins or phage-like particles, they can disrupt the membranes of both related and unrelated bacteria, but which normally have no effect on the host strain (Michel-Briand and Baysse, 2002). Three principal pyocin types have been described, with the R-type (rod-shaped) pyocins closely resembling the contractile tails of bacteriophage. Specifically, R-type pyocins are related to φCTX bacteriophages (Hayashi et al., 1994), with core OS serving as a receptor. Five subtypes of R-type pyocins (R1–R5) have been described, each with different killing spectra. Using a collection of well-defined LPS-deficient *P. aeruginosa* knockout mutants generated by our group, the Rha^{A}, Glc^{II}, and Glc^{I}/Glc^{III} core sugars (**Figure 5**) were identified as receptors for R1, R2, and R5 R-type pyocins, respectively. Therefore, spontaneous truncations of the core OS provide resistance to some of the R pyocins. The presence of OSA on the surface of *P. aeruginosa* was demonstrated to provide a "shield" against pyocin-mediated killing (Köhler et al., 2010).

TREATMENT AND PREVENTION OF INFECTION

Upon infection with *P. aeruginosa*, leading treatment regimens often involve doses of antibiotics such as oral ciprofloxacin, or aerosolized drugs including TOBI® (inhaled tobramycin therapeutic), or colymycin (inhaled colistin–lysin) that are currently being used to treat CF patients nowadays (Anderson, 2010). Tobramycin belongs to the family of aminoglycosides that inhibits bacterial protein biosynthesis via irreversible binding to the 30S bacterial ribosome (Edson and Terrell, 1999). Aminoglycosides such as gentamicin have also been shown to disrupt the cell envelope of *P. aeruginosa* (Martin and Beveridge, 1986; Walker and Beveridge, 1988), leading to lysis of the bacterium (Kadurugamuwa et al., 1993a). Irrespective of its mechanism of bactericidal activity, the initial event in gentamicin-mediated treatment of infection involves ionic binding of the drug to the surface of *P. aeruginosa*. However, comparison of a wildtype strain with isogenic mutants lacking various LPS glycoforms indicated a higher affinity of OMs containing OSA for gentamicin. Furthermore, OSA-expressing strains were more susceptible to killing via gentamicin treatment, with viability reduced almost 50% in strains simultaneously expressing OSA and CPA as a result of higher antibiotic binding than in any other strain (Kadurugamuwa et al., 1993b). The importance of OSA in gentamicin binding has direct implications for the treatment of CF patients, as chronic *P. aeruginosa* isolates often lose the ability to synthesize OSA, while LPS capped with CPA is maintained (Hancock et al., 1983; Lam et al., 1989).

Given the ability of various phages to specifically and selectively target their respective host bacterium, combined with their overall lack of eukaryotic epitope recognition, the use of phage to control bacterial infection is once again gaining attention in the medical community after a prolonged period of research dormancy in the field (Hanlon, 2007) This is of particular interest in light of the

continued development of widespread resistance to antibiotics, including isolates of *P. aeruginosa*, a bacterium already known to possess high intrinsic antibiotic resistance. As such, with a range of *P. aeruginosa*-specific bacteriophages identified (Ceyssens and Lavigne, 2010), phage therapy could serve as an alternative or complementary method to the treatment and management of infections with this bacterium. One such example is the recently identified LPS-specific phage JG024 which was found to efficiently lyse a wide range of environmental and clinical isolates of *P. aeruginosa* (Garbe et al., 2010).

Rather than intervention through therapeutic measures after an infection has occurred, researchers have pursued prevention of *P. aeruginosa* colonization through the use of immunogenic vaccines to foster sustained adaptive immunity (Stanislavsky and Lam, 1997). Various vaccine formulations have been tested in a range of healthy and compromised individuals, using mixtures of LPS from different serotypes, different LPS components, immunogenic toxins conjugated to LPS components, and inactivated whole-cell preparations, all traditionally administered via injection (Stanislavsky and Lam, 1997; Sedlak-Weinstein et al., 2005). While certain vaccine trials involving whole-molecule LPS preparations have yielded increased anti-LPS antibody titres, most LPS vaccines have been characterized by various toxic side effects, yielding inconsistent results with varying degrees of sustained protection (Sedlak-Weinstein et al., 2005). Furthermore, certain instances of non-*Pseudomonas* microbe replacement have also been identified following testing of LPS vaccines (Pennington et al., 1975; Jones et al., 1979). To circumvent issues of pyrogenicity associated with purified LPS used for injection, lipid A-core OS from *P. aeruginosa* (and three other species) was reconstituted in liposomes and used to immunize rabbits, resulting in a well-tolerated vaccine that induced the production of cross-reactive anti-core OS antibodies against a large panel of pathogenic Gram-negative bacteria expressing both rough and smooth LPS (Bennett-Guerrero et al., 2000); this same liposomal formulation was later demonstrated to reduce the induction of TNF-α production *in vitro* when compared to the equivalent amounts of the purified LPS constituents (Erridge et al., 2002). As such, liposomal reconstitution may provide a means of reducing the endotoxic effects of LPS vaccine while still maintaining their protective efficacy.

In comparison to immunization with whole-molecule LPS, OSA vaccines are well tolerated. Correlation between the length of the OSA polymer and the production of protective antibodies was demonstrated in mice, with OSA containing over 18 repeat units inducing 50- to 100-fold increases (Macintyre et al., 1986). In CF patients not previously infected by *P. aeruginosa*, immunization with an octavalent OSA vaccine conjugated to exotoxin A was found to elicit high levels of anti-OSA antibodies in the serum (Lang et al., 1995), a trend sustained with yearly immunizations for a decade (Zuercher et al., 2006). These regular immunizations with the octavalent OSA-exotoxin A conjugate vaccine were found to delay the occurrence of colonization and reduce the frequency of chronic infection in young CF patients (Lang et al., 2004), demonstrating promise as a potential commercialized vaccine to prevent *P. aeruginosa* infection. Another method to induce protective immunity against *P. aeruginosa* has been through the generation of recombinant immunogens involving the OSA from certain serotypes. Heterologous expression of *P. aeruginosa* O11 OSA in an attenuated strain of *S. enterica* sv. Typhimurium, followed by intranasal immunization, resulted in the production of OSA-specific antibodies in the serum of immunized mice. Complete protection with the immunization was also provided against respiratory challenges until 6 months post-vaccination as well as against infection from a burn wound, while only partial protection was provided against corneal infection (Digiandomenico et al., 2007). This is a novel method for the stimulation of adaptive immunity against *P. aeruginosa*. However, despite the data from some of these studies demonstrating efficacy of the vaccine formulations in animal models, obvious hurdles must be overcome before the vaccines can be administered to CF patients. One of these would be the development of an effective and accurate diagnostic method to determine whether or not a young CF patient has been colonized by *P. aeruginosa*. Infected patients produce high amounts of antibodies against LPS. Therefore, administering a vaccine to a patient who has previously been colonized by *P. aeruginosa* can have deleterious effects due to the formation of immune complexes between specific antibodies against *P. aeruginosa* surface antigens, such as LPS, and the vaccine antigens. The presence of these immune complexes could lead to serum sickness. Ultimately, the continued pursuit of an effective anti-*P. aeruginosa* vaccine involving LPS (whole or in part) is an important avenue of research that will undoubtedly benefit millions of people suffering from a range of medical conditions.

Considering all of the aforementioned biological implications of LPS diversity in *P. aeruginosa*, it is indisputable that the bacterium follows a very complex infection dynamic, involving a multitude of simultaneous interactions with the host, many of which it can exploit to further pathogenesis and its continued survival upon infection; yet a majority of these virulence mechanisms are affected, either directly or indirectly, by the LPS of the pathogen. As such, continued research into the biosynthesis and assembly of this important cell-surface virulence factor is essential to clearly understanding the colonization process and eventually controlling or inhibiting infection by *P. aeruginosa*.

CONCLUSION

In this review, we have provided a comprehensive account of the genetics for the biosynthesis of each of the three regions of *P. aeruginosa* LPS. We have also provided a critical review of the information in the literature pertaining to knowledge of how the variation in producing each region of the LPS can impart diversity to this species. External factors such as temperate bacteriophages that recognize LPS epitopes as receptors, followed by internalization and propagating the viral life cycle, also influence diversity of LPS. All of these different factors account for the heterogeneity of *P. aeruginosa*, not only of the chain length of the O-Ag, but also of the chemical make up that accounts for the different serotypes. We close out the discussion by addressing the importance of LPS in host–pathogen interactions and the strategies that researchers use to target this cell-surface glycolipid to develop effective vaccines or novel antimicrobial interventions against *P. aeruginosa* infections. Suggestions for future research have been integrated throughout the various sections.

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REFERENCES

Abeyrathne, P. D., Daniels, C., Poon, K. K., Matewish, M. J., and Lam, J. S. (2005). Functional characterization of WaaL, a ligase associated with linking O-antigen polysaccharide to the core of *Pseudomonas aeruginosa* lipopolysaccharide. *J. Bacteriol.* 187, 3002–3012.

Abeyrathne, P. D., and Lam, J. S. (2007). WaaL of *Pseudomonas aeruginosa* utilizes ATP in in vitro ligation of O antigen onto lipid A-core. *Mol. Microbiol.* 65, 1345–1359.

Anderson, P. (2010). Emerging therapies in cystic fibrosis. *Ther. Adv. Respir. Dis.* 4, 177–185.

Arsenault, T. L., Hughes, D. W., Maclean, D. B., Szarek, W. A., Kropinski, A. M. B., and Lam, J. S. (1991). Structural studies on the polysaccharide portion of 'A-band' lipopolysaccharide from a mutant (AK14O1) of *Pseudomonas aeruginosa* PAO1. *Can. J. Chem.* 69, 1273–1280.

Augustin, D. K., Song, Y., Baek, M. S., Sawa, Y., Singh, G., Taylor, B., Rubio-Mills, A., Flanagan, J. L., Wiener-Kronish, J. P., and Lynch, S. V. (2007). Presence or absence of lipopolysaccharide O antigens affects type III secretion by *Pseudomonas aeruginosa*. *J. Bacteriol.* 189, 2203–2209.

Backhed, F., Normark, S., Schweda, E. K., Oscarson, S., and Richter-Dahlfors, A. (2003). Structural requirements for TLR4-mediated LPS signalling: a biological role for LPS modifications. *Microbes Infect.* 5, 1057–1063.

Bagdian, G., Luderitz, O., and Staub, A. M. (1966). Immunochemical studies on *Salmonella*: XI chemical modification correlated with conversion of group B *Salmonella* by bacteriophage 27. *Ann. N. Y. Acad. Sci.* 133, 405–424.

Bajmoczi, M., Gadjeva, M., Alper, S. L., Pier, G. B., and Golan, D. E. (2009). Cystic fibrosis transmembrane conductance regulator and caveolin-1 regulate epithelial cell internalization of *Pseudomonas aeruginosa*. *Am. J. Physiol. Cell Physiol.* 297, C263–C277.

Beckmann, F., Moll, H., Jager, K. E., and Zahringer, U. (1995). Preliminary communication 7-*O*-carbamoyl-L-glycero-D-manno-heptose: a new core constituent in the lipopolysaccharide of *Pseudomonas aeruginosa*. *Carbohydr. Res.* 267, C3–C7.

Bedoux, G., Vallee-Rehel, K., Kooistra, O., Zahringer, U., and Haras, D. (2004). Lipid A components from *Pseudomonas aeruginosa* PAO1 (serotype O5) and mutant strains investigated by electrospray ionization ion-trap mass spectrometry. *J. Mass. Spectrom.* 39, 505–513.

Belanger, M., Burrows, L. L., and Lam, J. S. (1999). Functional analysis of genes responsible for the synthesis of the B-band O-antigen of *Pseudomonas aeruginosa* serotype O6 lipopolysaccharide. *Microbiology* 145, 3505–3521.

Bennett-Guerrero, E., Mcintosh, T. J., Barclay, G. R., Snyder, D. S., Gibbs, R. J., Mythen, M. G., and Poxton, I. R. (2000). Preparation and preclinical evaluation of a novel liposomal complete-core lipopolysaccharide vaccine. *Infect. Immun.* 68, 6202–6208.

Bhat, R., Marx, A., Galanos, C., and Conrad, R. S. (1990). Structural studies of lipid A from *Pseudomonas aeruginosa* PAO1: occurrence of 4-amino-4-deoxyarabinose. *J. Bacteriol.* 172, 6631–6636.

Bishop, R. E. (2005). Fundamentals of endotoxin structure and function. *Contrib. Microbiol.* 12, 1–27.

Bufler, P., Schmidt, B., Schikor, D., Bauernfeind, A., Crouch, E. C., and Griese, M. (2003). Surfactant protein A and D differently regulate the immune response to nonmucoid *Pseudomonas aeruginosa* and its lipopolysaccharide. *Am. J. Respir. Cell Mol. Biol.* 28, 249–256.

Burrows, L. L., Charter, D. F., and Lam, J. S. (1996). Molecular characterization of the *Pseudomonas aeruginosa* serotype O5 (PAO1) B-band lipopolysaccharide gene cluster. *Mol. Microbiol.* 22, 481–495.

Burrows, L. L., Chow, D., and Lam, J. S. (1997). *Pseudomonas aeruginosa* B-band O-antigen chain length is modulated by Wzz (Ro1). *J. Bacteriol.* 179, 1482–1489.

Burrows, L. L., and Lam, J. S. (1999). Effect of wzx (rfbX) mutations on A-band and B-band lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa* O5. *J. Bacteriol.* 181, 973–980.

Bystrova, O. V., Knirel, Y. A., Lindner, B., Kocharova, N. A., Kondakova, A. N., Zahringer, U., and Pier, G. B. (2006). Structures of the core oligosaccharide and O-units in the R- and SR-type lipopolysaccharides of reference strains of *Pseudomonas aeruginosa* O-serogroups. *FEMS Immunol. Med. Microbiol.* 46, 85–99.

Cavenagh, M. M., and Miller, R. V. (1986). Specialized transduction of *Pseudomonas aeruginosa* PAO by bacteriophage D3. *J. Bacteriol.* 165, 448–452.

Cerantola, S., and Montrozier, H. (1997). Structural elucidation of two polysaccharides present in the lipopolysaccharide of a clinical isolate of *Burkholderia cepacia*. *Eur. J. Biochem.* 246, 360–366.

Ceyssens, P.-J., and Lavigne, R. (2010). Bacteriophages of *Pseudomonas*. *Future Microbiol.* 5, 1041–1055.

Choudhury, B., Carlson, R. W., and Goldberg, J. B. (2005). The structure of the lipopolysaccharide from a galU mutant of *Pseudomonas aeruginosa* serogroup-O11. *Carbohydr. Res.* 340, 2761–2772.

Choudhury, B., Carlson, R. W., and Goldberg, J. B. (2008). Characterization of the lipopolysaccharide from a wbjE mutant of the serogroup O11 *Pseudomonas aeruginosa* strain, PA103. *Carbohydr. Res.* 343, 238–248.

Creuzenet, C., and Lam, J. S. (2001). Topological and functional characterization of WbpM, an inner membrane UDP-GlcNAc C6 dehydratase essential for lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 41, 1295–1310.

Cryz, S. J., Jr., Pitt, T. L., Furer, E., and Germanier, R. (1984). Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* 44, 508–513.

Currie, H. L., Lightfoot, J., and Lam, J. S. (1995). Prevalence of gca, a gene involved in synthesis of A-band common antigen polysaccharide in *Pseudomonas aeruginosa*. *Clin. Diagn. Lab. Immunol.* 2, 554–562.

Da Silva Correia, J., Soldau, K., Christen, U., Tobias, P. S., and Ulevitch, R. J. (2001). Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR4 and MD-2. *J. Biol. Chem.* 276, 21129–21135.

Daniels, C., Griffiths, C., Cowles, B., and Lam, J. S. (2002). *Pseudomonas aeruginosa* O-antigen chain length is determined before ligation to lipid A core. *Environ. Microbiol.* 4, 883–897.

Dasgupta, T., De Kievit, T. R., Masoud, H., Altman, E., Richards, J. C., Sadovskaya, I., Speert, D. P., and Lam, J. S. (1994). Characterization of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* derived from serotypes O3, O5, and O6. *Infect. Immun.* 62, 809–817.

De Kievit, T. R., Dasgupta, T., Schweizer, H., and Lam, J. S. (1995). Molecular cloning and characterization of the rfc gene of *Pseudomonas aeruginosa* (serotype O5). *Mol. Microbiol.* 16, 565–574.

De Kievit, T. R., and Lam, J. S. (1994). Monoclonal antibodies that distinguish inner core, outer core, and lipid A regions of *Pseudomonas aeruginosa* lipopolysaccharide. *J. Bacteriol.* 176, 7129–7139.

De Kievit, T. R., and Lam, J. S. (1997). Isolation and characterization of two genes, waaC (rfaC) and waaF (rfaF), involved in *Pseudomonas aeruginosa* serotype O5 inner-core biosynthesis. *J. Bacteriol.* 179, 3451–3457.

Dean, C. R., Franklund, C. V., Retief, J. D., Coyne, M. J. Jr., Hatano, K., Evans, D. J., Pier, G. B., and Goldberg, J. B. (1999). Characterization of the serogroup O11 O-antigen locus of *Pseudomonas aeruginosa* PA103. *J. Bacteriol.* 181, 4275–4284.

Dean, C. R., and Goldberg, J. B. (2000). The wbpM gene in *Pseudomonas aeruginosa* serogroup O17 resides on a cryptic copy of the serogroup O11 O antigen gene locus. *FEMS Microbiol. Lett.* 187, 59–63.

Digiandomenico, A., Rao, J., Harcher, K., Zaidi, T. S., Gardner, J., Neely, A. N., Pier, G. B., and Goldberg, J. B. (2007). Intranasal immunization with heterologously expressed polysaccharide protects against multiple *Pseudomonas aeruginosa* infections. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4624–4629.

Dotson, G. D., Kaltashov, I. A., Cotter, R. J., and Raetz, C. R. (1998). Expression cloning of a *Pseudomonas* gene encoding a hydroxydecanoyl-acyl carrier protein-dependent UDP-GlcNAc acyltransferase. *J. Bacteriol.* 180, 330–337.

Dunkelberger, J. R., and Song, W. C. (2010). Complement and its role in innate and adaptive immune responses. *Cell Res.* 20, 34–50.

Edson, R. S., and Terrell, C. L. (1999). The aminoglycosides. *Mayo Clin. Proc.* 74, 519–528.

Ernst, R. K., Adams, K. N., Moskowitz, S. M., Kraig, G. M., Kawasaki, K., Stead, C. M., Trent, M. S., and Miller, S. I. (2006). The *Pseudomonas aeruginosa* lipid A deacylase: selection for expression and loss within the cystic fibrosis airway. *J. Bacteriol.* 188, 191–201.

Ernst, R. K., Hajjar, A. M., Tsai, J. H., Moskowitz, S. M., Wilson, C. B., and Miller, S. I. (2003). *Pseudomonas aeruginosa* lipid A diversity and its recognition by Toll-like receptor 4. *J. Endotoxin. Res.* 9, 395–400.

Ernst, R. K., Moskowitz, S. M., Emerson, J. C., Kraig, G. M., Adams, K. N., Harvey, M. D., Ramsey, B., Speert, D. P., Burns, J. L., and Miller, S. I. (2007). Unique lipid A modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. *J. Infect. Dis.* 196, 1088–1092.

Ernst, R. K., Yi, E. C., Guo, L., Lim, K. B., Burns, J. L., Hackett, M., and Miller, S. I. (1999). Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* 286, 1561–1565.

Erridge, C., Stewart, J., Bennett-Guerrero, E., Mcintosh, T. J., and Poxton, I. R. (2002). The biological activity of a liposomal complete core lipopolysaccharide vaccine. *J. Endotoxin. Res.* 8, 39–46.

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., and Prince, A. (1998). Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect. Immun.* 66, 43–51.

Fuqua, W. C., Winans, S. C., and Greenberg, E. P. (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* 176, 269–275.

Garbe, J., Wesche, A., Bunk, B., Kazmierczak, M., Selezska, K., Rohde, C., Sikorski, J., Rohde, M., Jahn, D., and Schobert, M. (2010). Characterization of JG024, a *Pseudomonas aeruginosa* PB1-like broad host range phage under simulated infection conditions. *BMC Microbiol.* 10, 301. doi: 10.1186/1471-2180-10-301

Ghanei, H., Abeyrathne, P. D., and Lam, J. S. (2007). Biochemical characterization of MsbA from *Pseudomonas aeruginosa*. *J. Biol. Chem.* 282, 26939–26947.

Gibbons, H. S., Lin, S., Cotter, R. J., and Raetz, C. R. (2000). Oxygen requirement for the biosynthesis of the *S*-2-hydroxymyristate moiety in *Salmonella* typhimurium lipid A. Function of LpxO, A *new* Fe2+/alpha-ketoglutarate-dependent dioxygenase homologue. *J. Biol. Chem.* 275, 32940–32949.

Gibbons, H. S., Reynolds, C. M., Guan, Z., and Raetz, C. R. (2008). An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of *Salmonella* lipid A. *Biochemistry* 47, 2814–2825.

Gibson, R. L., Burns, J. L., and Ramsey, B. W. (2003). Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 168, 918–951.

Goldman, R. C., Doran, C. C., Kadam, S. K., and Capobianco, J. O. (1988). Lipid A precursor from *Pseudomonas aeruginosa* is completely acylated prior to addition of 3-deoxy-D-manno-octulosonate. *J. Biol. Chem.* 263, 5217–5223.

Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997). Regulation of lipid A modifications by *Salmonella* typhimurium virulence genes phoP-phoQ. *Science* 276, 250–253.

Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998). Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* 95, 189–198.

Gupta, S. K., Masinick, S., Garrett, M., and Hazlett, L. D. (1997). *Pseudomonas aeruginosa* lipopolysaccharide binds galectin-3 and other human corneal epithelial proteins. *Infect. Immun.* 65, 2747–2753.

Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B., and Miller, S. I. (2002). Human toll-like receptor 4 recognizes host-specific LPS modifications. *Nat. Immunol.* 3, 354–359.

Hall-Stoodley, L., Costerton, J. W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108.

Hancock, R. E., and Diamond, G. (2000). The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol.* 8, 402–410.

Hancock, R. E., Mutharia, L. M., Chan, L., Darveau, R. P., Speert, D. P., and Pier, G. B. (1983). *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect. Immun.* 42, 170–177.

Hanlon, G. W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int. J. Antimicrob. Agents* 30, 118–128.

Hauser, A. R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat. Rev. Microbiol.* 7, 654–665.

Hayashi, T., Matsumoto, H., Ohnishi, M., Yokota, S., Shinomiya, T., Kageyama, M., and Terawaki, Y. (1994). Cytotoxin-converting phages, phi CTX and PS21, are R pyocin-related phages. *FEMS Microbiol. Lett.* 122, 239–244.

Hoiby, N., Ciofu, O., and Bjarnsholt, T. (2010). *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol.* 5, 1663–1674.

Holloway, B. W., and Cooper, G. N. (1962). Lysogenic conversion in *Pseudomonas aeruginosa*. *J. Bacteriol.* 84, 1321–1324.

Holmskov, U., Thiel, S., and Jensenius, J. C. (2003). Collectins and ficolins: humoral lectins of the innate immune defense. *Annu. Rev. Immunol.* 21, 547–578.

Islam, S. T., Gold, A. C., Taylor, V. L., Anderson, E. M., Ford, R. C., and Lam, J. S. (2011). Dual conserved periplasmic loops possess essential charge characteristics that support a catch-and-release mechanism of O-antigen polymerization by Wzy in *Pseudomonas aeruginosa* PAO1. *J. Biol. Chem.* 286, 20600–20605.

Islam, S. T., Taylor, V. L., Qi, M., and Lam, J. S. (2010). Membrane topology mapping of the O-antigen flippase (Wzx), polymerase (Wzy), and ligase (WaaL) from *Pseudomonas aeruginosa* PAO1 reveals novel domain architectures. *mBio* 1, e00189-10.

Ivanov, I. E., Kintz, E. N., Porter, L. A., Goldberg, J. B., Burnham, N. A., and Camesano, T. A. (2011). Relating the physical properties of *Pseudomonas aeruginosa* lipopolysaccharides to virulence by atomic force microscopy. *J. Bacteriol.* 193, 1259–1266.

Janeway, C. A. Jr., and Medzhitov, R. (2002). Innate immune recognition. *Annu. Rev. Immunol.* 20, 197–216.

Jarrell, K. F., and Kropinski, A. M. (1981a). Isolation and characterization of a bacteriophage specific for the lipopolysaccharide of rough derivatives of *Pseudomonas aeruginosa* strain PAO. *J. Virol.* 38, 529–538.

Jarrell, K. F., and Kropinski, A. M. (1981b). *Pseudomonas aeruginosa* bacteriophage phi PLS27-lipopolysaccharide interactions. *J. Virol.* 40, 411–420.

Jones, R. J., Roe, E. A., and Gupta, J. L. (1979). Controlled trials of a polyvalent *Pseudomonas* vaccine in burns. *Lancet* 314, 977–983.

Kadurugamuwa, J. L., Clarke, A. J., and Beveridge, T. J. (1993a). Surface action of gentamicin on *Pseudomonas aeruginosa*. *J. Bacteriol.* 175, 5798–5805.

Kadurugamuwa, J. L., Lam, J. S., and Beveridge, T. J. (1993b). Interaction of gentamicin with the A band and B band lipopolysaccharides of *Pseudomonas aeruginosa* and its possible lethal effect. *Antimicrob. Agents Chemother.* 37, 715–721.

Kaluzny, K., Abeyrathne, P. D., and Lam, J. S. (2007). Coexistence of two distinct versions of O-antigen polymerase, Wzy-alpha and Wzy-beta, in *Pseudomonas aeruginosa* serogroup O2 and their contributions to cell surface diversity. *J. Bacteriol.* 189, 4141–4152.

Kidd, T. J., Grimwood, K., Ramsay, K. A., Rainey, P. B., and Bell, S. C. (2011). Comparison of three molecular techniques for typing *Pseudomonas aeruginosa* isolates in sputum samples from patients with cystic fibrosis. *J. Clin. Microbiol.* 49, 263–268.

King, J. D., Kocincova, D., Westman, E. L., and Lam, J. S. (2009). Review: lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. *Innate Immun.* 15, 261–312.

King, J. D., Vinogradov, E., Tran, V., and Lam, J. S. (2010). Biosynthesis of uronamide sugars in *Pseudomonas aeruginosa* O6 and *Escherichia coli* O121 O-Antigens. *Environ. Microbiol.* 12, 1531–1544.

Kintz, E., Scarff, J. M., Digiandomenico, A., and Goldberg, J. B. (2008). Lipopolysaccharide O-antigen chain length regulation in *Pseudomonas aeruginosa* serogroup O11 strain PA103. *J. Bacteriol.* 190, 2709–2716.

Kishore, U., Wang, J.-Y., Hoppe, H.-J., and Reid, K. B. M. (1996). The (-helical neck region of human lung surfactant protein D is essential for the binding of the carbohydrate recognition domains to lipopolysaccharides and phospholipids. *Biophys. J.* 318, 505–511.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jørgensen, A., Molin, S., and Tolker-Nielsen, T. (2003). Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol. Microbiol.* 48, 1511–1524.

Kneidinger, B., O'riordan, K., Li, J., Brisson, J. -R., Lee, J. C., and Lam, J. S. (2003). Three highly conserved proteins catalyze the conversion of UDP-*N*-acetyl-D-glucosamine to precursors for the biosynthesis of O-Antigen in *Pseudomonas aeruginosa* O11 and capsule in *Staphylococcus aureus* Type 5. *J. Biol. Chem.* 278, 3615–3627.

Knirel, Y. A., Bystrova, O. V., Kocharova, N. A., Zahringer, U., and Pier, G. B. (2006). Conserved and variable structural features in the lipopolysaccharide of *Pseudomonas aeruginosa*. *J. Endotoxin. Res.* 12, 324–336.

Kocharova, N. A., Knirel' Iu, A., Kochetkov, N. K., and Stanislavskii, E. S. (1988). Characteristics of rhamnan isolated from preparations of *Pseudomonas aeruginosa* lipopolysaccharides. *Bioorg. Khim.* 14, 701–703.

Kocíncová, D., Hao, Y., Vinogradov, E., and Lam, J. S. (2011). Evidence that WapB is an 1,2 glucosyltransferase

of *Pseudomonas aeruginosa* involved in LPS outer core biosynthesis. *J. Bacteriol.* 193, 2708–2716.

Kocíncová, D., and Lam, J. S. (2011). Structural diversity of core oligosaccharide domain of *P. aeruginosa* lipopolysaccharide. *Biochemistry (Mosc.)* (accepted).

Köhler, T., Curty, L. K., Barja, F., Van Delden, C., and Pechère, J. -C. (2000). Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J. Bacteriol.* 182, 5990–5996.

Köhler, T., Donner, V., and Van Delden, C. (2010). Lipopolysaccharide as shield and receptor for R-pyocin-mediated killing in *Pseudomonas aeruginosa*. *J. Bacteriol.* 192, 1921–1928.

Kooistra, O., Bedoux, G., Brecker, L., Lindner, B., Sanchez Carballo, P., Haras, D., and Zahringer, U. (2003). Structure of a highly phosphorylated lipopolysaccharide core in the Delta algC mutants derived from *Pseudomonas aeruginosa* wild-type strains PAO1 (serogroup O5) and PAC1R (serogroup O3). *Carbohydr. Res.* 338, 2667–2677.

Koval, S. F., and Meadow, P. M. (1977). The isolation and characterization of lipopolysaccharide-defective mutants of *Pseudomonas aeruginosa* PAC1. *J. Gen. Microbiol.* 98, 387–398.

Kresse, A. U., Dinesh, S. D., Larbig, K., and Römling, U. (2003). Impact of large chromosomal inversions on the adaptation and evolution of *Pseudomonas aeruginosa* chronically colonizing cystic fibrosis lungs. *Mol. Microbiol.* 47, 145–158.

Kropinski, A. M., Farinha, M. A., and Jansons, I. (1994). Nucleotide sequence of the *Pseudomonas aeruginosa* insertion sequence IS222: another member of the IS3 family. *Plasmid* 31, 222–228.

Kulshin, V. A., Zahringer, U., Lindner, B., Jager, K. E., Dmitriev, B. A., and Rietschel, E. T. (1991). Structural characterization of the lipid A component of *Pseudomonas aeruginosa* wild-type and rough mutant lipopolysaccharides. *Eur. J. Biochem.* 198, 697–704.

Kunkel, T. A., and Erie, D. A. (2005). DNA mismatch repair. *Annu. Rev. Biochem.* 74, 681–710.

Kuzio, J., and Kropinski, A. M. (1983). O-antigen conversion in *Pseudomonas aeruginosa* PAO1 by bacteriophage D3. *J. Bacteriol.* 155, 203–212.

Lam, J. S., Macdonald, L. A., and Lam, M. Y. (1987a). Production of monoclonal antibodies against serotype strains of *Pseudomonas aeruginosa*. *Infect. Immun.* 55, 2854–2856.

Lam, J. S., Macdonald, L. A., Lam, M. Y., Duchesne, L. G., and Southam, G. G. (1987b). Production and characterization of monoclonal antibodies against serotype strains of *Pseudomonas aeruginosa*. *Infect. Immun.* 55, 1051–1057.

Lam, M. Y., Mcgroarty, E. J., Kropinski, A. M., Macdonald, L. A., Pedersen, S. S., Hoiby, N., and Lam, J. S. (1989). Occurrence of a common lipopolysaccharide antigen in standard and clinical strains of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 27, 962–967.

Lang, A. B., Rüdeberg, A., Schöni, M. H., Que, J. U., Fürer, E., and Schaad, U. B. (2004). Vaccination of cystic fibrosis patients against *Pseudomonas aeruginosa* reduces the proportion of patients infected and delays time to infection. *Pediatr. Infect. Dis. J.* 23, 504–510.

Lang, A. B., Schaad, U. B., Rüdeberg, A., Wedgwood, J., Que, J. U., Fürer, E., and Cryz, J. S. J. (1995). Effect of high-affinity anti-*Pseudomonas aeruginosa* lipopolysaccharide antibodies induced by immunization on the rate of *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. *J. Pediatr.* 127, 711–717.

Lau, P. C. Y., Dutcher, J. R., Beveridge, T. J., and Lam, J. S. (2009a). Absolute quantitation of bacterial biofilm adhesion and viscoelasticity by microbead force spectroscopy. *Biophys. J.* 96, 2935–2948.

Lau, P. C. Y., Lindhout, T., Beveridge, T. J., Dutcher, J. R., and Lam, J. S. (2009b). Differential lipopolysaccharide core capping leads to quantitative and correlated modifications of mechanical and structural properties in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* 191, 6618–6631.

Leclerc, J. E., Li, B., Payne, W. L., and Cebula, T. A. (1996). High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. *Science* 274, 1208–1211.

Lee, D. G., Urbach, J. M., Wu, G., Liberati, N. T., Feinbaum, R. L., Miyata, S., Diggins, L. T., He, J., Saucier, M., Deziel, E., Friedman, L., Li, L., Grills, G., Montgomery, K., Kucherlapati, R., Rahme, L. G., and Ausubel, F. M. (2006). Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biol.* 7, R90.

Levine, A. M., Kurak, K. E., Bruno, M. D., Stark, J. M., Whitsett, J. A., and Korfhagen, T. R. (1998). Surfactant protein-A-deficient mice are susceptible to *Pseudomonas aeruginosa* infection. *Am. J. Respir. Cell Mol. Biol.* 19, 700–708.

Lightfoot, J., and Lam, J. S. (1991). Molecular cloning of genes involved with expression of A-band lipopolysaccharide, an antigenically conserved form, in *Pseudomonas aeruginosa*. *J. Bacteriol.* 173, 5624–5630.

Lightfoot, J., and Lam, J. S. (1993). Chromosomal mapping, expression and synthesis of lipopolysaccharide in *Pseudomonas aeruginosa*: a role for guanosine diphospho (GDP)-D-mannose. *Mol. Microbiol.* 8, 771–782.

Lindhout, T., Lau, P. C. Y., Brewer, D., and Lam, J. S. (2009). Truncation in the core oligosaccharide of lipopolysaccharide affects flagella-mediated motility in *Pseudomonas aeruginosa* PAO1 via modulation of cell surface attachment. *Microbiology* 155, 3449–3460.

Liu, P. V., Matsumoto, H., Kusama, H., and Bergan, T. O. M. (1983). Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. *Int. J. Syst. Bacteriol.* 33, 256–264.

Liu, P. V., and Wang, S. (1990). Three new major somatic antigens of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 28, 922–925.

Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2000). Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect.* 2, 1051–1060.

Ma, S., Selvaraj, U., Ohman, D. E., Quarless, R., Hassett, D. J., and Wozniak, D. J. (1998). Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.* 180, 956–968.

Macfarlane, E. L., Kwasnicka, A., and Hancock, R. E. (2000). Role of *Pseudomonas aeruginosa* PhoP-phoQ in resistance to antimicrobial cationic peptides and aminoglycosides. *Microbiology* 146(Pt 10), 2543–2554.

Macintyre, S., Lucken, R., and Owen, P. (1986). Smooth lipopolysaccharide is the major protective antigen for mice in the surface extract from IATS serotype 6 contributing to the polyvalent *Pseudomonas aeruginosa* vaccine PEV. *Infect. Immun.* 52, 76–84.

Mariencheck, W. I., Savov, J., Dong, Q., Tino, M. J., and Wright, J. R. (1999). Surfactant protein A enhances alveolar macrophage phagocytosis of a live, mucoid strain of *P. aeruginosa*. *Am. J. Physiol.* 277, L777–L786.

Martin, N. L., and Beveridge, T. J. (1986). Gentamicin interaction with *Pseudomonas aeruginosa* cell envelope. *Antimicrob. Agents Chemother.* 29, 1079–1087.

Masoud, H., Altman, E., Richards, J. C., and Lam, J. S. (1994). General strategy for structural analysis of the oligosaccharide region of lipooligosaccharides. Structure of the oligosaccharide component of *Pseudomonas aeruginosa* IATS serotype 06 mutant R5 rough-type lipopolysaccharide. *Biochemistry* 33, 10568–10578.

Matewish, M. (2004). *The Functional Role of Lipopolysaccharide in the Cell Envelope and Surface Proteins of Pseudomonas aeruginosa*. Ph.D. thesis, University of Guelph, Guelph, ON, Canada.

Mcphee, J. B., Bains, M., Winsor, G., Lewenza, S., Kwasnicka, A., Brazas, M. D., Brinkman, F. S., and Hancock, R. E. (2006). Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg2+-induced gene regulation in *Pseudomonas aeruginosa*. *J. Bacteriol.* 188, 3995–4006.

Mcphee, J. B., Lewenza, S., and Hancock, R. E. (2003). Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 50, 205–217.

Meadow, P. M., and Wells, P. L. (1978). Receptor sites for R-type pyocins and bacteriophage E79 in the Core Part of the lipopolysaccharide of *Pseudomonas aeruginosa* PAC1. *J. Gen. Microbiol.* 108, 339–343.

Mena, A., Smith, E. E., Burns, J. L., Speert, D. P., Moskowitz, S. M., Perez, J. L., and Oliver, A. (2008). Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. *J. Bacteriol.* 190, 7910–7917.

Michel-Briand, Y., and Baysse, C. (2002). The pyocins of *Pseudomonas aeruginosa*. *Biochimie* 84, 499–510.

Miller, R. V., Pemberton, J. M., and Richards, K. E. (1974). F116, D3 and G101: temperate bacteriophages of *Pseudomonas aeruginosa*. *Virology* 59, 566–569.

Miller, S. I., Ernst, R. K., and Bader, M. W. (2005). LPS, TLR4 and infectious disease diversity. *Nat. Rev. Microbiol.* 3, 36–46.

Miller, W. L., Wenzel, C. Q., Daniels, C., Larocque, S., Brisson, J. R., and Lam, J. S. (2004). Biochemical characterization of WbpA, a UDP-*N*-acetyl-D-glucosamine 6-dehydrogenase involved in O-antigen biosynthesis in *Pseudomonas aeruginosa* PAO1. *J. Biol. Chem.* 279, 37551–37558.

Mohan, S., Kelly, T. M., Eveland, S. S., Raetz, C. R., and Anderson, M. S. (1994). An *Escherichia coli* gene (fabZ) encoding (3R)-hydroxymyristoyl acyl carrier protein dehydrase. Relation to fabA and suppression of mutations in lipid A biosynthesis. *J. Biol. Chem.* 269, 32896–32903.

Moskowitz, S. M., and Ernst, R. K. (2010). The role of *Pseudomonas* lipopolysaccharide in cystic fibrosis airway infection. *Subcell. Biochem.* 53, 241–253.

Moskowitz, S. M., Ernst, R. K., and Miller, S. I. (2004). PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *J. Bacteriol.* 186, 575–579.

Mueller-Ortiz, S. L., Drouin, S. M., and Wetsel, R. A. (2004). The alternative activation pathway and complement component C3 are critical for a protective immune response against *Pseudomonas aeruginosa* in a murine model of pneumonia. *Infect. Immun.* 72, 2899–2906.

Mutharia, L. M., and Lam, J. S. (2007). "Antigen-antibody reactions," in *Methods for General and Molecular Microbiology*, 3rd Edn, eds C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. A. Marzluf, T. M. Schmidt, and L. R. Snyder (Washington, DC: ASM Press), 138–167.

Newton, G. J., Daniels, C., Burrows, L. L., Kropinski, A. M., Clarke, A. J., and Lam, J. S. (2001). Three-component-mediated serotype conversion in *Pseudomonas aeruginosa* by bacteriophage D3. *Mol. Microbiol.* 39, 1237–1247.

Ni, M., Evans, D. J., Hawgood, S., Anders, E. M., Sack, R. A., and Fleiszig, S. M. J. (2005). Surfactant protein D is present in human tear fluid and the cornea and inhibits epithelial cell invasion by *Pseudomonas aeruginosa*. *Infect. Immun.* 73, 2147–2156.

O'toole, G. A., and Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* 30, 295–304.

Oberhardt, M. A., Goldberg, J. B., Hogardt, M., and Papin, J. A. (2010). Metabolic network analysis of *Pseudomonas aeruginosa* during chronic cystic fibrosis lung infection. *J. Bacteriol.* 192, 5534–5548.

Ojeniyi, B., Lam, J. S., Hoiby, N., and Rosdahl, V. T. (1989). A comparison of the efficiency in serotyping of *Pseudomonas aeruginosa* from cystic fibrosis patients using monoclonal and polyclonal antibodies. *APMIS* 97, 631–636.

Ojeniyi, B., Wolz, C., Doring, G., Lam, J. S., Rosdahl, V. T., and Hoiby, N. (1990). Typing of polyagglutinable *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *APMIS* 98, 423–431.

Oliver, A., Baquero, F., and Blázquez, J. (2002). The mismatch repair system (mutS, mutL and uvrD genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol. Microbiol.* 43, 1641–1650.

Oliver, A., Cantón, R., Campo, P., Baquero, F., and Blázquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288, 1251–1253.

Ovod, V., Rudolph, K., Knirel, Y., and Krohn, K. (1996). Immunochemical characterization of *O* polysaccharides composing the alpha-D-rhamnose backbone of lipopolysaccharide of *Pseudomonas syringae* and classification of bacteria into serogroups O1 and O2 with monoclonal antibodies. *J. Bacteriol.* 178, 6459–6465.

Palsson-Mcdermott, E. M., and O'neill, L. A. (2004). Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113, 153–162.

Pennington, J. E., Reynolds, H. Y., Wood, R. E., Robinson, R. A., and Levine, A. S. (1975). Use of a *Pseudomonas aeruginosa* vaccine in patients with acute leukemia and cystic fibrosis. *Am. J. Med.* 58, 629–636.

Pier, G. B., Grout, M., and Zaidi, T. S. (1997). Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of *Pseudomonas aeruginosa* from the lung. *Proc. Natl. Acad. Sci. U.S.A.* 94, 12088–12093.

Pier, G. B., Grout, M., Zaidi, T. S., Olsen, J. C., Johnson, L. G., Yankaskas, J. R., and Goldberg, J. B. (1996). Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. *Science* 271, 64–67.

Poon, K. K., Westman, E. L., Vinogradov, E., Jin, S., and Lam, J. S. (2008). Functional characterization of MigA and WapR: putative rhamnosyltransferases involved in outer core oligosaccharide biosynthesis of *Pseudomonas aeruginosa*. *J. Bacteriol.* 190, 1857–1865.

Postle, A. D., Mander, A., Reid, K. B. M., Wang, J. -Y., Wright, S. M., Moustaki, M., and Warner, J. O. (1999). Deficient hydrophilic lung surfactant proteins A and D with normal surfactant phospholipid molecular species in cystic fibrosis. *Am. J. Respir. Cell Mol. Biol.* 20, 90–98.

Raetz, C. R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71, 635–700.

Raymond, C. K., Sims, E. H., Kas, A., Spencer, D. H., Kutyavin, T. V., Ivey, R. G., Zhou, Y., Kaul, R., Clendenning, J. B., and Olson, M. V. (2002). Genetic variation at the O-antigen biosynthetic locus in *Pseudomonas aeruginosa*. *J. Bacteriol.* 184, 3614–3622.

Restrepo, C. I., Dong, Q., Savov, J., Mariencheck, W. I., and Wright, J. R. (1999). Surfactant protein D stimulates phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 21, 576–585.

Rivera, M., Bryan, L. E., Hancock, R. E., and Mcgroarty, E. J. (1988). Heterogeneity of lipopolysaccharides from *Pseudomonas aeruginosa*: analysis of lipopolysaccharide chain length. *J. Bacteriol.* 170, 512–521.

Rivera, M., Chivers, T. R., Lam, J. S., and Mcgroarty, E. J. (1992). Common antigen lipopolysaccharide from *Pseudomonas aeruginosa* AK1401 as a receptor for bacteriophage A7. *J. Bacteriol.* 174, 2407–2411.

Robbins, P. W., Keller, J. M., Wright, A., and Bernstein, R. L. (1965). Enzymatic and kinetic studies on the mechanism of O-Antigen conversion by bacteriophage epsilon-15. *J. Biol. Chem.* 240, 384–390.

Robertson, D. M., Petroll, W. M., Jester, J. V., and Cavanagh, H. D. (2007). Current concepts: contact lens related *Pseudomonas keratitis*. *Cont. Lens Anterior Eye* 30, 94–107.

Rocchetta, H. L., Burrows, L. L., Pacan, J. C., and Lam, J. S. (1998a). Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. *Mol. Microbiol.* 28, 1103–1119.

Rocchetta, H. L., and Lam, J. S. (1997). Identification and functional characterization of an ABC transport system involved in polysaccharide export of A-band lipopolysaccharide in *Pseudomonas aeruginosa*. *J. Bacteriol.* 179, 4713–4724.

Rocchetta, H. L., Pacan, J. C., and Lam, J. S. (1998b). Synthesis of the A-band polysaccharide sugar D-rhamnose requires Rmd and WbpW: identification of multiple AlgA homologues, WbpW and ORF488, in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 29, 1419–1434.

Roy, P. H., Tetu, S. G., Larouche, A., Elbourne, L., Tremblay, S., Ren, Q., Dodson, R., Harkins, D., Shay, R., Watkins, K., Mahamoud, Y., and Paulsen, I. T. (2010). Complete genome sequence of the multiresistant taxonomic outlier *Pseudomonas aeruginosa* PA7. *PLoS ONE* 5, e8842. doi: 10.1371/journal.pone.0008842

Sadovskaya, I., Brisson, J. R., Lam, J. S., Richards, J. C., and Altman, E. (1998). Structural elucidation of the lipopolysaccharide core regions of the wild-type strain PAO1 and O-chain-deficient mutant strains AK1401 and AK1012 from *Pseudomonas aeruginosa* serotype O5. *Eur. J. Biochem.* 255, 673–684.

Sanchez Carballo, P. M., Rietschel, E. T., Kosma, P., and Zahringer, U. (1999). Elucidation of the structure of an alanine-lacking core tetrasaccharide trisphosphate from the lipopolysaccharide of *Pseudomonas aeruginosa* mutant H4. *Eur. J. Biochem.* 261, 500–508.

Sawada, S., Kawamura, T., Masuho, Y., and Tomibe, K. (1985). A new common polysaccharide antigen of strains of *Pseudomonas aeruginosa* detected with a monoclonal antibody. *J. Infect. Dis.* 152, 1290–1299.

Schiller, N., Hackley, D., and Morrison, A. (1984). Isolation and characterization of serum-resistant strains of *Pseudomonas aeruginosa* derived from serum-sensitive parental strains. *Curr. Microbiol.* 10, 185–189.

Schiller, N. L., Hatch, R. A., and Joiner, K. A. (1989). Complement activation and C3 binding by serum-sensitive and serum-resistant strains of *Pseudomonas aeruginosa*. *Infect. Immun.* 57, 1707–1713.

Schmidt, K. D., Tümmler, B., and Römling, U. (1996). Comparative genome mapping of *Pseudomonas aeruginosa* PAO with *P. aeruginosa* C, which belongs to a major clone in cystic fibrosis patients and aquatic habitats. *J. Bacteriol.* 178, 85–93.

Sedlak-Weinstein, E., Cripps, A. W., Kyd, J. M., and Foxwell, A. R. (2005). *Pseudomonas aeruginosa*: the potential to immunise against infection. *Expert Opin. Biol. Ther.* 5, 967–982.

Senchenkova, S. N., Huang, X., Laux, P., Knirel, Y. A., Shashkov, A. S., and Rudolph, K. (2002). Structures of the *O*-polysaccharide chains of the lipopolysaccharides of *Xanthomonas campestris* pv phaseoli var fuscans GSPB 271 and *X. campestris* pv malvacearum GSPB 1386 and GSPB 2388. *Carbohydr. Res.* 337, 1723–1728.

Silby, M. W., Cerdeno-Tarraga, A. M., Vernikos, G. S., Giddens, S. R., Jackson, R. W., Preston, G. M., Zhang, X. X., Moon, C. D., Gehrig, S. M., Godfrey, S. A., Knight, C. G., Malone, J. G., Robinson, Z., Spiers, A. J., Harris, S., Challis, G. L., Yaxley, A. M., Harris, D., Seeger, K., Murphy, L., Rutter, S., Squares, R., Quail, M. A., Saunders, E., Mavromatis, K., Brettin, T. S., Bentley, S. D., Hothersall, J., Stephens, E., Thomas, C. M., Parkhill, J., Levy, S. B., Rainey, P. B., and Thomson, N. R. (2009). Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. *Genome Biol.* 10, R51.

Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P., Moskowitz, S.

M., Burns, J. L., Kaul, R., and Olson, M. V. (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8487–8492.

Speert, D. P., Campbell, M. L., Puterman, J., Govan, J., Doherty, C., Hoiby, N., Ojeniyi, B., Lam, J. S., Ogle, J. W., Johnson, Z., Paranchych, W., Sastry, P. A., Pitt, T. L., and Lawrence, L. (1994). A multicenter comparison of methods for typing strains of *Pseudomonas aeruginosa* predominantly from patients with cystic fibrosis. The International *Pseudomonas aeruginosa* Typing Study Group. *J. Infect. Dis.* 169, 134–142.

Spencer, D. H., Kas, A., Smith, E. E., Raymond, C. K., Sims, E. H., Hastings, M., Burns, J. L., Kaul, R., and Olson, M. V. (2003). Whole-genome sequence variation among multiple isolates of *Pseudomonas aeruginosa*. *J. Bacteriol.* 185, 1316–1325.

Stanislavsky, E. S., and Lam, J. S. (1997). *Pseudomonas aeruginosa* antigens as potential vaccines. *FEMS Microbiol. Rev.* 21, 243–277.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., and Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959–964.

Takada, H., and Kotani, S. (1992). "Structure-function relationships of lipid A," in *Bacterial Endotoxic Lipopolysaccharides*, Vol. I, *Molecular Biochemistry and Cellular Biology*, eds D. C. Morrison and J. L. Ryan (Boca Raton. FL: CRC press), 107–134.

Temple, G. S., Ayling, P. D., and Wilkinson, S. G. (1986). Isolation and characterization of a lipopolysaccharide-specific bacteriophage of *Pseudomonas aeruginosa*. *Microbios* 45, 81–91.

To, T. (2006). *Purification and Characterization of WapP of Pseudomonas aeruginosa, a Putative Lipopolysaccharide Kinase*. M.Sc. thesis, University of Guelph, Guelph.

Trent, M. S. (2004). Biosynthesis, transport, and modification of lipid A. *Biochem. Cell Biol.* 82, 71–86.

Walker, S. G., and Beveridge, T. J. (1988). Amikacin disrupts the cell envelope of *Pseudomonas aeruginosa* ATCC 9027. *Can. J. Microbiol.* 34, 12–18.

Walsh, A. G., Matewish, M. J., Burrows, L. L., Monteiro, M. A., Perry, M. B., and Lam, J. S. (2000). Lipopolysaccharide core phosphates are required for viability and intrinsic drug resistance in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 35, 718–727.

Wang, J., Lory, S., Ramphal, R., and Jin, S. (1996). Isolation and characterization of *Pseudomonas aeruginosa* genes inducible by respiratory mucus derived from cystic fibrosis patients. *Mol. Microbiol.* 22, 1005–1012.

Weisner, A. M., Chart, H., Bush, A., Davies, J. C., and Pitt, T. L. (2007). Detection of antibodies to *Pseudomonas aeruginosa* in serum and oral fluid from patients with cystic fibrosis. *J. Med. Microbiol.* 56, 670–674.

Wenzel, C. Q., Daniels, C., Keates, R. A., Brewer, D., and Lam, J. S. (2005). Evidence that WbpD is an *N*-acetyltransferase belonging to the hexapeptide acyltransferase superfamily and an important protein for O-antigen biosynthesis in *Pseudomonas aeruginosa* PAO1. *Mol. Microbiol.* 57, 1288–1303.

Westman, E. L., Mcnally, D. J., Charchoglyan, A., Brewer, D., Field, R. A., and Lam, J. S. (2009). Characterization of WbpB, WbpE, and WbpD and reconstitution of a pathway for the biosynthesis of UDP-2,3-diacetamido-2,3-dideoxy-D-mannuronic acid in *Pseudomonas aeruginosa*. *J. Biol. Chem.* 284, 11854–11862.

Westman, E. L., Preston, A., Field, R. A., and Lam, J. S. (2008). Biosynthesis of a rare Di-*N*-Acetylated Sugar in the lipopolysaccharides of both *Pseudomonas aeruginosa* and *Bordetella pertussis* occurs via an identical scheme despite different gene clusters. *J. Bacteriol.* 190, 6060–6069.

Whiteley, M., Lee, K. M., and Greenberg, E. P. (1999). Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13904–13909.

Whitfield, C. (1995). Biosynthesis of lipopolysaccharide O antigens. *Trends Microbiol.* 3, 178–185.

Winn, A. M., and Wilkinson, S. G. (1998). The O7 antigen of *Stenotrophomonas maltophilia* is a linear D-rhamnan with a trisaccharide repeating unit that is also present in polymers for some *Pseudomonas* and *Burkholderia* species. *FEMS Microbiol. Lett.* 166, 57–61.

Winstanley, C., Langille, M. G., Fothergill, J. L., Kukavica-Ibrulj, I., Paradis-Bleau, C., Sanschagrin, F., Thomson, N. R., Winsor, G. L., Quail, M. A., Lennard, N., Bignell, A., Clarke, L., Seeger, K., Saunders, D., Harris, D., Parkhill, J., Hancock, R. E., Brinkman, F. S., and Levesque, R. C. (2009). Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool epidemic strain of *Pseudomonas aeruginosa*. *Genome Res.* 19, 12–23.

Wolfgang, M. C., Kulasekara, B. R., Liang, X., Boyd, D., Wu, K., Yang, Q., Miyada, C. G., and Lory, S. (2003). Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8484–8489.

Wyckoff, T. J., Lin, S., Cotter, R. J., Dotson, G. D., and Raetz, C. R. (1998). Hydrocarbon rulers in UDP-*N*-acetylglucosamine acyltransferases. *J. Biol. Chem.* 273, 32369–32372.

Yang, H., Matewish, M., Loubens, I., Storey, D. G., Lam, J. S., and Jin, S. (2000). migA, a quorum-responsive gene of *Pseudomonas aeruginosa*, is highly expressed in the cystic fibrosis lung environment and modifies low-molecular-mass lipopolysaccharide. *Microbiology* 146, 2509–2519.

Yokota, S., Hayashi, T., and Matsumoto, H. (1994). Identification of the lipopolysaccharide core region as the receptor site for a cytotoxin-converting phage, phi CTX, of *Pseudomonas aeruginosa*. *J. Bacteriol.* 176, 5262–5269.

Yokota, S., Kaya, S., Sawada, S., Kawamura, T., Araki, Y., and Ito, E. (1987). Characterization of a polysaccharide component of lipopolysaccharide from *Pseudomonas aeruginosa* IID 1008 (ATCC 27584) as D-rhamnan. *Eur. J. Biochem.* 167, 203–209.

Zaidi, T. S., Lyczak, J., Preston, M., and Pier, G. B. (1999). Cystic fibrosis transmembrane conductance regulator-mediated corneal epithelial cell ingestion of *Pseudomonas aeruginosa* is a key component in the pathogenesis of experimental murine keratitis. *Infect. Immun.* 67, 1481–1492.

Zhao, X., and Lam, J. S. (2002). WaaP of *Pseudomonas aeruginosa* is a novel eukaryotic type protein-tyrosine kinase as well as a sugar kinase essential for the biosynthesis of core lipopolysaccharide. *J. Biol. Chem.* 277, 4722–4730.

Zhao, X., Wenzel, C. Q., and Lam, J. S. (2002). Nonradiolabeling assay for WaaP, an essential sugar kinase involved in biosynthesis of core lipopolysaccharide of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 46, 2035–2037.

Zuercher, A. W., Horn, M. P., Que, J. U., Ruedeberg, A., Schoeni, M. H., Schaad, U. B., Marcus, P., and Lang, A. B. (2006). Antibody responses induced by long-term vaccination with an octovalent conjugate *Pseudomonas aeruginosa* vaccine in children with cystic fibrosis. *FEMS Immunol. Med. Microbiol.* 47, 302–308.

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Pseudomonas aeruginosa: resistance to the max

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Pseudomonas aeruginosa is intrinsically resistant to a variety of antimicrobials and can develop resistance during anti-pseudomonal chemotherapy both of which compromise treatment of infections caused by this organism. Resistance to multiple classes of antimicrobials (multidrug resistance) in particular is increasingly common in *P. aeruginosa*, with a number of reports of pan-resistant isolates treatable with a single agent, colistin. Acquired resistance in this organism is multifactorial and attributable to chromosomal mutations and the acquisition of resistance genes via horizontal gene transfer. Mutational changes impacting resistance include upregulation of multidrug efflux systems to promote antimicrobial expulsion, derepression of *ampC*, AmpC alterations that expand the enzyme's substrate specificity (i.e., extended-spectrum AmpC), alterations to outer membrane permeability to limit antimicrobial entry and alterations to antimicrobial targets. Acquired mechanisms contributing to resistance in *P. aeruginosa* include β-lactamases, notably the extended-spectrum β-lactamases and the carbapenemases that hydrolyze most β-lactams, aminoglycoside-modifying enzymes, and 16S rRNA methylases that provide high-level pan-aminoglycoside resistance. The organism's propensity to grow *in vivo* as antimicrobial-tolerant biofilms and the occurrence of hypermutator strains that yield antimicrobial resistant mutants at higher frequency also compromise anti-pseudomonal chemotherapy. With limited therapeutic options and increasing resistance will the untreatable *P. aeruginosa* infection soon be upon us?

Keywords: *Pseudomonas aeruginosa*, antimicrobial, resistance, β-lactamase, efflux, biofilm, hypermutability

INTRODUCTION

Pseudomonas aeruginosa is a common nosocomial pathogen (Hidron et al., 2008; Jones et al., 2009; Zhanel et al., 2010) that causes infections with a high mortality rate (Mutlu and Wunderink, 2006; Kerr and Snelling, 2009; Mahar et al., 2010; Lambert et al., 2011). This latter is, in part, attributable to the organism's intrinsically high resistance to many antimicrobials (Poole, 2002) and the development of increased, particularly multidrug resistance in healthcare settings (Rossolini and Mantengoli, 2005; Ferrara, 2006; Giamarellos-Bourboulis et al., 2006; Paterson, 2006; Kerr and Snelling, 2009; Shorr, 2009; Hirsch and Tam, 2010; Kallen et al., 2010; Keen III, et al., 2010), both of which complicate anti-pseudomonal chemotherapy. Indeed, numerous studies point to a link between multidrug resistance and increased morbidity/mortality, as well as increased length of hospital stay and increased hospital costs (Slama, 2008; Kerr and Snelling, 2009; Mauldin et al., 2010; Tumbarello et al., 2011). While acquisition of resistance genes [e.g., those encoding β-lactamases (Gupta, 2008; Zhao and Hu, 2010) and aminoglycoside-modifying enzymes (Poole, 2005; Ramirez and Tolmasky, 2010)] via horizontal gene transfer can and do drive antimicrobial/multidrug resistance development in *P. aeruginosa* (Strateva and Yordanov, 2009), more commonly mutations of chromosomal genes (target site, efflux mutations) explain resistance in this organism (Lister et al., 2009; Strateva and Yordanov, 2009). This review provides an overview of antimicrobial resistance in *P. aeruginosa* that is acquired, either via mutation of endogenous genes or via acquisition of exogenous resistance genes.

RESISTANCE TO β-LACTAMS

β-Lactams, including penicillins (e.g., ticarcillin, piperacillin), cephalosporins (e.g., ceftazidime, cefepime), carbapenems (e.g., imipenem, meropenem), and monobactams (e.g., aztreonam) are commonly used in the treatment of *P. aeruginosa* infections (Paul et al., 2010). Resistance to these agents is increasing (Jones et al., 2009; Zilberberg et al., 2010) and mediated by a variety of mechanisms, most commonly antibiotic cleavage by β-lactamase enzymes, antibiotic expulsion by chromosomally encoded efflux mechanisms and reduced drug uptake owing to loss of outer membrane porin proteins (Poole, 2004b; Pfeifer et al., 2010).

β-LACTAMASES

β-Lactamases, hydrolytic enzymes that disrupt the amide bond of the classical four-membered β-lactam ring thus rendering the antimicrobial ineffective, are a major determinant of resistance in Gram-negative bacteria, including *P. aeruginosa*. Four molecular classes of these enzymes have been described (A–D) and include metal dependent (Zn^{2+}-requiring; class B) and metal-independent (active site serine; classes A, C, and D) β-lactamases (reviewed in Helfand and Bonomo, 2003), all of which have been reported in *P. aeruginosa* (Zhao and Hu, 2010).

Endogenous β-lactamases

Pseudomonas aeruginosa typically carries chromosomal genes for two β-lactamases, a class C cephalosporinase, AmpC (Lodge et al., 1990), and a class D oxacillinase, PoxB (Girlich et al., 2004; Kong et al., 2005). AmpC is a well-characterized β-lactamase

(Jacoby, 2009) commonly linked to β-lactam resistance in clinical isolates (Arora and Bal, 2005; Bratu et al., 2007; Reinhardt et al., 2007; Tam et al., 2007, 2010; Drissi et al., 2008; Vettoretti et al., 2009; Upadhyay et al., 2010; Xavier et al., 2010) while PoxB activity was only detected in lab mutants lacking AmpC and its clinical significance is uncertain. AmpC, which is a common chromosomally encoded enzyme in many Gram-negative bacteria (Poole, 2004b; Jacoby, 2009), is inducible by a number of β-lactam antibiotics (e.g., benzyl penicillin and narrow-spectrum cephalosporins) and thus contributes to intrinsic (i.e., natural, non-mutational) resistance to these (Livermore, 1991). It is not, however, inducible by monobactams (aztreonam; Sakurai et al., 1990), the anti-pseudomonal penicillin piperacillin (Livermore, 1995), and many of the newer cephalosporins (e.g., cefotaxime, ceftriaxone, ceftazidime; Livermore and Yang, 1987; Livermore, 1995; Poole, 2004b) that are, nonetheless, good substrates for the enzyme and as such resistance is dependent upon mutational derepression of *ampC*. Indeed, mutational derepression of *ampC* is the most common mechanism of resistance to β-lactams in *P. aeruginosa* (Arora and Bal, 2005; Tam et al., 2007; Drissi et al., 2008; Xavier et al., 2010), including expanded-spectrum cephalosporins (e.g., ceftazidime; Juan et al., 2005; Picao et al., 2009a; Queenan et al., 2010) and penicillins (e.g., ticarcillin; Cavallo et al., 2007; Dubois et al., 2008). Interestingly, while carbapenems (e.g., imipenem) are excellent inducers of *ampC*, their rapid bactericidal activity and stability to hydrolysis renders them effective against AmpC$^+$ *P. aeruginosa* (Jones, 1998) although derepressed AmpC appears to contribute to carbapenem resistance in conjunction with other mechanisms of resistance (e.g., loss of porin protein D; see below). Recently, the production of AmpC variants with improved activity against oxyiminocephalosporins (e.g., ceftazidime), cefepime, and carbapenems (including imipenem), first described in the Enterobacteriacae and referred to as extended-spectrum AmpC (ESAC; Nordmann and Mammeri, 2007), have been reported in clinical isolates of *P. aeruginosa* (Rodriguez-Martinez et al., 2009a,b). These, too, appear to contribute to carbapenem resistance in conjunction with loss of OprD (Rodriguez-Martinez et al., 2009b).

Acquired β-lactamases

While the original β-lactamases were plasmid-encoded restricted-spectrum class A enzymes that only hydrolyzed penicillins and older, narrow-spectrum cephalosporins, more recently described acquired β-lactamases in *P. aeruginosa* include the extended-spectrum β-lactamase (ESBL) enzymes (classes A and D) able to hydrolyze a wider range of β-lactams, including the broad-spectrum cephalosporins and monobactams, and the carbapenemases (classes A, B, and D) that hydrolyze most β-lactams, including the carbapenems, but not aztreonam (Zhao and Hu, 2010). ESBLs and carbapenemases are typically encoded by plasmid- or transposon-bone genes, often on integrons (Poirel and Nordmann, 2002; Castanheira et al., 2004; Walsh et al., 2005; Naas et al., 2006; Bogaerts et al., 2007; Gupta, 2008; Li et al., 2008; Castanheira et al., 2009; Zhao et al., 2009; Kotsakis et al., 2010; Poirel et al., 2010b), genetic elements capable of capturing, and subsequently mobilizing resistance genes (Cambray et al., 2010), although some β-lactamase genes are associated with novel mobile insertion sequences termed IS*CR* elements (Poirel et al., 2004; Picao et al., 2009a,b; Kotsakis et al., 2010).

Extended-spectrum β-lactamases. More commonly reported in the Enterobacteriaceae, though present also in *P. aeruginosa*, ESBLs typically hydrolyze and, so, provide resistance to broad-spectrum cephalosporins (e.g., the third generation oxyiminocephalosporins cefotaxime and ceftazidime) and aztreonam, in addition to penicillins and narrow-spectrum cephalosporins (reviewed in Paterson and Bonomo, 2005; Bush, 2008). Classical ESBLS have evolved from restricted-spectrum class A TEM and SHV β-lactamases although a variety of non-TEM, non-SHV class A ESBLS have been described (e.g., CTX-M, PER, VEB, GES, BEL; Poole, 2004b; Paterson and Bonomo, 2005) and class D ESBLs derived from narrow-spectrum OXA β-lactamases are also well-known (Paterson and Bonomo, 2005; Poirel et al., 2010b).

Class A ESBLs are typically identified in *P. aeruginosa* isolates showing resistance to ceftazidime (e.g., De Champs et al., 2002; Girlich et al., 2002; Strateva et al., 2007; Hocquet et al., 2010]. VEB-type ESBLs were the predominant ESBL reported in *P. aeruginosa* in a number of studies where ESBLs were commonly seen (Jiang et al., 2006; Strateva et al., 2007; Woodford et al., 2008; Shahcheraghi et al., 2009) although PER-type ESBLs were also well-represented (Celenza et al., 2006; Endimiani et al., 2006; Shahcheraghi et al., 2009; Glupczynski et al., 2010). While BEL-1 (Poirel et al., 2005; Bogaerts et al., 2007) and CTX-M (al Naiemi et al., 2006; Picao et al., 2009b) ESBLs are not frequently observed in *P. aeruginosa*, they were the predominant ESBLs reported in ESBL$^+$ *P. aeruginosa* in a Belgium study (Glupczynski et al., 2010) and a Bolivian study (Celenza et al., 2006), respectively. Recently, a second BEL ESBL, BEL-2 with enhanced activity against expanded-spectrum cephalosporins was recovered in Belgium (Poirel et al., 2010a). Similarly, a high prevalence of an SHV ESBL was reported in one study (Shahcheraghi et al., 2009) although this β-lactamase is seldom reported in *P. aeruginosa* (Mansour et al., 2009; Hocquet et al., 2010). TEM-(Dubois et al., 2005; Shahcheraghi et al., 2009) and GES-(Labuschagne et al., 2008; Picao et al., 2009a; Viedma et al., 2009; Kotsakis et al., 2010) type ESBLs have also been described in *P. aeruginosa*.

Class D OXA enzymes (so named because of their preference for oxacillin and cloxacillin over benzylpenicillin, though not all class D enzymes show this property), are mostly narrow-spectrum β-lactamases that confer resistance to amino- and carboxypenicillins and narrow-spectrum cephalosporins (Poirel et al., 2010b) although several OXA-type enzymes are ESBLs (reviewed in Poirel et al., 2010b). Occurring predominantly in *P. aeruginosa* these confer resistance to cefotaxime (Danel et al., 1999; Aubert et al., 2001; Fournier et al., 2010) or ceftazidime (Toleman et al., 2003; Juan et al., 2009; Fournier et al., 2010; Hocquet et al., 2010), with some OXA β-lactamases also linked to resistance and/or reduced susceptibility to cefepime (Aubert et al., 2001; Toleman et al., 2003; Juan et al., 2009; Fournier et al., 2010; Liu et al., 2010) and/or aztreonam (Toleman et al., 2003; Juan et al., 2009; Fournier et al., 2010).

Carbapenemases. Carbapenems (e.g., meropenem, imipenem) are an important class of anti-pseudomonal β-lactam owing to their stability to most β-lactamases (see El Gamal and Oh, 2010 for a recent review of carbapenems) and are of particular use in treating infections associated with ESBL- and AmpC-producers. β-lactamases capable of hydrolyzing carbapenems are known

(reviewed in Queenan and Bush, 2007; Walsh, 2010) and include class A and class D carbapenemases (the latter also referred to as carbapenem-hydrolyzing class D β-lactamases, CHDLs; Poirel et al., 2010b) and class B metallo-β-lactamases (MBLs; reviewed in Walsh et al., 2005), though there are no hitherto reports of CHDLs in *P. aeruginosa*.

Class A β-lactamases with activity against carbapenems are uncommon and can be divided into five groups (GES, IMI, KPC, NMC-A, and SME; reviewed in Walther-Rasmussen and Hoiby, 2007) of which only GES and KPC enzymes have been described to date in *P. aeruginosa* (Zhao and Hu, 2010). KPC enzymes show activity against most β-lactams including oxyiminocephalosporins, monobactams, and carbapenems and while they occur as yet rarely in *P. aeruginosa* (only KPC-2 and KPC-5 have been reported in this organism) the number of reports of KPC-producing *P. aeruginosa* is increasing (Villegas et al., 2007; Akpaka et al., 2009; Wolter et al., 2009a; Poirel et al., 2010c). Interestingly, KPC-2 is more active against carbapenems than is KPC-5 while the latter shows better activity against ceftazidime (Wolter et al., 2009b). Of note, too, the presence of KPC enzymes in carbapenem-resistant isolates is often coupled with loss of the OprD outer membrane porin (Villegas et al., 2007; Wolter et al., 2009a) that is the primary route of entry of these agents into *P. aeruginosa* (Trias and Nikaido, 1990). While all GES enzymes are ESBLs three of these also show reasonable activity against carbapenems (GES-2, -4, and -5), with GES-2 and -5 having been reported in *P. aeruginosa* (Walther-Rasmussen and Hoiby, 2007; Viedma et al., 2009; Wang et al., 2010).

Class B MBLs are by far the major determinants of β-lactamase-mediated resistance to carbapenems and the major cause of high-level resistance to these agents. Acquired MBLs include the VIM and IMP enzymes, of which there are numerous variants of the original VIM-1 and IMP-1 MBLs, as well as the SPM-1, GIM-1, NDM-1, AIM-1, and SIM-1 enzymes (Gupta, 2008; Walsh, 2010). The VIM and IMP enzymes are by far the most common MBLs found in carbapenem-resistant bacteria (Walsh et al., 2005), including carbapenem-resistant *P. aeruginosa* (Gupta, 2008). The predominance of VIM vs. IMP in *P. aeruginosa* appears to be geographical, with IMP-type MBLs predominating in Asia where it was first discovered and VIM-type enzymes predominating in Europe though both enzymes are now disseminated globally, with VIM-2 in particular well established on five continents (Gupta, 2008; Walsh, 2010; Zhao and Hu, 2010). There are single reports, only, of the GIM-1 (found in five isolates from Germany; Castanheira et al., 2004) and the AIM-1 (Gupta, 2008) MBLs in *P. aeruginosa*. SPM-1 is the predominant MBL in Brazil (Sader et al., 2005; Picao et al., 2009a) and while previously found only in Brazilian clinical isolates it has now been reported in Europe (Salabi et al., 2010).

EFFLUX

Five families of efflux systems that export and provide resistance to antimicrobials in bacteria have been described (Li and Nikaido, 2009) although members of the Resistance Nodulation Division (RND) family appear to be the most significant contributors to antimicrobial resistance in *P. aeruginosa* (Poole, 2004a, 2007). There are 12 RND-type efflux systems present in *P. aeruginosa* of which three, MexAB-OprM, MexCD-OprJ, and MexXY-OprM have been shown to accommodate and provide resistance to β-lactams (Poole, 2004b). MexAB-OprM accommodates the broadest range of β-lactams (amongst these pumps) and is most frequently linked to β-lactam resistance in clinical isolates (Drissi et al., 2008; Tomas et al., 2010). The MexXY-OprM efflux system has also been linked to β-lactam resistance in clinical isolates of *P. aeruginosa* (as one of several contributors; Maniati et al., 2007; Vettoretti et al., 2009). While MexAB-OprM, MexCD-OprJ, and MexXY-OprM have all been shown to accommodate carbapenems (except imipenem; Okamoto et al., 2002) MexAB-OprM is by far the better exporter of these agents and the pump has been shown to contribute to reduced susceptibility to meropenem in clinical isolates (Pai et al., 2001; Pournaras et al., 2005). Still, efflux appears to be a minor contributor to carbapenem resistance in this organism, typically operating in conjunction with other mechanisms (Quale et al., 2006; Dotsch et al., 2009; Hammami et al., 2009; Wang et al., 2010). MexAB-OprM has also been implicated in resistance to the penicillin ticarcillin (Boutoille et al., 2004; Cavallo et al., 2007; Hocquet et al., 2007) and its expression linked statistically to aztreonam resistance (Quale et al., 2006). MexXY production, too, has been noted in ticarcillin-resistant *P. aeruginosa* (Hocquet et al., 2007) although a contribution to resistance was not proven and this efflux system is more commonly associated with resistance to the fourth generation cephalosporin cefepime in clinical isolates (Hocquet et al., 2006; Pena et al., 2009). Indeed, cefepime commonly selects for MexXY-derepressed mutants *in vitro* (Queenan et al., 2010). MexXY-OprM was also responsible for reduced susceptibility to ceftobiprole in a clinical study of this the novel broad-spectrum cephalosporin (Baum et al., 2009) and mutants expressing *mexXY* are readily selected by this β-lactam *in vitro* (Queenan et al., 2010). Although MexCD-OprJ accommodates cefepime (Masuda et al., 2000) it has rarely been linked to resistance to this agent in clinical isolates (Jeannot et al., 2008).

PERMEABILITY

By far the most common mechanism of resistance to the carbapenems (including imipenem) in *P. aeruginosa* is loss or alteration of the outer membrane porin protein OprD (Rodriguez-Martinez et al., 2009b; Wang et al., 2010), the major portal for entry for carbapenems (Trias and Nikaido, 1990). While not providing the high-level resistance seen in MBL-producers, loss of OprD function is the major determinant of non-MBL-mediated resistance to these agents (Gutierrez et al., 2007; Rodriguez-Martinez et al., 2009b; Tomas et al., 2010; Wang et al., 2010), often seen operating in conjunction with other mechanisms [e.g., derepressed *ampC* (Gutierrez et al., 2007; Rodriguez-Martinez et al., 2009b; Tomas et al., 2010; Wang et al., 2010) or MexAB-OprM (Gutierrez et al., 2007; Tomas et al., 2010; Wang et al., 2010)]. Indeed, carbapenem resistance resulting from loss of OprD requires the presence of AmpC (inducible or stably derepressed; Livermore, 1992).

RESISTANCE TO FLUOROQUINOLONES

Fluoroquinolones (FQs), particularly ciprofloxacin, are commonly used in the treatment of *P. aeruginosa* infections. Resistance to these agents, particularly high-level resistance, is predominantly mediated by mutations in the DNA gyrase and topoismerase IV enzymes that are the targets of the FQs, though efflux is a significant

contributing actor (Jacoby, 2005; Drlica et al., 2009) often in combination with target site mutations (Higgins et al., 2003; Henrichfreise et al., 2007; Rejiba et al., 2008; Tam et al., 2010).

TARGET SITE MUTATIONS

The FQ class of antimicrobial acts on bacterial topoisomerases [topoisomerase II (a.k.a. gyrase) and topoisoemrase IV] that are responsible for the introduction and/or removal of supercoils in, as well as catenation/decatenation of DNA and, thus, play an essential role in DNA replication, transcription, recombination, and repair (Drlica and Zhao, 1997). In Gram-negative bacteria, gyrase is the preferred target of FQs, and resistance mutations thus tend to occur in this enzyme first with additional mutations in topoisomerase IV seen in some highly resistant isolates (Jacoby, 2005). DNA gyrase (GyrA and GyrB) and topoisomerase (ParC and ParE) are each comprised of two subunits, with FQ resistance mutations typically occurring in the so-called "quinolone resistance determining region" (QRDR) of GyrA and/or ParC (Jacoby, 2005; Drlica et al., 2009). Such mutations are common in FQ-resistant *P. aeruginosa* (Higgins et al., 2003; Lee et al., 2005; Muramatsu et al., 2005; Henrichfreise et al., 2007; Rejiba et al., 2008) with highly resistant isolates carrying multiple mutations in *gyrA* and/or *parC* (Nakano et al., 1997; Higgins et al., 2003; Lee et al., 2005; Muramatsu et al., 2005), with mutations in *gyrB* (Lee et al., 2005; Muramatsu et al., 2005; Schwartz et al., 2006) and *parE* (Lee et al., 2005; Rejiba et al., 2008) less common.

EFFLUX

Four members of the RND family of multidrug efflux systems, MexAB-OprM, MexCD-OprJ, MexEF-oprN, and MexXY-OprM are known to accommodate FQs (Poole, 2000) and these efflux systems have been implicated in FQ resistance in clinical isolates (Poole, 2000; Wolter et al., 2004; Zhanel et al., 2004; Reinhardt et al., 2007). Expression of *mexAB-oprM* is controlled directly or indirectly by three repressors, MexR (Srikumar et al., 2000), NalD (Morita et al., 2006) and NalC (Cao et al., 2004), and mutations in *mexR* (Henrichfreise et al., 2007), *nalC* (Henrichfreise et al., 2007) and *nalD* (Tomas et al., 2010) have been reported in FQ-resistant clinical isolates. *mexCD-oprJ* expression is controlled by a single known regulator, the NfxB repressor (Poole et al., 1996), and lab (Poole et al., 1996) and clinical (Jalal et al., 2000; Higgins et al., 2003; Henrichfreise et al., 2007) isolates expressing this efflux system and resistant to FQs invariably contain mutations in *nfxB* (Jalal et al., 2000; Higgins et al., 2003; Henrichfreise et al., 2007). Still, *mexCD-oprJ*-expressing mutants appear to be rare in a clinical setting (Jeannot et al., 2008; Kiser et al., 2010). Unlike the other FQ-exporting RND-type efflux systems, expression of *mexEF-oprN* is regulated by a transcriptional activator, MexT (Köhler et al., 1999; Ochs et al., 1999). Unusually, many wild type stains carry inactivating mutations in *mexT* (Maseda et al., 2000), with *mexEF-oprN* expression and resistance resulting from reversion of these mutations (Maseda et al., 2000). These so-called *nfxC* mutants (Köhler et al., 1997), which have been described in the clinic (Fukuda et al., 1995; Jalal et al., 2000), also show resistance to carbapenems such as imipenem, though not because MexEF-orpN accommodates these agents but because of a coordinate, MexT-dependent reduction of OprD in such mutants (Köhler et al., 1999; Ochs et al., 1999). Hyperexpression of this efflux system (and reduction in OprD production) is also seen in lab isolates disrupted in the *mexS* gene encoding a putative oxidoreductase (a.k.a qrh; Köhler et al., 1999) of unknown function (Sobel et al., 2005). Expression of *mexXY* is controlled by a single known regulator, the MexZ repressor (Matsuo et al., 2004), and *mexZ* mutations have been reported in lab-selected FQ-resistant isolates hyperexpressing *mexXY* (Hocquet et al., 2008). *mexXY*-hyperexpressing FQ-resistant isolates lacking mutations in *mexZ* have also been described although the mutation(s) responsible were not identified (Hocquet et al., 2008). Despite its ability to accommodate FQs, however, MexXY-OprM has seldom been linked to FQ resistance in clinical isolates (Wolter et al., 2004).

RESISTANCE TO AMINOGLYCOSIDES

A number of aminoglycosides are commonly used in the treatment of *P. aeruginosa* infections (e.g., tobramycin, gentamicin, amikacin; Gilbert et al., 2003; Bartlett, 2004), particularly pulmonary infections in patients with cystic fibrosis (CF) where amikacin and, in particular, tobramycin are routinely employed (Canton et al., 2005; Taccetti et al., 2008). Their use is, however, linked to resistance development, with acquired aminoglycoside-modifying enzymes (AMEs) and rRNA methylases, and endogenous efflux mechanisms typically responsible (Poole, 2005).

AMINOGLYCOSIDE-MODIFYING ENZYMES

Aminoglycoside modification leading to antibiotic inactivation typically involves their phosphorylation (by aminoglycoside phosphoryltransferases, APHs), acetylation (by aminoglycoside acetyltransferases, AACs), or adenylation (by aminoglycoside nucleotidyltransferases, ANTs; aka. aminoglycoside adenylyltransferase, AAD; see Ramirez and Tolmasky, 2010 for a recent review of these modifying enzymes). AMEs are common determinants of aminoglycoside resistance in *P. aeruginosa* (reviewed in detail in Poole, 2005) except in CF isolates where these mechanisms are almost unknown (Shawar et al., 1999; Henrichfreise et al., 2007; Islam et al., 2009). Genes for AMEs are typically found on integrons with other resistance genes (Poole, 2005; Ramirez and Tolmasky, 2010) and, as such, AME-haboring isolates are often multidrug-resistant.

Aminoglycoside acetyltransferases

Acetylation of aminoglycosides can occur at 1-, 3-, 6′-, and 2′-amino groups and involve virtually all medically useful compounds (e.g., gentamicin, tobramycin, and amikacin; Ramirez and Tolmasky, 2010). Enzymes that modify the 3 [3-*N*-aminoglycoside acetyltransferases, AAC(3)] (Biddlecome et al., 1976) and 6′ [6′-*N*-aminoglycoside acetyltransferases, AAC(6′)] (Haas et al., 1976) positions are the most common acetyltransferases (Ramirez and Tolmasky, 2010) and, with ANT(2″) (see below) the most common enzymes providing for aminoglycoside resistance in this organism (Poole, 2005; Shahid and Malik, 2005; Dubois et al., 2008). The AAC(3) family, of which five subfamilies have been described in *P. aeruginosa* (I, II, II, IV, and VI; Kim et al., 2008; Zhao et al., 2009; Ramirez and Tolmasky, 2010), is a common determinant of gentamicin resistance in this organism, less commonly contributing to tobramycin resistance (subfamilies II, III, and VI; Poole, 2005). The AAC(6′) family, of which two major subfamilies have

been described in *P. aeruginosa* (I and II; and many variants of the I subfamily; Ramirez and Tolmasky, 2010), is the major AAC family contributing to aminoglycoside resistance in *P. aeruginosa*, with subfamily II predominating (Poole, 2005). AAC(6′) enzymes are major determinants of resistance to tobramycin and amikacin (subfamily I) and tobramycin and gentamicin (subfamily II; Poole, 2005), although some subfamily I variants lack activity against amikacin (e.g., Ib, Ib′; Galimand et al., 1993; MacLeod et al., 2000). Owing to irregularities in AAC(6′) nomenclature, several of these enzymes that have been reported in *P. aeruginosa* lack a roman numeral subclass designation (e.g., AAC(6′)-29a, -29b, -30, -32, -33; Ramirez and Tolmasky, 2010) and of these AAC(6′)-29a and -29b provide resistance to amikacin and tobramycin (Poirel et al., 2001) while AAC(6′)-30 exists as part of a bifunctional AAC(6′)-30/AAC(6′)-Ib′ enzyme that promotes resistance to tobramycin and only reduced susceptibility to amikacin and gentamicin (Mendes et al., 2004). A novel aminoglycoside acetyltransferase that exhibits FQ-acetylating activity, AAC(6′)-Ib-cr, has also been described in *P. aeruginosa* (Libisch et al., 2008).

Aminoglycoside nucleotidyltransferases

The most prevalent nucleotidyltransferase in *P. aeruginosa* is the ANT(2′)-I enzyme which inactivates gentamicin and tobramycin but not amikacin and is, thus, found in gentamicin- and tobramycin-resistant clinical isolates (Poole, 2005). A less common nucleotidyltransferases associated with aminoglycoside resistance in *P. aeruginosa* is ANT(4′)-II which provides resistance to tobramycin and amikacin (Poole, 2005; Ramirez and Tolmasky, 2010). Two variants of this enzyme, ANT(4′)-IIa (Shaw et al., 1993) and -IIb (Sabtcheva et al., 2003) have been described in amikacin-resistant clinical isolates and there is a report of an *ant(4′)-I* gene in *P. aeruginosa* although its contribution to resistance was not established (Jin et al., 2009). While there are a number of reports of the ANT(3′) nucleotidyltransferase in *P. aeruginosa* (Ramirez and Tolmasky, 2010) this enzyme is active against streptomycin and none of the clinically used anti-pseudomonal aminoglycosides.

Aminoglycoside phosphoryltransferases

Aminoglycoside phosphoryltransferases found in *P. aeruginosa* are almost invariably 3′ enzymes that act on the 3-OH of target aminoglycosides and generally provide resistance to aminoglycosides not typically used to treat *P. aeruginosa* infections (kanamycin, neomycin, and streptomycin; Poole, 2005). APH(3′)-II predominates in clinical isolates resistant to kanamycin (and neomycin; Miller et al., 1994; Poole, 2005) and, indeed, a chromosomal *aphA*-encoded APH(3′)-II type enzyme, APH(3′)-IIb (Hachler et al., 1996) is likely responsible for the general insensitivity of *P. aeruginosa* to kanamycin. APH enzymes that provide resistance to other aminoglycosides have also been described in *P. aeruginosa* and include APH(3′)-VI (amikacin; Kettner et al., 1995; Kim et al., 2008; Jin et al., 2009), APH(3′)-IIb-like (amikacin, weakly; Riccio et al., 2001), and APH(2″) (gentamicin and tobramycin; Kettner et al., 1995).

EFFLUX

Aminoglycoside resistance independent of inactivating enzymes has been known for some time in *P. aeruginosa* (Bryan et al., 1976). Characterized by resistance to all aminoglycosides and often associated with reduced aminoglycoside accumulation (Bryan et al., 1976) such resistance, particularly common in CF isolates (reviewed in Poole, 2005), was attributed to reduced uptake owing to reduced permeability and, as such, was typically referred to as impermeability resistance. It is now known, however, that this resistance was likely due to efflux mediated by the MexXY-OprM multidrug efflux system. Indeed, this efflux system has been implicated in aminoglycoside resistance in clinical isolates, particularly CF isolates, in a number of studies (Sobel et al., 2003; Hocquet et al., 2006; Henrichfreise et al., 2007; Islam et al., 2009).

The MexXY-OprM system is encoded by the *mexXY* operon that is under the control of the MexZ repressor (Matsuo et al., 2004) and the *oprM* gene of the *mexAB-oprM* multidrug efflux operon. Mutations in *mexZ* are common in pan-aminoglycoside-resistant CF isolates of *P. aeruginosa* expressing *mexXY* (Poole, 2005; Hocquet et al., 2006; Henrichfreise et al., 2007; Islam et al., 2009; Feliziani et al., 2010) with *mexZ*, in fact, identified as the most commonly mutated gene in CF isolates (Smith et al., 2006; Feliziani et al., 2010). A number of studies highlight, however, the absence of mutations in *mexZ* or the *mexXY* promoter region in *mexXY*-expressing aminoglycoside-resistant CF isolates (Sobel et al., 2003; Hocquet et al., 2006; Islam et al., 2009), indicating that additional genes/mutations are linked to expression of this efflux locus in *P. aeruginosa*. A recent report of an *in vitro*-selected *mexXY*-expressing aminoglycoside-resistant mutant lacking a *mexZ* mutation identified a novel gene, *parR*, as the site of mutation (Muller et al., 2010). *parR* forms part of a two-gene operon, *parRS*, encoding a two-component regulatory systems that impacts expression of several antimicrobial resistance determinants in *P. aeruginosa* (e.g., *oprD*), including *mexXY*. Significantly, mutations in *parR* are present in some clinical isolates that express *mexXY* but lack mutations in *mexZ* (Muller et al., 2010).

16S rRNA METHYLASES

A more recently discovered aminoglycoside resistance mechanism involves methylation of the 16S rRNA of the A site of the 30S ribosomal subunit, which interferes with aminoglycoside binding and so promotes high-level resistance to clinically relevant aminoglycosides like gentamicin, tobramycin, and amikacin (reviewed in Doi and Arakawa, 2007). A number of different pan-aminoglycoside resistance-promoting 16S rRNA methylases have been described in *P. aeruginosa*, including RmtA (Yamane et al., 2004; Jin et al., 2009), RmtB (Zhou et al., 2010), RmtD (Doi et al., 2007; Lincopan et al., 2010), and ArmA (Gurung et al., 2010; Zhou et al., 2010). RmtD is frequently co-produced with the SPM-1 MLB that predominates in Brazil (Doi et al., 2007; Lincopan et al., 2010) and co-carriage of ArmA and the IMP-1 MBL has also been reported in *P. aeruginosa* isolates from Korea (Gurung et al., 2010).

RESISTANCE TO POLYCATIONIC ANTIMICROBIALS

Owing to the increased prevalence of multidrug-resistant *P. aeruginosa*, "older" antimicrobials like the polymyxins (polymyxin B and colistin) are back in favor, with earlier issues surrounding nephrotoxicity largely dealt with (Zavascki et al., 2007; Molina et al., 2009). While these agents, and colistin in particular, are quite efficacious in the treatment of multidrug-resistant *P. aeruginosa* infections (Montero et al., 2009; Falagas et al., 2010) there are

reports of resistance to both polymyxin B (Landman et al., 2005; Abraham and Kwon, 2009; Barrow and Kwon, 2009) and colistin (Johansen et al., 2008; Matthaiou et al., 2008; Samonis et al., 2010) in clinical isolates. While in many cases the mechanism(s) of clinical polymyxin resistance are unknown, substitution of LPS lipid A with aminoarabinose has been shown to contribute to polymyxin resistance in *P. aeruginosa in vitro* (Moskowitz et al., 2004) and in CF isolates (Ernst et al., 1999). This modification is carried out by the products of the *arnBCADTEF-ugd* locus (a.k.a. *pmrHFIJKLM-ugd* and PA3552–59) that is regulated both by PhoPQ (Macfarlane et al., 2000) and a second two-component regulatory system, PmrAB (McPhee et al., 2003; Moskowitz et al., 2004), with mutations in *phoQ* and *pmrB* shown to promote ArnBCADTEF-dependent polymyxin B resistance in clinical isolates (Abraham and Kwon, 2009; Barrow and Kwon, 2009). A third two-component system, ParRS, also controls *arnBCADTEF-ugd* expression (Fernandez et al., 2010), with a mutation in *parR* linked to ArnBCADTEF-mediated polymyxin resistance in a lab isolate (Muller et al., 2010). *parR* (and *parS*) mutations have been noted in clinical isolates, although there was no indication that the *arn* locus was upregulated, and the polymyxin resistance of these isolates was minimal (Muller et al., 2010).

BIOFILM RESISTANCE

Biofilms, surface-attached three-dimensional structures in which bacteria are imbedded in a matrix comprised of polysaccharide, protein, and DNA, are increasingly recognized as the preferred mode of bacterial growth in nature and infectious disease (Lopez et al., 2010). This is true of *P. aeruginosa* (Harmsen et al., 2010), particularly in the case of pulmonary infections in patients with CF (Wagner and Iglewski, 2008; Davies and Bilton, 2009). An important consequence of *P. aeruginosa* biofilm growth and one that is particularly relevant in a clinical context is marked resistance to antimicrobial agents (Davies and Bilton, 2009; Hoiby et al., 2010). Antimicrobial resistance of *P. aeruginosa* biofilms appears to be complex, multifactorial, and in many instances not well understood (Drenkard, 2003; Hoiby et al., 2010). Some studies indicate that *P. aeruginosa* within biofilms are metabolically less active and grow more slowly than cells at the biofilm periphery (owing to limited access to nutrients and oxygen; Werner et al., 2004), which may contribute to increasing biofilm tolerance to antimicrobials since antimicrobials often target metabolically active cells (Pamp et al., 2008). Certainly, the suggestion that biofilm-grown *P. aeruginosa* from CF patients are anaerobic (Hassett et al., 2009) is likely to be significant in the context of antimicrobial resistance since many agents are inactive or less active under anaerobiosis (Schobert and Tielen, 2010). Oxygen limitation has, in fact, been shown to contribute significantly to the antimicrobial resistance of *in vitro*-grown *P. aeruginosa* biofilms (Borriello et al., 2004).

One explanation for biofilms being generally refractory to antimicrobial chemotherapy is the presence, in biofilms, of a highly resistant sub-population of cells called persisters (Lewis, 2008). Intriguingly, "late" isolates of *P. aeruginosa* in CF (those recovered later in infection) produce increased levels of drug-tolerant persister cells, which may be the primary "mechanism" for surviving chemotherapy and, so, may explain the general recalcitrance of *P. aeruginosa* infections in CF (Mulcahy et al., 2010). The idea of a sub-population of biofilm cells displaying different patterns of antimicrobial susceptibility is supported by a recent study showing that only the mobile cells responsible for forming the "cap" component of the typical *P. aeruginosa* biofilm mushroom structures exhibited tolerance to colistin, as a result of colistin triggering PmrAB-dependent expression of the *arn* LPS modification locus (Haagensen et al., 2007; Pamp et al., 2008). While the details of persister formation and the mechanism(s) responsible for persister resistance remain unknown, a preliminary screen of a transposon insertion mutant library for mutants showed altered persister formation identified several genes whose disruption either increased or decreased persister formation (De Groote et al., 2009).

Aminoglycosides have been shown to induce biofilm formation by *P. aeruginosa*, in a process that requires a gene, *arr* (aminoglycoside response regulator; Hoffman et al., 2005). *arr* encodes a phosphodiesterase that impacts the levels of bis-(3′,5′)-cyclic-di-guanidine monophosphate (c-di-GMP; Hoffman et al., 2005), a second messenger known to influence biofilm formation (Harmsen et al., 2010) and lack of *arr* compromises biofilm resistance to aminoglycosides (Hoffman et al., 2005). Given that c-di-GMP production is generally correlated with biofilm formation (Harmsen et al., 2010) it is unclear how Arr-promoted turnover of this second messenger would promote biofilm formation. A second gene linked to biofilm-specific resistance to aminoglycosides in some strains only, *ndvB*, is involved in the synthesis of periplasmic (and intracellular) glucans that bind aminoglycosides (tobramycin), suggestive of a mechanism of resistance whereby aminoglycosides are sequestered and prevented from reaching their targets in the cytosol (Mah et al., 2003). These glucans, which have recently been purified and identified as highly glycerol-phosphorylated β-(1 → 3) glucans, actually form part of the biofilm matrix where they do, indeed, bind aminoglycosides (Sadovskaya et al., 2010). A tripartite ABC-family efflux system that is preferentially expressed in biofilm vs. planktonic cells, PA1875-PA1876-PA1877, has also been linked to biofilm-specific aminoglycoside résistance (Zhang and Mah, 2008). Efflux (mediated by MexCD-OprJ) has also been linked to biofilm-specific resistance to azithromycin in *P. aeruginosa* (Gillis et al., 2005; Mulet et al., 2009).

HYPERMUTATION AND RESISTANCE

Hypermutable (or mutator) *P. aeruginosa* exhibiting increased mutation rates are common in chronic infections such as those that occur in the lungs of CF patients (see Oliver, 2010; Oliver and Mena, 2010; for reviews of hypermutation in CF isolates). The hypermutation phenotype of mutator stains results from defects in DNA repair, predominantly in the mismatch repair (MMR) system (Oliver, 2010), with mutations in *mutS* (Oliver et al., 2002; Macia et al., 2005; Feliziani et al., 2010), *mutL* (Oliver et al., 2002; Feliziani et al., 2010), and *uvrD* (a.k.a *mutU*; Oliver et al., 2002) typically responsible. Significantly from an antimicrobial resistance standpoint, mutator strains show higher rates of antimicrobial resistance development than non-mutator strains (Oliver et al., 2000; Ferroni et al., 2009), with the mutator phenotype of CF isolates often correlating with antimicrobial, including multidrug, resistance (Macia et al., 2005; Waine et al., 2008; Ferroni et al., 2009; Feliziani et al., 2010; Tam et al., 2010; reviewed in Oliver, 2010).

A second DNA repair system less commonly linked to the mutator phenotype in *P. aeruginosa* is the DNA oxidative repair (GO) system charged with repairing and preventing incorporation into DNA of an oxidatively damaged form of guanosine (8-oxo-2′-deoxyguanosine, 8-oxodG; Oliver and Mena, 2010). *In vitro* studies have shown that knockouts in the GO genes *mutT* and *mutY* yield increased mutation rates concomitant with increased oxidative damage of DNA (Mandsberg et al., 2009), with *mutT* (Mandsberg et al., 2009; Morero and Argarana, 2009) and *mutY* (Mandsberg et al., 2009) strains also showing higher rates of antimicrobial resistance. Given that the characteristically chronically inflamed CF lung is an environment rich in reactive oxygen species (ROS) that can damage DNA, the potential for ROS-promoted hypermutability owing to defects in the GO system is certainly real. Although uncommon, mutator strains with lesions in *mutT* and *mutY* have been recovered from CF patients (Mandsberg et al., 2009).

CONCLUDING REMARKS

Rates of infection and resistance are increasing in *P. aeruginosa* (Talbot et al., 2006; Kerr and Snelling, 2009), and with reports of colistin-only sensitive *P. aeruginosa* and the presence of colistin-resistance in this organism the untreatable *P. aeruginosa* infection may be imminent. Compounding the increasing lack of effective anti-pseudomonal agents is the paucity of new drugs being developed that are active against *P. aeruginosa* and, indeed, the absence of any late-stage agents effective against pan-resistant *P. aeruginosa* (Talbot et al., 2006; Boucher et al., 2009; Page and Heim, 2009). The few novel agents with anti-pseudomonad activity (e.g., the siderophore–monobactam hybrid, BAL30072, the anti-pseudomonal cephalosporin CXA-101, and the MBL inhibitor ME1071) are, unfortunately, negatively impacted by known resistance mechanisms (Page and Heim, 2009). While the lack of classical antimicrobial options has prompted research into novel anti-pseudomonal strategies/agents, including a humaneered anti-*P. aeruginosa* Fab antibody fragment, KB001, cationic antimicrobial peptides, efflux pump inhibitors, modulators of virulence (Page and Heim, 2009; Veesenmeyer et al., 2009), and phage therapy (Wright et al., 2009), only KB001 is in later stage clinical trials (Page and Heim, 2009). Clearly, more therapeutic options are needed. Given the resistance armamentarium available to *P. aeruginosa* and the observation that drug use begets resistance, more also needs to be done in the areas of antimicrobial stewardship, resistance surveillance, and infection control (Kerr and Snelling, 2009). With limited (and shrinking) options, and an environment where anti-infectives, generally, are not being developed and fewer and fewer resources are being devoted to this therapeutic area by the major pharmaceutical companies (Boucher et al., 2009) prudent management of available agents and more robust resistance monitoring and infection control practices are essential. While these will likely not prevent the rise of untreatable pan-resistant *P. aeruginosa*, hopefully their numbers and impact can be limited.

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REFERENCES

Abraham, N., and Kwon, D. H. (2009). A single amino acid substitution in PmrB is associated with polymyxin B resistance in clinical isolate of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* 298, 249–254.

Akpaka, P. E., Swanston, W. H., Ihemere, H. N., Correa, A., Torres, J. A., Tafur, J. D., Montealegre, M. C., Quinn, J. P., and Villegas, M. V. (2009). Emergence of KPC-producing *Pseudomonas aeruginosa* in Trinidad and Tobago. *J. Clin. Microbiol.* 47, 2670–2671.

al Naiemi, N., Duim, B., and Bart, A. (2006). A CTX-M extended-spectrum β-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. *J. Med. Microbiol.* 55, 1607–1608.

Arora, S., and Bal, M. (2005). AmpC β-lactamase producing bacterial isolates from Kolkata hospital. *Indian J. Med. Res.* 122, 224–233.

Aubert, D., Poirel, L., Chevalier, J., Leotard, S., Pages, J. M., and Nordmann, P. (2001). Oxacillinase-mediated resistance to cefepime and susceptibility to ceftazidime in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 45, 1615–1620.

Barrow, K., and Kwon, D. H. (2009). Alterations in two-component regulatory systems of *phoPQ* and *pmrAB* are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 5150–5154.

Bartlett, J. G. (2004). *2004 Pocket Book of Infectious Disease Therapy*. Baltimore: Lippincott Williams & Wilkins.

Baum, E. Z., Crespo-Carbone, S. M., Morrow, B. J., Davies, T. A., Foleno, B. D., He, W., Queenan, A. M., and Bush, K. (2009). Effect of MexXY overexpression on ceftobiprole susceptibility in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 2785–2790.

Biddlecome, S., Haas, M., Davies, J., Miller, G. H., Rane, D. F., and Daniels, P. J. (1976). Enzymatic modification of aminoglycoside antibiotics: a new 3-N-acetylating enzyme from a *Pseudomonas aeruginosa* isolate. *Antimicrob. Agents Chemother.* 9, 951–955.

Bogaerts, P., Bauraing, C., Deplano, A., and Glupczynski, Y. (2007). Emergence and dissemination of BEL-1-producing *Pseudomonas aeruginosa* isolates in Belgium. *Antimicrob. Agents Chemother.* 51, 1584–1585.

Borriello, G., Werner, E., Roe, F., Kim, A. M., Ehrlich, G. D., and Stewart, P. S. (2004). Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob. Agents Chemother.* 48, 2659–2664.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B., and Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* 48, 1–12.

Boutoille, D., Corvec, S., Caroff, N., Giraudeau, C., Espaze, E., Caillon, J., Plesiat, P., and Reynaud, A. (2004). Detection of an IS21 insertion sequence in the *mexR* gene of *Pseudomonas aeruginosa* increasing β-lactam resistance. *FEMS Microbiol. Lett.* 230, 143–146.

Bratu, S., Landman, D., Gupta, J., and Quale, J. (2007). Role of AmpD, OprF and penicillin-binding proteins in β-lactam resistance in clinical isolates of *Pseudomonas aeruginosa*. *J. Med. Microbiol.* 56, 809–814.

Bryan, L. E., Haraphongse, R., and Van den Elzen, H. M. (1976). Gentamicin resistance in clinical-isolates of *Pseudomonas aeruginosa* associated with diminished gentamicin accumulation and no detectable enzymatic modification. *J. Antibiot.* 29, 743–753.

Bush, K. (2008). Extended-spectrum β-lactamases in North America, 1987–2006. *Clin. Microbiol. Infect.* 14(Suppl. 1), 134–143.

Cambray, G., Guerout, A. M., and Mazel, D. (2010). Integrons. *Annu. Rev. Genet.* 44, 141–166.

Canton, R., Cobos, N., de Gracia, J., Baquero, F., Honorato, J., Gartner, S., Alvarez, A., Salcedo, A., Oliver, A., and Garcia-Quetglas, E. (2005). Antimicrobial therapy for pulmonary pathogenic colonisation and infection by *Pseudomonas aeruginosa* in cystic fibrosis patients. *Clin. Microbiol. Infect.* 11, 690–703.

Cao, L., Srikumar, R., and Poole, K. (2004). MexAB-OprM hyperexpression in NalC type multidrug resistant *Pseudomonas aeruginosa*: identification and characterization of the *nalC* gene encoding a repressor of PA3720-PA3719. *Mol. Microbiol.* 53, 1423–1436.

Castanheira, M., Bell, J. M., Turnidge, J. D., Mathai, D., and Jones, R. N. (2009). Carbapenem resistance among *Pseudomonas aeruginosa* strains from India: evidence for nationwide endemicity of multiple metallo-β-lactamase clones (VIM-2, -5, -6, and -11 and the newly characterized VIM-18). *Antimicrob. Agents Chemother.* 53, 1225–1227.

Castanheira, M., Toleman, M. A., Jones, R. N., Schmidt, F. J., and Walsh, T. R.

(2004). Molecular characterization of a β-lactamase gene, blaGIM-1, encoding a new subclass of metallo-β-lactamase. *Antimicrob. Agents Chemother.* 48, 4654–4661.

Cavallo, J. D., Hocquet, D., Plesiat, P., Fabre, R., and Roussel-Delvallez, M. (2007). Susceptibility of *Pseudomonas aeruginosa* to antimicrobials: a 2004 French multicentre hospital study. *J. Antimicrob. Chemother.* 59, 1021–1024.

Celenza, G., Pellegrini, C., Caccamo, M., Segatore, B., Amicosante, G., and Perilli, M. (2006). Spread of *bla*(CTX-M-type) and *bla*(PER-2) β-lactamase genes in clinical isolates from Bolivian hospitals. *J. Antimicrob. Chemother.* 57, 975–978.

Danel, F., Hall, L. M., Duke, B., Gur, D., and Livermore, D. M. (1999). OXA-17, a further extended-spectrum variant of OXA-10 β-lactamase, isolated from *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 43, 1362–1366.

Davies, J. C., and Bilton, D. (2009). Bugs, biofilms, and resistance in cystic fibrosis. *Respir. Care* 54, 628–640.

De Champs, C., Poirel, L., Bonnet, R., Sirot, D., Chanal, C., Sirot, J., and Nordmann, P. (2002). Prospective survey of β-lactamases produced by ceftazidime- resistant *Pseudomonas aeruginosa* isolated in a French hospital in 2000. *Antimicrob. Agents Chemother.* 46, 3031–3034.

De Groote, V. N., Verstraeten, N., Fauvart, M., Kint, C. I., Verbeeck, A. M., Beullens, S., Cornelis, P., and Michiels, J. (2009). Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. *FEMS Microbiol. Lett.* 297, 73–79.

Doi, Y., and Arakawa, Y. (2007). 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. *Clin. Infect. Dis.* 45, 88–94.

Doi, Y., Ghilardi, A. C., Adams, J., de Oliveira, G. D., and Paterson, D. L. (2007). High prevalence of metallo-β-lactamase and 16S rRNA methylase coproduction among imipenem-resistant *Pseudomonas aeruginosa* isolates in Brazil. *Antimicrob. Agents Chemother.* 51, 3388–3390.

Dotsch, A., Becker, T., Pommerenke, C., Magnowska, Z., Jansch, L., and Haussler, S. (2009). Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 2522–2531.

Drenkard, E. (2003). Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes Infect.* 5, 1213–1219.

Drissi, M., Ahmed, Z. B., Dehecq, B., Bakour, R., Plesiat, P., and Hocquet, D. (2008). Antibiotic susceptibility and mechanisms of β-lactam resistance among clinical strains of *Pseudomonas aeruginosa*: first report in Algeria. *Med. Mal. Infect.* 38, 187–191.

Drlica, K., Hiasa, H., Kerns, R., Malik, M., Mustaev, A., and Zhao, X. (2009). Quinolones: action and resistance updated. *Curr. Top. Med. Chem.* 9, 981–998.

Drlica, K., and Zhao, X. (1997). DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev.* 61, 377–392.

Dubois, V., Arpin, C., Dupart, V., Scavelli, A., Coulange, L., Andre, C., Fischer, I., Grobost, F., Brochet, J. P., Lagrange, I., Dutilh, B., Jullin, J., Noury, P., Larribet, G., and Quentin, C. (2008). β-lactam and aminoglycoside resistance rates and mechanisms among *Pseudomonas aeruginosa* in French general practice (community and private healthcare centres). *J. Antimicrob. Chemother.* 62, 316–323.

Dubois, V., Arpin, C., Noury, P., Andre, C., Coulange, L., and Quentin, C. (2005). Prolonged outbreak of infection due to TEM-21-producing strains of *Pseudomonas aeruginosa* and enterobacteria in a nursing home. *J Clin. Microbiol.* 43, 4129–4138.

El Gamal, M. I., and Oh, C. H. (2010). Current Status of carbapenem antibiotics. *Curr. Top. Med. Chem.* 10, 1882–1897.

Endimiani, A., Luzzaro, F., Pini, B., Amicosante, G., Rossolini, G. M., and Toniolo, A. Q. (2006). *Pseudomonas aeruginosa* bloodstream infections: risk factors and treatment outcome related to expression of the PER-1 extended-spectrum β-lactamase. *BMC Infect. Dis.* 6, 52. doi: 10.1186/1471-2334-6-52

Ernst, R. K., Yi, E. C., Guo, L., Lim, K. B., Burns, J. L., Hackett, M., and Miller, S. I. (1999). Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* 286, 1561–1565.

Falagas, M. E., Rafailidis, P. I., Ioannidou, E., Alexiou, V. G., Matthaiou, D. K., Karageorgopoulos, D. E., Kapaskelis, A., Nikita, D., and Michalopoulos, A. (2010). Colistin therapy for microbiologically documented multidrug-resistant Gram-negative bacterial infections: a retrospective cohort study of 258 patients. *Int. J. Antimicrob. Agents* 35, 194–199.

Feliziani, S., Lujan, A. M., Moyano, A. J., Sola, C., Bocco, J. L., Montanaro, P., Canigia, L. F., Argarana, C. E., and Smania, A. M. (2010). Mucoidy, quorum sensing, mismatch repair and antibiotic resistance in *Pseudomonas aeruginosa* from cystic fibrosis chronic airways infections. *PLoS ONE* 5, e12669. doi: 10.1371/journal.pone.0012669

Fernandez, L., Gooderham, W. J., Bains, M., McPhee, J. B., Wiegand, I., and Hancock, R. E. (2010). Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob. Agents Chemother.* 54, 3372–3382.

Ferrara, A. M. (2006). Potentially multidrug-resistant non-fermentative Gram-negative pathogens causing nosocomial pneumonia. *Int. J. Antimicrob. Agents* 27, 183–195.

Ferroni, A., Guillemot, D., Moumile, K., Bernede, C., Le Bourgeois, M., Waernessyckle, S., Descamps, P., Sermet-Gaudelus, I., Lenoir, G., Berche, P., and Taddei, F. (2009). Effect of mutator *P. aeruginosa* on antibiotic resistance acquisition and respiratory function in cystic fibrosis. *Pediatr. Pulmonol.* 44, 820–825.

Fournier, D., Hocquet, D., Dehecq, B., Cholley, P., and Plesiat, P. (2010). Detection of a new extended-spectrum oxacillinase in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 65, 364–365.

Fukuda, H., Hosaka, M., Iyobe, S., Gotoh, N., Nishino, T., and Hirai, K. (1995). *nfxC*-type quinolone resistance in a clinical isolate of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 39, 790–792.

Galimand, M., Lambert, T., Gerbaud, G., and Courvalin, P. (1993). Characterization of the *aac(6′)-Ib* gene encoding an aminoglycoside 6′-*N*-acetyltransferase in *Pseudomonas aeruginosa* BM2656. *Antimicrob. Agents Chemother.* 37, 1456–1462.

Giamarellos-Bourboulis, E. J., Papadimitriou, E., Galanakis, N., Antonopoulou, A., Tsaganos, T., Kanellakopoulou, K., and Giamarellou, H. (2006). Multidrug resistance to antimicrobials as a predominant factor influencing patient survival. *Int. J. Antimicrob. Agents* 27, 476–481.

Gilbert, D. N., Moellering, R. C. Jr., and Sande, M. A. (2003). *The Sanford Guide to Antimicrobial Therapy 2003*. Hyde Park: Antimicrobial Therapy, Inc.

Gillis, R. J., White, K. G., Choi, K. H., Wagner, V. E., Schweizer, H. P., and Iglewski, B. H. (2005). Molecular basis of azithromycin-resistant *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 49, 3858–3867.

Girlich, D., Naas, T., Leelaporn, A., Poirel, L., Fennewald, M., and Nordmann, P. (2002). Nosocomial spread of the integron-located *veb-1*-like cassette encoding an extended-spectrum β-lactamase in *Pseudomonas aeruginosa* in Thailand. *Clin. Infect. Dis.* 34, 603–611.

Girlich, D., Naas, T., and Nordmann, P. (2004). Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 48, 2043–2048.

Glupczynski, Y., Bogaerts, P., Deplano, A., Berhin, C., Huang, T. D., Van Eldere, J., and Rodriguez-Villalobos, H. (2010). Detection and characterization of class A extended-spectrum-β-lactamase-producing *Pseudomonas aeruginosa* isolates in Belgian hospitals. *J. Antimicrob. Chemother.* 65, 866–871.

Gupta, V. (2008). Metallo β-lactamases in *Pseudomonas aeruginosa* and *Acinetobacter* species. *Expert Opin. Investig. Drugs* 17, 131–143.

Gurung, M., Moon, D. C., Tamang, M. D., Kim, J., Lee, Y. C., Seol, S. Y., Cho, D. T., and Lee, J. C. (2010). Emergence of 16S rRNA methylase gene *armA* and cocarriage of bla_{IMP-1} in *Pseudomonas aeruginosa* isolates from South Korea. *Diagn. Microbiol. Infect Dis.* 68, 468–470.

Gutierrez, O., Juan, C., Cercenado, E., Navarro, F., Bouza, E., Coll, P., Perez, J. L., and Oliver, A. (2007). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. *Antimicrob. Agents Chemother.* 51, 4329–4335.

Haagensen, J. A., Klausen, M., Ernst, R. K., Miller, S. I., Folkesson, A., Tolker-Nielsen, T., and Molin, S. (2007). Differentiation and distribution of colistin- and sodium dodecyl sulfate-tolerant cells in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* 189, 28–37.

Haas, M., Biddlecome, S., Davies, J., Luce, C. E., and Daniels, P. J. (1976). Enzymatic modification of aminoglycoside antibiotics: a new 6′-N-acetylating enzyme from a *Pseudomonas aeruginosa* isolate. *Antimicrob. Agents Chemother.* 9, 945–950.

Hachler, H., Santanam, P., and Kayser, F. H. (1996). Sequence and characterization of a novel chromosomal aminoglycoside phosphotransferase gene, *aph (3′)-IIb*, in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 40, 1254–1256.

Hammami, S., Ghozzi, R., Burghoffer, B., Arlet, G., and Redjeb, S. (2009). Mechanisms of carbapenem resistance in non-metallo-β-lactamase-producing clinical isolates of *Pseudomonas aeruginosa* from a Tunisian hospital. *Pathol. Biol.* 57, 530–535.

Harmsen, M., Yang, L., Pamp, S. J., and Tolker-Nielsen, T. (2010). An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. *FEMS Immunol. Med. Microbiol.* 59, 253–268.

Hassett, D. J., Sutton, M. D., Schurr, M. J., Herr, A. B., Caldwell, C. C., and Matu, J. O. (2009). *Pseudomonas aeruginosa* hypoxic or anaerobic biofilm infections within cystic fibrosis airways. *Trends Microbiol.* 17, 130–138.

Helfand, M. S., and Bonomo, R. A. (2003). β-lactamases: a survey of protein diversity. *Curr. Drug Targets Infect. Disord.* 3, 9–23.

Henrichfreise, B., Wiegand, I., Pfister, W., and Wiedemann, B. (2007). Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. *Antimicrob. Agents Chemother.* 51, 4062–4070.

Hidron, A. I., Edwards, J. R., Patel, J., Horan, T. C., Sievert, D. M., Pollock, D. A., and Fridkin, S. K. (2008). NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infect. Control Hosp. Epidemiol.* 29, 996–1011.

Higgins, P. G., Fluit, A. C., Milatovic, D., Verhoef, J., and Schmitz, F. J. (2003). Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 21, 409–413.

Hirsch, E. B., and Tam, V. H. (2010). Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. *Expert Rev. Pharmacoecon. Outcomes Res.* 10, 441–451.

Hocquet, D., Muller, A., Blanc, K., Plesiat, P., Talon, D., Monnet, D. L., and Bertrand, X. (2008). Relationship between antibiotic use and incidence of MexXY-OprM overproducers among clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 52, 1173–1175.

Hocquet, D., Nordmann, P., El Garch, F., Cabanne, L., and Plesiat, P. (2006). Involvement of the MexXY-OprM efflux system in emergence of cefepime resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 50, 1347–1351.

Hocquet, D., Plesiat, P., Dehecq, B., Mariotte, P., Talon, D., and Bertrand, X. (2010). Nationwide investigation of extended-spectrum β-lactamases, metallo-β-lactamases, and extended-spectrum oxacillinases produced by ceftazidime-resistant *Pseudomonas aeruginosa* strains in France. *Antimicrob. Agents Chemother.* 54, 3512–3515.

Hocquet, D., Roussel-Delvallez, M., Cavallo, J. D., and Plesiat, P. (2007). MexAB-OprM- and MexXY-overproducing mutants are very prevalent among clinical strains of *Pseudomonas aeruginosa* with reduced susceptibility to ticarcillin. *Antimicrob. Agents Chemother.* 51, 1582–1583.

Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A., and Miller, S. I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436, 1171–1175.

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* 35, 322–332.

Islam, S., Oh, H., Jalal, S., Karpati, F., Ciofu, O., Hoiby, N., and Wretlind, B. (2009). Chromosomal mechanisms of aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Clin. Microbiol. Infect.* 15, 60–66.

Jacoby, G. A. (2005). Mechanisms of resistance to quinolones. *Clin. Infect. Dis.* 41(Suppl. 2), S120–S126.

Jacoby, G. A. (2009). AmpC β-lactamases. *Clin. Microbiol. Rev.* 22, 161–182.

Jalal, S., Ciofu, O., Hoiby, N., Gotoh, N., and Wretlind, B. (2000). Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosos. *Antimicrob. Agents Chemother.* 44, 710–712.

Jeannot, K., Elsen, S., Kohler, T., Attree, I., Van Delden, C., and Plesiat, P. (2008). Resistance and virulence of *Pseudomonas aeruginosa* clinical strains overproducing the MexCD-OprJ efflux pump. *Antimicrob. Agents Chemother.* 52, 2455–2462.

Jiang, X., Zhang, Z., Li, M., Zhou, D., Ruan, F., and Lu, Y. (2006). Detection of extended-spectrum β-lactamases in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 50, 2990–2995.

Jin, J. S., Kwon, K. T., Moon, D. C., and Lee, J. C. (2009). Emergence of 16S rRNA methylase rmtA in colistin-only-sensitive *Pseudomonas aeruginosa* in South Korea. *Int. J. Antimicrob. Agents* 33, 490–491.

Johansen, H. K., Moskowitz, S. M., Ciofu, O., Pressler, T., and Hoiby, N. (2008). Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients. *J. Cyst. Fibros.* 7, 391–397.

Jones, R. N. (1998). Important and emerging β-lactamase-mediated resistances in hospital-based pathogens: the Amp C enzymes. *Diagn. Microbiol. Infect. Dis.* 31, 461–466.

Jones, R. N., Stilwell, M. G., Rhomberg, P. R., and Sader, H. S. (2009). Antipseudomonal activity of piperacillin/tazobactam: more than a decade of experience from the SENTRY Antimicrobial Surveillance Program (1997–2007). *Diagn. Microbiol. Infect. Dis.* 65, 331–334.

Juan, C., Macia, M. D., Gutierrez, O., Vidal, C., Perez, J. L., and Oliver, A. (2005). Molecular mechanisms of β-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. *Antimicrob. Agents Chemother.* 49, 4733–4738.

Juan, C., Mulet, X., Zamorano, L., Alberti, S., Perez, J. L., and Oliver, A. (2009). Detection of the novel extended-spectrum β-lactamase OXA-161 from a plasmid-located integron in *Pseudomonas aeruginosa* clinical isolates from Spain. *Antimicrob. Agents Chemother.* 53, 5288–5290.

Kallen, A. J., Hidron, A. I., Patel, J., and Srinivasan, A. (2010). Multidrug resistance among Gram-negative pathogens that caused healthcare-associated infections reported to the National Healthcare Safety Network, 2006–2008. *Infect. Control Hosp. Epidemiol.* 31, 528–531.

Keen, E. F., III, Robinson, B. J., Hospenthal, D. R., Aldous, W. K., Wolf, S. E., Chung, K. K., and Murray, C. K. (2010). Prevalence of multidrug-resistant organisms recovered at a military burn center. *Burns* 36, 819–825.

Kerr, K. G., and Snelling, A. M. (2009). *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *J. Hosp. Infect.* 73, 338–344.

Kettner, M., Milosovic, P., Hletkova, M., and Kallova, J. (1995). Incidence and mechanisms of aminoglycoside resistance in *Pseudomonas aeruginosa* serotype O11 isolates. *Infection* 23, 380–383.

Kim, J. Y., Park, Y. J., Kwon, H. J., Han, K., Kang, M. W., and Woo, G. J. (2008). Occurrence and mechanisms of amikacin resistance and its association with β-lactamases in *Pseudomonas aeruginosa*: a Korean nationwide study. *J. Antimicrob. Chemother.* 62, 479–483.

Kiser, T. H., Obritsch, M. D., Jung, R., Maclaren, R., and Fish, D. N. (2010). Efflux pump contribution to multidrug resistance in clinical isolates of *Pseudomonas aeruginosa*. *Pharmacotherapy* 30, 632–638.

Köhler, T., Epp, S. F., Curty, L. K., and Pechére, J.-C. (1999). Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *J. Bacteriol.* 181, 6300–6305.

Köhler, T., Michea-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L. K., and Pechere, J.-C. (1997). Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 23, 345–354.

Kong, K. F., Jayawardena, S. R., Del Puerto, A., Wiehlmann, L., Laabs, U., Tummler, B., and Mathee, K. (2005). Characterization of *poxB*, a chromosomal-encoded *Pseudomonas aeruginosa* oxacillinase. *Gene* 358, 82–92.

Kotsakis, S. D., Papagiannitsis, C. C., Tzelepi, E., Legakis, N. J., Miriagou, V., and Tzouvelekis, L. S. (2010). GES-13, a β-lactamase variant possessing Lys-104 and Asn-170 in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 54, 1331–1333.

Labuschagne, C. J., Weldhagen, G. F., Ehlers, M. M., and Dove, M. G. (2008). Emergence of class 1 integron-associated GES-5 and GES-5-like extended-spectrum β-lactamases in clinical isolates of *Pseudomonas aeruginosa* in South Africa. *Int. J. Antimicrob. Agents* 31, 527–530.

Lambert, M. L., Suetens, C., Savey, A., Palomar, M., Hiesmayr, M., Morales, I., Agodi, A., Frank, U., Mertens, K., Schumacher, M., and Wolkewitz, M. (2011). Clinical outcomes of health-care-associated infections and antimicrobial resistance in patients admitted to European intensive-care units: a cohort study. *Lancet Infect. Dis.* 11, 30–38.

Landman, D., Bratu, S., Alam, M., and Quale, J. (2005). Citywide emergence of *Pseudomonas aeruginosa* strains with reduced susceptibility to polymyxin B. *J. Antimicrob. Chemother.* 55, 954–957.

Lee, J. K., Lee, Y. S., Park, Y. K., and Kim, B. S. (2005). Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 25, 290–295.

Lewis, K. (2008). Multidrug tolerance of biofilms and persister cells. *Curr. Top. Microbiol. Immunol.* 322, 107–131.

Li, H., Toleman, M. A., Bennett, P. M., Jones, R. N., and Walsh, T. R. (2008). Complete Sequence of p07-406, a 24,179-base-pair plasmid harboring the bla_{VIM-7} metallo-β-lactamase gene in a *Pseudomonas aeruginosa* isolate from the United States. *Antimicrob. Agents Chemother.* 52, 3099–3105.

Li, X. Z., and Nikaido, H. (2009). Efflux-mediated drug resistance in bacteria: an update. *Drugs* 69, 1555–1623.

Libisch, B., Poirel, L., Lepsanovic, Z., Mirovic, V., Balogh, B., Paszti, J., Hunyadi, Z., Dobak, A., Fuzi, M., and Nordmann, P. (2008). Identification of PER-1 extended-spectrum β-lactamase producing *Pseudomonas aeruginosa* clinical isolates of the international clonal complex CC11 from Hungary and Serbia. *FEMS Immunol. Med. Microbiol.* 54, 330–338.

Lincopan, N., Neves, P., Mamizuka, E. M., and Levy, C. E. (2010). Balanoposthitis caused by *Pseudomonas aeruginosa* co-producing metallo-β-lactamase and 16S rRNA methylase in children with hematological malignancies. *Int. J. Infect. Dis.* 14, e344–e347.

Lister, P. D., Wolter, D. J., and Hanson, N. D. (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin. Microbiol. Rev.* 22, 582–610.

Liu, W., Liu, X., Liao, J., Zhang, Y., and Liang, X. (2010). Identification of *bla* OXA-128 and *bla* OXA-129, two novel OXA-type extended-spectrum-β-lactamases in *Pseudomonas aeruginosa*, in Hunan Province, China. *J. Basic Microbiol.* 50(Suppl. 1), S116–S119.

Livermore, D. M. (1991). β-lactamases of *Pseudomonas aeruginosa*. *Antibiot. Chemother.* 44, 215–220.

Livermore, D. M. (1992). Interplay of impermeability and chromosomal β-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 36, 2046–2048.

Livermore, D. M. (1995). β-lactamases in laboratory and clinical resistance. *Clin. Microbiol. Rev.* 8, 557–584.

Livermore, D. M., and Yang, Y. J. (1987). β-lactamase lability and inducer power of newer β-lactam antibiotics in relation to their activity against β-lactamase-inducibility mutants of *Pseudomonas aeruginosa*. *J. Infect. Dis.* 155, 775–782.

Lodge, J. M., Minchin, S. D., Piddock, L. J., and Busby, J. W. (1990). Cloning, sequencing and analysis of the structural gene and regulatory region of the *Pseudomonas aeruginosa* chromosomal *ampC* β-lactamase. *Biochem. J.* 272, 627–631.

Lopez, D., Vlamakis, H., and Kolter, R. (2010). Biofilms. *Cold Spring Harb. Perspect. Biol.* 2, a000398.

Macfarlane, E. L., Kwasnicka, A., and Hancock, R. E. (2000). Role of *Pseudomonas aeruginosa* PhoP-phoQ in resistance to antimicrobial cationic peptides and aminoglycosides. *Microbiology* 146, 2543–2554.

Macia, M. D., Blanquer, D., Togores, B., Sauleda, J., Perez, J. L., and Oliver, A. (2005). Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrob. Agents Chemother.* 49, 3382–3386.

MacLeod, D. L., Nelson, L. E., Shawar, R. M., Lin, B. B., Lockwood, L. G., Dirk, J. E., Miller, G. H., Burns, J. L., and Garber, R. L. (2000). Aminoglycoside-resistance mechanisms for cystic fibrosis *Pseudomonas aeruginosa* isolates are unchanged by long-term, intermittent, inhaled tobramycin treatment. *J. Infect. Dis.* 181, 1180–1184.

Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S., and O'Toole, G. A. (2003). A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426, 306–310.

Mahar, P., Padiglione, A. A., Cleland, H., Paul, E., Hinrichs, M., and Wasiak, J. (2010). *Pseudomonas aeruginosa* bacteraemia in burns patients: risk factors and outcomes. *Burns* 36, 1228–1233.

Mandsberg, L. F., Ciofu, O., Kirkby, N., Christiansen, L. E., Poulsen, H. E., and Hoiby, N. (2009). Antibiotic resistance in *Pseudomonas aeruginosa* strains with increased mutation frequency due to inactivation of the DNA oxidative repair system. *Antimicrob. Agents Chemother.* 53, 2483–2491.

Maniati, M., Ikonomidis, A., Mantzana, P., Daponte, A., Maniatis, A. N., and Pournaras, S. (2007). A highly carbapenem-resistant *Pseudomonas aeruginosa* isolate with a novel $bla_{VIM-4/blaP1b}$ integron overexpresses two efflux pumps and lacks OprD. *J. Antimicrob. Chemother.* 60, 132–135.

Mansour, W., Dahmen, S., Poirel, L., Charfi, K., Bettaieb, D., Boujaafar, N., and Bouallegue, O. (2009). Emergence of SHV-2a extended-spectrum β-lactamases in clinical isolates of *Pseudomonas aeruginosa* in a university hospital in Tunisia. *Microb. Drug Resist.* 15, 295–301.

Maseda, H., Saito, K., Nakajima, A., and Nakae, T. (2000). Variation of the *mexT* gene, a regulator of the MexEF-oprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* 192, 107–112.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., and Nishino, T. (2000). Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 44, 3322–3327.

Matsuo, Y., Eda, S., Gotoh, N., Yoshihara, E., and Nakae, T. (2004). MexZ-mediated regulation of *mexXY* multidrug efflux pump expression in *Pseudomonas aeruginosa* by binding on the *mexZ-mexX* intergenic DNA. *FEMS Microbiol. Lett.* 238, 23–28.

Matthaiou, D. K., Michalopoulos, A., Rafailidis, P. I., Karageorgopoulos, D. E., Papaioannou, V., Ntani, G., Samonis, G., and Falagas, M. E. (2008). Risk factors associated with the isolation of colistin-resistant Gram-negative bacteria: a matched case-control study. *Crit. Care Med.* 36, 807–811.

Mauldin, P. D., Salgado, C. D., Hansen, I. S., Durup, D. T., and Bosso, J. A. (2010). Attributable hospital cost and length of stay associated with health care-associated infections caused by antibiotic-resistant Gram-negative bacteria. *Antimicrob. Agents Chemother.* 54, 109–115.

McPhee, J. B., Lewenza, S., and Hancock, R. E. (2003). Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 50, 205–217.

Mendes, R. E., Toleman, M. A., Ribeiro, J., Sader, H. S., Jones, R. N., and Walsh, T. R. (2004). Integron carrying a novel metallo-β-lactamase gene, bla_{IMP-16}, and a fused form of aminoglycoside-resistant gene aac(6′)-30/aac(6′)-Ib′: report from the SENTRY Antimicrobial Surveillance Program. *Antimicrob. Agents Chemother.* 48, 4693–4702.

Miller, G. H., Sabatelli, F. J., Naples, L., Hare, R. S., and Shaw, K. J. (1994). Resistance to aminoglycosides in *Pseudomonas*. Aminoglycoside Resistance Study Groups. *Trends Microbiol.* 2, 347–353.

Molina, J., Cordero, E., and Pachon, J. (2009). New information about the polymyxin/colistin class of antibiotics. *Expert Opin. Pharmacother.* 10, 2811–2828.

Montero, M., Horcajada, J. P., Sorli, L., Alvarez-Lerma, F., Grau, S., Riu, M., Sala, M., and Knobel, H. (2009). Effectiveness and safety of colistin for the treatment of multidrug-resistant *Pseudomonas aeruginosa* infections. *Infection* 37, 461–465.

Morero, N. R., and Argarana, C. E. (2009). *Pseudomonas aeruginosa* deficient in 8-oxodeoxyguanine repair system shows a high frequency of resistance to ciprofloxacin. *FEMS Microbiol. Lett.* 290, 217–226.

Morita, Y., Cao, L., Gould, G., Avison, M. B., and Poole, K. (2006). nalD encodes a second repressor of the mexAB-oprM multidrug efflux operon of *Pseudomonas aeruginosa*. *J. Bacteriol.* 188, 8649–8654.

Moskowitz, S. M., Ernst, R. K., and Miller, S. I. (2004). PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *J. Bacteriol.* 186, 575–579.

Mulcahy, L. R., Burns, J. L., Lory, S., and Lewis, K. (2010). Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J. Bacteriol.* 192, 6191–6199.

Mulet, X., Macia, M. D., Mena, A., Juan, C., Perez, J. L., and Oliver, A. (2009). Azithromycin in *Pseudomonas aeruginosa* biofilms: bactericidal activity and selection of nfxB mutants. *Antimicrob. Agents Chemother.* 53, 1552–1560.

Muller, C., Plesiat, P., and Jeannot, K. (2010). A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 55, 1211–1221.

Muramatsu, H., Horii, T., Takeshita, A., Hashimoto, H., and Maekawa, M. (2005). Characterization of fluoroquinolone and carbapenem susceptibilities in clinical isolates of levofloxacin-resistant *Pseudomonas aeruginosa*. *Chemotherapy* 51, 70–75.

Mutlu, G. M., and Wunderink, R. G. (2006). Severe pseudomonal infections. *Curr. Opin. Crit. Care* 12, 458–463.

Naas, T., Aubert, D., Lambert, T., and Nordmann, P. (2006). Complex genetic structures with repeated elements, a sul-type class 1 integron, and the blaVEB extended-spectrum β-lactamase gene. *Antimicrob. Agents Chemother.* 50, 1745–1752.

Nakano, M., Deguchi, T., Kawamura, T., Yasuda, M., Kimura, M., Okano, Y., and Kawada, Y. (1997). Mutations in the *gyrA* and *parC* genes in fluoroquinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 41, 2289–2291.

Nordmann, P., and Mammeri, H. (2007). Extended-spectrum cephalosporinases: structure, detection and epidemiology. *Future Microbiol.* 2, 297–307.

Ochs, M. M., McCusker, M. P., Bains, M., and Hancock, R. E. (1999). Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. *Antimicrob. Agents Chemother.* 43, 1085–1090.

Okamoto, K., Gotoh, N., and Nishino, T. (2002). Alterations of susceptibility of *Pseudomonas aeruginosa* by overproduction of multidrug efflux systems, MexAB-OprM, MexCD-OprJ, and MexXY/OprM to carbapenems:

substrate specificities of the efflux systems. *J. Infect. Chemother.* 8, 371–373.
Oliver, A. (2010). Mutators in cystic fibrosis chronic lung infection: prevalence, mechanisms, and consequences for antimicrobial therapy. *Int. J. Med. Microbiol.* 300, 563–572.
Oliver, A., Baquero, F., and Blazquez, J. (2002). The mismatch repair system (*mutS*, *mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol. Microbiol.* 43, 1641–1650.
Oliver, A., Canton, R., Campo, P., Baquero, F., and Blazquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288, 1251–1254.
Oliver, A., and Mena, A. (2010). Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. *Clin. Microbiol. Infect.* 16, 798–808.
Page, M. G., and Heim, J. (2009). Prospects for the next anti-*Pseudomonas* drug. *Curr. Opin. Pharmacol.* 9, 558–565.
Pai, H., Kim, J., Kim, J., Lee, J. H., Choe, K. W., and Gotoh, N. (2001). Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother.* 45, 480–484.
Pamp, S. J., Gjermansen, M., Johansen, H. K., and Tolker-Nielsen, T. (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Mol. Microbiol.* 68, 223–240.
Paterson, D. L. (2006). The epidemiological profile of infections with multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species. *Clin. Infect. Dis.* 43(Suppl. 2), S43–S48.
Paterson, D. L., and Bonomo, R. A. (2005). Extended-spectrum β-lactamases: a clinical update. *Clin. Microbiol. Rev.* 18, 657–686.
Paul, M., Yahav, D., Bivas, A., Fraser, A., and Leibovici, L. (2010). Anti-pseudomonal β-lactams for the initial, empirical, treatment of febrile neutropenia: comparison of β-lactams. *Cochrane Database Syst. Rev.* 11, CD005197.
Pena, C., Suarez, C., Tubau, F., Juan, C., Moya, B., Dominguez, M. A., Oliver, A., Pujol, M., and Ariza, J. (2009). Nosocomial outbreak of a non-cefepime-susceptible ceftazidime-susceptible *Pseudomonas aeruginosa* strain overexpressing MexXY-OprM and producing an integron-borne PSE-1 β-lactamase. *J. Clin. Microbiol.* 47, 2381–2387.
Pfeifer, Y., Cullik, A., and Witte, W. (2010). Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. *Int. J. Med. Microbiol.* 300, 371–379.
Picao, R. C., Poirel, L., Gales, A. C., and Nordmann, P. (2009a). Diversity of β-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolates causing bloodstream infections in Brazil. *Antimicrob. Agents Chemother.* 53, 3908–3913.
Picao, R. C., Poirel, L., Gales, A. C., and Nordmann, P. (2009b). Further identification of CTX-M-2 extended-spectrum β-lactamase in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 2225–2226.
Poirel, L., Brinas, L., Verlinde, A., Ide, L., and Nordmann, P. (2005). BEL-1, a novel clavulanic acid-inhibited extended-spectrum β-lactamase, and the class 1 integron In120 in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 49, 3743–3748.
Poirel, L., Docquier, J. D., De Luca, F., Verlinde, A., Ide, L., Rossolini, G. M., and Nordmann, P. (2010a). BEL-2, an extended-spectrum β-lactamase with increased activity toward expanded-spectrum cephalosporins in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 54, 533–535.
Poirel, L., Naas, T., and Nordmann, P. (2010b). Diversity, epidemiology, and genetics of class D β-lactamases. *Antimicrob. Agents Chemother.* 54, 24–38.
Poirel, L., Nordmann, P., Lagrutta, E., Cleary, T., and Munoz-Price, L. S. (2010c). Emergence of KPC-producing *Pseudomonas aeruginosa* in the United States. *Antimicrob. Agents Chemother.* 54, 3072.
Poirel, L., Lambert, T., Turkoglu, S., Ronco, E., Gaillard, J., and Nordmann, P. (2001). Characterization of Class 1 integrons from *Pseudomonas aeruginosa* that contain the bla_{VIM-2} carbapenem-hydrolyzing β-lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrob. Agents Chemother.* 45, 546–552.
Poirel, L., Magalhaes, M., Lopes, M., and Nordmann, P. (2004). Molecular analysis of metallo-β-lactamase gene bla_{SPM-1}-surrounding sequences from disseminated *Pseudomonas aeruginosa* isolates in Recife, Brazil. *Antimicrob. Agents Chemother.* 48, 1406–1409.
Poirel, L., and Nordmann, P. (2002). Acquired carbapenem-hydrolyzing β-lactamases and their genetic support. *Curr. Pharm. Biotechnol.* 3, 117–127.
Poole, K. (2000). Efflux-mediated resistance to fluoroquinolones in Gram-negative bacteria. *Antimicrob. Agents Chemother.* 44, 2233–2241.
Poole, K. (2002). Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. *Curr. Pharm. Biotechnol.* 3, 77–98.
Poole, K. (2004a). "Efflux pumps," in *Pseudomonas, Vol. 1. Genomics, life style and molecular architecture*, ed. J. L. Ramos (New York, NY: Kluwer Academic, Plenum Publishers), 635–674.
Poole, K. (2004b). Resistance to β-lactam antibiotics. *Cell. Mol. Life Sci.* 61, 2200–2223.
Poole, K. (2005). Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 49, 479–487.
Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms. *Ann. Med.* 39, 162–176.
Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., Yamasaki, T., Neshat, S., Yamagishi, J.-I., Li, X.-Z., and Nishino, T. (1996). Overexpression of the *mexC-mex-oprJ* efflux operon in *nfxB* multidrug resistant strains of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 21, 713–724.
Pournaras, S., Maniati, M., Spanakis, N., Ikonomidis, A., Tassios, P. T., Tsakris, A., Legakis, N. J., and Maniatis, A. N. (2005). Spread of efflux pump-overexpressing, non-metallo-β-lactamase-producing, meropenem-resistant but ceftazidime-susceptible *Pseudomonas aeruginosa* in a region with bla_{VIM} endemicity. *J. Antimicrob. Chemother.* 56, 761–764.
Quale, J., Bratu, S., Gupta, J., and Landman, D. (2006). Interplay of efflux system, *ampC*, and *oprD* expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother.* 50, 1633–1641.
Queenan, A. M., and Bush, K. (2007). Carbapenemases: the versatile β-lactamases. *Clin. Microbiol. Rev.* 20, 440–458.
Queenan, A. M., Shang, W., Bush, K., and Flamm, R. K. (2010). Differential selection of single-step AmpC or efflux mutants of *Pseudomonas aeruginosa* by using cefepime, ceftazidime, or ceftobiprole. *Antimicrob. Agents Chemother.* 54, 4092–4097.
Ramirez, M. S., and Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. *Drug Resist.* 13, 151–171.
Reinhardt, A., Kohler, T., Wood, P., Rohner, P., Dumas, J. L., Ricou, B., and Van Delden, C. (2007). Development and persistence of antimicrobial resistance in *Pseudomonas aeruginosa*: a longitudinal observation in mechanically ventilated patients. *Antimicrob. Agents Chemother.* 51, 1341–1350.
Rejiba, S., Aubry, A., Petitfrere, S., Jarlier, V., and Cambau, E. (2008). Contribution of *parE* mutation and efflux to ciprofloxacin resistance in *Pseudomonas aeruginosa* clinical isolates. *J. Chemother.* 20, 749–752.
Riccio, M. L., Pallecchi, L., Fontana, R., and Rossolini, G. M. (2001). In70 of plasmid pAX22, a bla_{VIM-1}-containing integron carrying a new aminoglycoside phosphotransferase gene cassette. *Antimicrob. Agents Chemother.* 45, 1249–1253.
Rodriguez-Martinez, J. M., Poirel, L., and Nordmann, P. (2009a). Extended-spectrum cephalosporinases in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 1766–1771.
Rodriguez-Martinez, J. M., Poirel, L., and Nordmann, P. (2009b). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 4783–4788.
Rossolini, G. M., and Mantengoli, E. (2005). Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. *Clin. Microbiol. Infect.* 11(Suppl. 4), 17–32.
Sabtcheva, S., Galimand, M., Gerbaud, G., Courvalin, P., and Lambert, T. (2003). Aminoglycoside resistance gene *ant(4′)-IIb* of *Pseudomonas aeruginosa* BM4492, a clinical isolate from Bulgaria. *Antimicrob. Agents Chemother.* 47, 1584–1588.
Sader, H. S., Reis, A. O., Silbert, S., and Gales, A. C. (2005). IMPs, VIMs and SPMs: the diversity of metallo-β-lactamases produced by carbapenem-resistant *Pseudomonas aeruginosa* in a Brazilian hospital. *Clin. Microbiol. Infect.* 11, 73–76.
Sadovskaya, I., Vinogradov, E., Li, J., Hachani, A., Kowalska, K., and Filloux, A. (2010). High-level antibiotic resistance in *Pseudomonas aeruginosa* biofilm: the *ndvB* gene is involved in the production of highly glycerol-phosphorylated β-(1->3)-glucans, which bind aminoglycosides. *Glycobiology* 20, 895–904.
Sakurai, Y., Yoshida, Y., Saitoh, K., Nemoto, M., Yamaguchi, A., and Sawai, T. (1990). Characteristics of aztreonam as a substrate, inhibitor and inducer for β-lactamases. *J. Antibiot.* 43, 403–410.
Salabi, A. E., Toleman, M. A., Weeks, J., Bruderer, T., Frei, R., and Walsh, T. R. (2010). First report of the metallo-β-lactamase SPM-1 in Europe. *Antimicrob. Agents Chemother.* 54, 582.
Samonis, G., Matthaiou, D. K., Kofteridis, D., Maraki, S., and Falagas, M. E. (2010). In vitro susceptibility to various antibiotics of colistin-resistant Gram-negative bacterial isolates in

a general tertiary hospital in Crete, Greece. *Clin. Infect. Dis.* 50, 1689–1691.
Schobert, M., and Tielen, P. (2010). Contribution of oxygen-limiting conditions to persistent infection in *Pseudomonas aeruginosa*. *Future Microbiol.* 5, 603–621.
Schwartz, T., Volkmann, H., Kirchen, S., Kohnen, W., Schon-Holz, K., Jansen, B., and Obst, U. (2006). Real-time PCR detection of *Pseudomonas aeruginosa* in clinical and municipal wastewater and genotyping of the ciprofloxacin-resistant isolates. *FEMS Microbiol. Ecol.* 57, 158–167.
Shahcheraghi, F., Nikbin, V. S., and Feizabadi, M. M. (2009). Prevalence of ESBLs genes among multidrug-resistant isolates of *Pseudomonas aeruginosa* isolated from patients in Tehran. *Microb. Drug Resist.* 15, 37–39.
Shahid, M., and Malik, A. (2005). Resistance due to aminoglycoside modifying enzymes in *Pseudomonas aeruginosa* isolates from burns patients. *Indian J. Med. Res.* 122, 324–329.
Shaw, K. J., Munayyer, H., Rather, P. N., Hare, R. S., and Miller, G. H. (1993). Nucleotide sequence analysis and DNA hybridization studies of the *ant(4′)-IIa* gene from *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 37, 708–714.
Shawar, R. M., MacLeod, D. L., Garber, R. L., Burns, J. L., Stapp, J. R., Clausen, C. R., and Tanaka, S. K. (1999). Activities of tobramycin and six other antibiotics against *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Antimicrob. Agents Chemother.* 43, 2877–2880.
Shorr, A. F. (2009). Review of studies of the impact on Gram-negative bacterial resistance on outcomes in the intensive care unit. *Crit. Care Med.* 37, 1463–1469.
Slama, T. G. (2008). Gram-negative antibiotic resistance: there is a price to pay. *Crit. Care* 12(Suppl. 4), S4.
Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P., Moskowitz, S. M., Burns, J. L., Kaul, R., and Olson, M. V. (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc. Natl. Acad. Sci U. S. A.* 103, 8487–8492.
Sobel, M. L., McKay, G. A., and Poole, K. (2003). Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother.* 47, 3202–3207.
Sobel, M. L., Poole, K., and Neshat, S. (2005). Mutations in PA2491 (*mexS*) promote MexT-dependent *mexEF-oprN* expression and multidrug resistance in a clinical strain of *Pseudomonas aeruginosa*. *J. Bacteriol.* 187, 1246–1253.
Srikumar, R., Paul, C. J., and Poole, K. (2000). Influence of mutations in the *mexR* repressor gene on expression of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa*. *J. Bacteriol.* 182, 1410–1414.
Strateva, T., Ouzounova-Raykova, V., Markova, B., Todorova, A., Marteva-Proevska, Y., and Mitov, I. (2007). Widespread detection of VEB-1-type extended-spectrum β-lactamases among nosocomial ceftazidime-resistant *Pseudomonas aeruginosa* isolates in Sofia, Bulgaria. *J. Chemother.* 19, 140–145.
Strateva, T., and Yordanov, D. (2009). *Pseudomonas aeruginosa* – a phenomenon of bacterial resistance. *J. Med. Microbiol.* 58, 1133–1148.
Taccetti, G., Campana, S., Neri, A. S., Boni, V., and Festini, F. (2008). Antibiotic therapy against *Pseudomonas aeruginosa* in cystic fibrosis. *J. Chemother.* 20, 166–169.
Talbot, G. H., Bradley, J., Edwards, J. E. Jr., Gilbert, D., Scheld, M., and Bartlett, J. G. (2006). Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. *Clin. Infect. Dis.* 42, 657–668.
Tam, V. H., Chang, K. T., Abdelraouf, K., Brioso, C. G., Ameka, M., McCaskey, L. A., Weston, J. S., Caeiro, J. P., and Garey, K. W. (2010). Prevalence, resistance mechanisms, and susceptibility of multidrug-resistant bloodstream isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 54, 1160–1164.
Tam, V. H., Schilling, A. N., LaRocco, M. T., Gentry, L. O., Lolans, K., Quinn, J. P., and Garey, K. W. (2007). Prevalence of AmpC over-expression in bloodstream isolates of *Pseudomonas aeruginosa*. *Clin. Microbiol. Infect.* 13, 413–418.
Toleman, M. A., Rolston, K., Jones, R. N., and Walsh, T. R. (2003). Molecular and biochemical characterization of OXA-45, an extended-spectrum class 2d′ β-lactamase in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 47, 2859–2863.
Tomas, M., Doumith, M., Warner, M., Turton, J. F., Beceiro, A., Bou, G., Livermore, D. M., and Woodford, N. (2010). Efflux pumps, OprD porin, AmpC β-lactamase, and multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob. Agents Chemother.* 54, 2219–2224.
Trias, J., and Nikaido, H. (1990). Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 34, 52–57.
Tumbarello, M., Repetto, E., Trecarichi, E. M., Bernardini, C., DE Pascale, G., Parisini, A., Rossi, M., Molinari, M. P., Spanu, T., Viscoli, C., Cauda, R., and Bassetti, M. (2011). Multidrug-resistant *Pseudomonas aeruginosa* bloodstream infections: risk factors and mortality. *Epidemiol. Infect.* (in press).
Upadhyay, S., Sen, M. R., and Bhattacharjee, A. (2010). Presence of different β-lactamase classes among clinical isolates of *Pseudomonas aeruginosa* expressing AmpC β-lactamase enzyme. *J. Infect. Dev. Ctries.* 4, 239–242.
Veesenmeyer, J. L., Hauser, A. R., Lisboa, T., and Rello, J. (2009). *Pseudomonas aeruginosa* virulence and therapy: evolving translational strategies. *Crit. Care Med.* 37, 1777–1786.
Vettoretti, L., Floret, N., Hocquet, D., Dehecq, B., Plesiat, P., Talon, D., and Bertrand, X. (2009). Emergence of extensive-drug-resistant *Pseudomonas aeruginosa* in a French university hospital. *Eur. J. Clin. Microbiol. Infect. Dis.* 28, 1217–1222.
Viedma, E., Juan, C., Acosta, J., Zamorano, L., Otero, J. R., Sanz, F., Chaves, F., and Oliver, A. (2009). Nosocomial spread of colistin-only-sensitive sequence type 235 *Pseudomonas aeruginosa* isolates producing the extended-spectrum β-lactamases GES-1 and GES-5 in Spain. *Antimicrob. Agents Chemother.* 53, 4930–4933.
Villegas, M. V., Lolans, K., Correa, A., Kattan, J. N., Lopez, J. A., and Quinn, J. P. (2007). First identification of *Pseudomonas aeruginosa* isolates producing a KPC-type carbapenem-hydrolyzing β-lactamase. *Antimicrob. Agents Chemother.* 51, 1553–1555.
Wagner, V. E., and Iglewski, B. H. (2008). *P. aeruginosa biofilms in CF infection. Clin. Rev. Allergy Immunol.* 35, 124–134.
Waine, D. J., Honeybourne, D., Smith, E. G., Whitehouse, J. L., and Dowson, C. G. (2008). Association between hypermutator phenotype, clinical variables, mucoid phenotype, and antimicrobial resistance in *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 46, 3491–3493.
Walsh, T. R. (2010). Emerging carbapenemases: a global perspective. *Int. J. Antimicrob. Agents* 36(Suppl. 3), S8–S14.
Walsh, T. R., Toleman, M. A., Poirel, L., and Nordmann, P. (2005). Metallo-β-lactamases: the quiet before the storm? *Clin. Microbiol. Rev.* 18, 306–325.
Walther-Rasmussen, J., and Hoiby, N. (2007). Class A carbapenemases. *J. Antimicrob. Chemother.* 60, 470–482.
Wang, J., Zhou, J. Y., Qu, T. T., Shen, P., Wei, Z. Q., Yu, Y. S., and Li, L. J. (2010). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Chinese hospitals. *Int. J. Antimicrob. Agents* 35, 486–491.
Werner, E., Roe, F., Bugnicourt, A., Franklin, M. J., Heydorn, A., Molin, S., Pitts, B., and Stewart, P. S. (2004). Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 70, 6188–6196.
Wolter, D. J., Khalaf, N., Robledo, I. E., Vazquez, G. J., Sante, M. I., Aquino, E. E., Goering, R. V., and Hanson, N. D. (2009a). Surveillance of carbapenem-resistant *Pseudomonas aeruginosa* isolates from Puerto Rican Medical Center Hospitals: dissemination of KPC and IMP-18 β-lactamases. *Antimicrob. Agents Chemother.* 53, 1660–1664.
Wolter, D. J., Kurpiel, P. M., Woodford, N., Palepou, M. F., Goering, R. V., and Hanson, N. D. (2009b). Phenotypic and enzymatic comparative analysis of the novel KPC variant KPC-5 and its evolutionary variants, KPC-2 and KPC-4. *Antimicrob. Agents Chemother.* 53, 557–562.
Wolter, D. J., Smith-Moland, E., Goering, R. V., Hanson, N. D., and Lister, P. D. (2004). Multidrug resistance associated with *mexXY* expression in clinical isolates of *Pseudomonas aeruginosa* from a Texas hospital. *Diagn. Microbiol. Infect. Dis.* 50, 43–50.
Woodford, N., Zhang, J., Kaufmann, M. E., Yarde, S., Tomas, M. M., Faris, C., Vardhan, M. S., Dawson, S., Cotterill, S. L., and Livermore, D. M. (2008). Detection of *Pseudomonas aeruginosa* isolates producing VEB-type extended-spectrum β-lactamases in the United Kingdom. *J. Antimicrob. Chemother.* 62, 1265–1268.
Wright, A., Hawkins, C. H., Anggard, E. E., and Harper, D. R. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. *Clin. Otolaryngol.* 34, 349–357.
Xavier, D. E., Picao, R. C., Girardello, R., Fehlberg, L. C., and Gales, A. C. (2010). Efflux pumps expression and its association with porin down-regulation and β-lactamase production among *Pseudomonas aeruginosa* causing bloodstream infections in

Brazil. *BMC Microbiol.* 10, 217. doi: 10.1186/1471-2180-10-217

Yamane, K., Doi, Y., Yokoyama, K., Yagi, T., Kurokawa, H., Shibata, N., Shibayama, K., Kato, H., and Arakawa, Y. (2004). Genetic environments of the *rmtA* gene in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother.* 48, 2069–2074.

Zavascki, A. P., Goldani, L. Z., Li, J., and Nation, R. L. (2007). Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J. Antimicrob. Chemother.* 60, 1206–1215.

Zhanel, G. G., Decorby, M., Adam, H., Mulvey, M. R., McCracken, M., Lagace-Wiens, P., Nichol, K. A., Wierzbowski, A., Baudry, P. J., Tailor, F., Karlowsky, J. A., Walkty, A., Schweizer, F., Johnson, J., and Hoban, D. J. (2010). Prevalence of antimicrobial-resistant pathogens in canadian hospitals: results of the Canadian Ward Surveillance Study (CANWARD 2008). *Antimicrob. Agents Chemother.* 54, 4684–4693.

Zhanel, G. G., Hoban, D. J., Schurek, K., and Karlowsky, J. A. (2004). Role of efflux mechanisms on fluoroquinolone resistance in *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 24, 529–535.

Zhang, L., and Mah, T. F. (2008). Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *J. Bacteriol.* 190, 4447–4452.

Zhao, W. H., Chen, G., Ito, R., and Hu, Z. Q. (2009). Relevance of resistance levels to carbapenems and integron-borne $bla_{\text{IMP-1}}$, $bla_{\text{IMP-7}}$, $bla_{\text{IMP-10}}$ and $bla_{\text{VIM-2}}$ in clinical isolates of *Pseudomonas aeruginosa*. *J. Med. Microbiol.* 58, 1080–1085.

Zhao, W. H., and Hu, Z. Q. (2010). β-lactamases identified in clinical isolates of *Pseudomonas aeruginosa*. *Crit. Rev. Microbiol.* 36, 245–258.

Zhou, Y., Yu, H., Guo, Q., Xu, X., Ye, X., Wu, S., Guo, Y., and Wang, M. (2010). Distribution of 16S rRNA methylases among different species of Gram-negative bacilli with high-level resistance to aminoglycosides. *Eur. J. Clin. Microbiol. Infect. Dis.* 29, 1349–1353.

Zilberberg, M. D., Chen, J., Mody, S. H., Ramsey, A. M., and Shorr, A. F. (2010). Imipenem resistance of *Pseudomonas* in pneumonia: a systematic literature review. *BMC Pulm. Med.* 10, 45. doi: 10.1186/1471-2466-10-45

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Susceptibility of *Pseudomonas aeruginosa* biofilm to alpha-helical peptides: D-enantiomer of LL-37

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Pseudomonas aeruginosa is a highly versatile opportunistic pathogen and its ability to produce biofilms is a direct impediment to the healing of wounds and recovery from infection. Interest in anti-microbial peptides (AMPs) has grown due to their potential therapeutic applications and their possible use against antibiotic resistant bacteria. LL-37 is the only cathelicidin expressed by humans. In this study, we tested LL-37 and the effect of a protease-resistant LL-37 peptide mimetic, the peptide enantiomer D-LL-37, for anti-microbial and anti-biofilm activity against *P. aeruginosa*. Both forms of the peptide were equally effective as AMPs with similar killing kinetics. Circular dichroism spectra were obtained to demonstrate the chirality of D- and L-LL-37, and the trypsin resistance of D-LL-37 was confirmed. The helical cathelicidin from the cobra *Naja atra* (NA-CATH), and synthetic peptide variations (ATRA-1, ATRA-2, NA-CATH:ATRA1-ATRA1) were also tested. Although the cobra cathelicidin and related peptides had strong anti-microbial activity, those tested did not inhibit *Pseudomonas* biofilm formation, neither did control peptides. Both D- and L-LL-37 inhibited the attachment of *Pseudomonas* to a 96-well plate and decreased the amount of pre-formed (established) biofilm. D-LL-37 is able to promote *Pseudomonas* motility and decrease biofilm formation by altering the rate of twitching as well as by downregulating the expression of the biofilm-related genes, *rhlA* and *rhlB*, similar to L-LL-37. Both L- and D-LL-37 protected *Galleria mellonella in vivo* against *Pseudomonas* infection, while NA-CATH:ATRA1-ATRA1 peptide did not. This study demonstrates the ability and equivalence of D-LL-37 compared to L-LL-37 to promote bacterial twitching motility and inhibit biofilm formation, and protect against *in vivo* infection, and suggests that this peptide could be a critical advancement in the development of new treatments for *P. aeruginosa* infection.

Keywords: *Pseudomonas*, biofilm, cathelicidin, enantiomer, mimetic, protease-resistance, chiral

INTRODUCTION

Pseudomonas aeruginosa, a Gram-negative bacterium, is a major cause of hospital-associated infections, causing ∼10% of nosocomial infections (Hancock and Speert, 2000), with a sepsis mortality rate of nearly 50% (Bone, 1993). This opportunistic pathogen can cause pneumonia, catheter-associated and urinary tract infections, and sepsis in wounded patients and those who are immunocompromised (Van Delden and Iglewski, 1998). *P. aeruginosa* is the most clinically relevant pulmonary pathogen in patients with cystic fibrosis, causing chronic infections, and is the most common cause of fatal lung disease (Richards et al., 1999), as well as the most frequently recorded cause of hospital-acquired pneumonia, with an increasing number of these cases involving antibiotic resistant strains (Rello et al., 1993; Heyland et al., 1999).

In order for the opportunistic pathogen *P. aeruginosa* to infect a host, it requires a break in the innate immunological defenses. Once established within the host, the ability of *P. aeruginosa* to form biofilm is a signature characteristic for it to become a chronic infection (Hoiby et al., 2001; Singh et al., 2002). This property has made *Pseudomonas* a model organism in the study of biofilm formation. Biofilms, non-motile communities enveloped in an exopolysaccaride and extracellular protein secretion that enhances survival, are often physically resistant to anti-microbial agents and host immune responses (Stewart et al., 2001; Donlan and Costerton, 2002; Drenkard and Ausubel, 2002). It has been shown that biofilm formation can be a pathogen's defensive reaction to the presence of antibiotics. Specifically, subinhibitory concentrations of antibiotics can initiate the production of the exopolysaccharide matrix by *P. aeruginosa*, and stimulate increases in biofilm volume (Rachid et al., 2000; Bagge et al., 2004). The clinical use of current antibiotics at concentrations effective for eliminating biofilms would result in significant systemic side effects, limiting their utility in such cases (Whatley et al., 2006). In addition to *P. aeruginosa* infections in burn victims (Ressner et al., 2008), examples of biofilm-associated infections include: pneumonia and cystic fibrosis (Hoiby et al., 2001; Singh et al., 2002), chronic

Abbreviations: AMP, anti-microbial peptide; CAMP, cationic anti-microbial peptide; CFU, colony forming unit; EC50, half-maximal effective concentration; MIC, minimum inhibitory concentration; TFE, trifluoroethanol.

wounds such as diabetic foot ulcers, sinus and ear infections, and endocarditis (Costerton et al., 1999; Prince, 2002; Dieter, 2004; Kaneko et al., 2007).

Anti-microbial peptides (AMPs) are critical components of innate immunity in humans, contributing to our first line of defense against infection (Zasloff, 2002). The cathelicidin family of AMPs range from 12 to 80 amino acid residues in length, and are identified based on a conserved N-terminal domain, the cathelin domain, present in the inactive precursor peptide (cleaved from the active peptide) within the granules of natural killer T cells, in the mucosal epithelia of the lungs, and other sites (Ganz and Weiss, 1997). The sequence diversity of cathelicidins translates into the peptides demonstrating structural diversity, which can be grouped into sub-classes based on shared structural features. Alpha-helical cathelicidins, which adopt amphipathic helical conformations when interacting with membranes, are the largest of the cathelicidin structural classes. Our knowledge of their structural and functional properties is largely based on observations from the single, highly studied human cathelicidin, LL-37 (Gennaro et al., 1998).

The single human cathelicidin LL-37 is derived from the C-terminus of the human CAP-18 protein. It is a 37 residue cationic peptide which forms an alpha-helix when in contact with bacterial membranes. This peptide has broad-spectrum anti-microbial activity against Gram-negative and Gram-positive bacteria, including against *P. aeruginosa* (EC50 of $2.8 \pm 1.3\,\mu g/ml$; Bals et al., 1998; Turner et al., 1998; Travis et al., 2000; Saiman et al., 2001; Bucki et al., 2004; Gordon et al., 2005). However, multi-drug resistant *P. aeruginosa* strains exhibit reduced sensitivity to several AMPs (Ouhara et al., 2008), demonstrating the need to identify more effective AMPs. In order to develop additional AMPs we decided to test the impact of peptide chirality on the anti-microbial effectiveness of LL-37. We synthesized a D-LL-37 peptide, in which each amino acid residue of the naturally occurring L-isomer had been replaced with the corresponding D-amino acid reside. This peptide exhibits immunostimulatory activity more potent than that of LL-37 (induction of IL-8 in keratinocytes and promoting fibroblast proliferation; Braff et al., 2005a), which suggests the interaction between the cathelicidin peptide and the host cellular membrane or intracellular targets may be complex, perhaps involving receptors that exhibit chiral discrimination and recognition.

There are only a few studies that show peptides with anti-biofilm activity against *P. aeruginosa*. We and others have demonstrated the anti-biofilm activity of synthetic peptide mimics of AMPs against *Staphylococcus aureus*, and other pathogens (Beckloff et al., 2007; Pollard et al., 2009; Amer et al., 2010; Dean et al., 2011; Leszczynska et al., 2011). *In vivo*, a 24-residue LL-37-derived AMP was shown to degrade established *P. aeruginosa* biofilm in an animals' nasopharynx, however the significant effect required 2.5 mg/ml of peptide, a high concentration for therapeutic use (Chennupati et al., 2009). Overhage et al. (2008) demonstrated that $4\,\mu g/ml$ LL-37 was able to reduce pre-formed *Pseudomonas* biofilm formation *in vitro*, and that LL-37 treatment affected gene expression of many biofilm-related genes in *P. aeruginosa* PAO1, including the significant downregulation of *rhlA* and *rhlB* expression.

A recently identified helical cathelicidin from the elapid snake *Bungarus fasciatus* (BF) was found to be antimicrobial against *P. aeruginosa* (MIC of $1.2\,\mu g/ml$; Wang et al., 2008). A related cathelicidin peptide that has shown to be a potent anti-microbial against many bacteria was discovered in the elapid snake *Naja atra* (NA), the Chinese Cobra, but has not been previously tested against *P. aeruginosa*. We previously observed that the 39 residue amino acid sequence of the NA cathelicidin (NA-CATH) contains an imperfect, repeated 11 residue motif (ATRA), larger than what was first described by Zhao et al. (Zhao et al., 2008; Amer et al., 2010; de Latour et al., 2010). We previously designed and synthesized a peptide called NA-CATH:ATRA1-ATRA1 to explore the significance of the conserved residues within the ATRA motif for anti-microbial and anti-biofilm activity against *Staphylococcus aureus* (Dean et al., 2011). In this study, we test the ATRA family of peptides against *Pseudomonas*. In addition, we have synthesized the D-form of LL-37 as a peptide mimetic to address the protease-sensitivity concern stated below.

Although AMPs are promising for potential clinical use, and their *in vivo* abilities to combat *in vivo* bacterial infections have been demonstrated (Wilson et al., 1999; Nizet et al., 2001; Moser et al., 2002; Yasin et al., 2004; Lopez-Leban et al., 2010) there are some challenges that have prevented their widespread use. First, mammalian peptides, such as the human cathelicidin LL-37, are commonly sensitive to high salt concentrations (Goldman et al., 1997; Johansson et al., 1998). Second, the feasibility of the use of AMPs in the presence of bacterial and host proteases is a considerable challenge. It was found that *Pseudomonas* biofilms continuously exposed to ciprofloxacin continuously secreted proteases over a period of 4 days (Braff et al., 2005b; Oldak and Trafny, 2005), thus potentially limiting the utility of AMPs. A significant improvement would be to design a protease-resistant peptide, such as D-LL-37, if the D-peptide has the same biological effect as the L-peptide.

Thus, this study will examine the ability of a family of cathelicidins and related small peptides to exert anti-microbial and anti-biofilm effects against *P. aeruginosa*. We will also examine the activity of both the D and L chiral enantiomers of several of the peptides. Finally, we will examine changes in *rhlA* and *rhlB* gene expression in the presence of D-LL-37, to determine if the chiral enantiomer is acting through the same mechanism as L-LL-37.

MATERIALS AND METHODS

BACTERIAL STRAINS AND MEDIA

Pseudomonas aeruginosa [ATCC 19429 and ATCC 27853 (a type strain blood isolate), American Type Culture Collection, Manassas, VA, USA] were grown in Nutrient Broth (Difco Laboratories, Detroit, MI, USA) at 37°C, 24 h with shaking at 200 rpm. The CFU/ml was determined by dilution plating on Nutrient Agar plates. For the anti-microbial assays, frozen enumerated aliquots were thawed immediately before use.

ANTI-MICROBIAL ASSAYS

The anti-microbial activity of the NA-CATH and NA-CATH:ATRA1-ATRA1 (AAPPTEC, Louisville, KY, USA), the variations on the ATRA peptides (Genscript, Piscataway, NJ, USA), LL-37 (AnaSpec 61302), and D-LL-37 (Lifetein, South Plainfield,

NJ, USA) against *P. aeruginosa* (ATCC 19429) were determined as previously described, with some modification (Han et al., 2008; Papanastasiou et al., 2009). The sequences and net charges of the peptides are shown in **Table 1**.

In a 96-well plate, 1×10^5 CFU per well of bacteria were incubated with different peptide concentrations (in serial dilutions of 1:10 or 1:5 per dilution) in a solution of buffer containing 10 mM sodium phosphate at pH 7.4 (3 h, 37°C). Serial dilutions were then carried out in 1× Dulbecco's PBS and plated in triplicate on Nutrient Agar plates, incubated (37°C, 24 h) and CFUs counted. Bacterial survival at each peptide concentration was calculated as previously described (Amer et al., 2010; de Latour et al., 2010) based on the percentage of colonies in each experimental plate relative to the average number of colonies observed for assay cultures lacking peptide. The peptide concentration required to kill 50% of the *P. aeruginosa* in the anti-microbial assay cultures (EC50) was determined by plotting percent survival as a function of the log of peptide concentration ($\log_{10}$) and fitting the data using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA), using the equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{[(\log EC50 - X) \times \text{Hill slope})]})$$

where Y corresponds to bacterial survival (in percentage) at a given peptide concentration (μg/ml), with X being the logarithm of that concentration. In the equation, "Top" and "Bottom" refers to the upper and lower boundaries, and were constrained to values <100% and >0%, respectively. For graphing purposes, samples that had no peptide were plotted at 10^{-9} μg/ml peptide. EC50 values were determined by fitting the data from the anti-microbial assays to a standard sigmoidal dose–response curve. In **Figure 1**, only the range from −2.5 to 2.5 on the x-axis is shown. Each experiment was repeated at least twice, and a representative experiment is shown, for clarity. 95% confidence intervals (CI) are reported to indicate the error of each EC50 determination.

In order to study the *in vitro* killing kinetics of the LL-37, D-LL-37, and NA-CATH:ATRA1-ATRA1 peptides, cultures of *P. aeruginosa* (ATCC 19429) were incubated with the peptides in 10 mM sodium phosphate. The concentrations used in the assay were: 1.0 μg/ml of LL-37, 1.0 μg/ml of NA-CATH, and 0.73 μg/ml of NA-CATH:ATRA1-ATRA1. The anti-microbial activity of the peptides was determined over a period of 3 h, and plated in triplicate, as previously described (Amer et al., 2010).

CD SPECTROSCOPY

Circular dichroism (CD) spectra of the peptides were collected using Jasco J-815 spectropolarimeter. Samples were allowed to equilibrate for 10 min at 25°C prior to data collection in a 0.1-cm path length cuvette, with a chamber temperature 25°C throughout each scan. Spectra were collected from 190 to 260 nm using 0.2-nm intervals; three scans per sample were averaged. All peptides were analyzed at 250 μg/ml in 10 mM sodium phosphate (pH 7), 50% (v/v) trifluoroethanol (TFE) in 10 mM sodium phosphate (pH 7; Lee et al., 2003).

BIOFILM PRODUCTION

Biofilm production was measured as previously described (Durham-Colleran et al.) with the following modifications. *P. aeruginosa* (ATCC 19429; 1×10^5 CFU) in 200 μl of Trypticase Soy Broth was incubated with peptide at concentrations of 1.0, 0.1, and 0.01 μg/ml for 24 h, 37°C. Optical density of the cultures (OD600 nm) at 24 h was determined prior to staining as a measure of bacterial growth. Six wells were used for each peptide concentration ($n = 6$). Biofilm production was measured using the crystal violet stain technique (Durham-Colleran et al.).

BIOFILM ATTACHMENT ASSAY

The biofilm attachment assay was performed using an overnight culture ($OD_{600} = 1.0$) *P. aeruginosa* (ATCC 19429 and ATCC 27853) in 200 μl of TSB was incubated with either D- or L-LL-37 at 1 μg/ml for 1 h, 37°C. The optical density of the cultures (570 nm) at 1 h was determined prior to staining as a measure of bacterial growth. Twelve wells were used for each peptide concentration ($n = 12$). Biofilm production was measured using the crystal violet stain technique (Durham-Colleran et al.).

PRE-FORMED BIOFILM ASSAY

The pre-formed biofilm assay was performed using *P. aeruginosa* (ATCC 19429 and ATCC 27853; 1×10^5 CFU) in 200 μl of TSB was incubated for 24 h, 37°C. Following the formation of an established biofilm at 24 h, the biofilm was treated with either D- or L-LL-37 at 1 μg/ml and incubated for 24 h, 37°C. The optical density of the cultures (570 nm) at 48 h was determined prior to staining as a measure of bacterial growth. Twelve wells were used for each peptide concentration ($n = 12$). Biofilm production was measured using the crystal violet stain technique (Durham-Colleran et al.).

Table 1 | Peptides used in this study.

Anti-microbial peptide	Sequence	Net charge
NA-CATH	**KRFKKFFKKLK**NSVK**KRAKKFFKKPK**VIGVTFPF	15
NA-CATH:ATRA1-ATRA1	**KRFKKFFKKLK**NSVK**KRFKKFFKKLK**VIGVTFPF	15
ATRA-1	KRFKKFFKKLK-NH2	8
ATRA-2	KRAKKFFKKPK-NH2	8
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	6
D-LL-37	*LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES*	6
mCRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	6
Scrambled LL-37	GLKLRFEFSKIKGEFLKTPEVRFRDIKLKDNRISVQR	6

This table indicates the sequence and charges of the anti-microbial peptides used. The ATRA motif is indicated in BOLD.

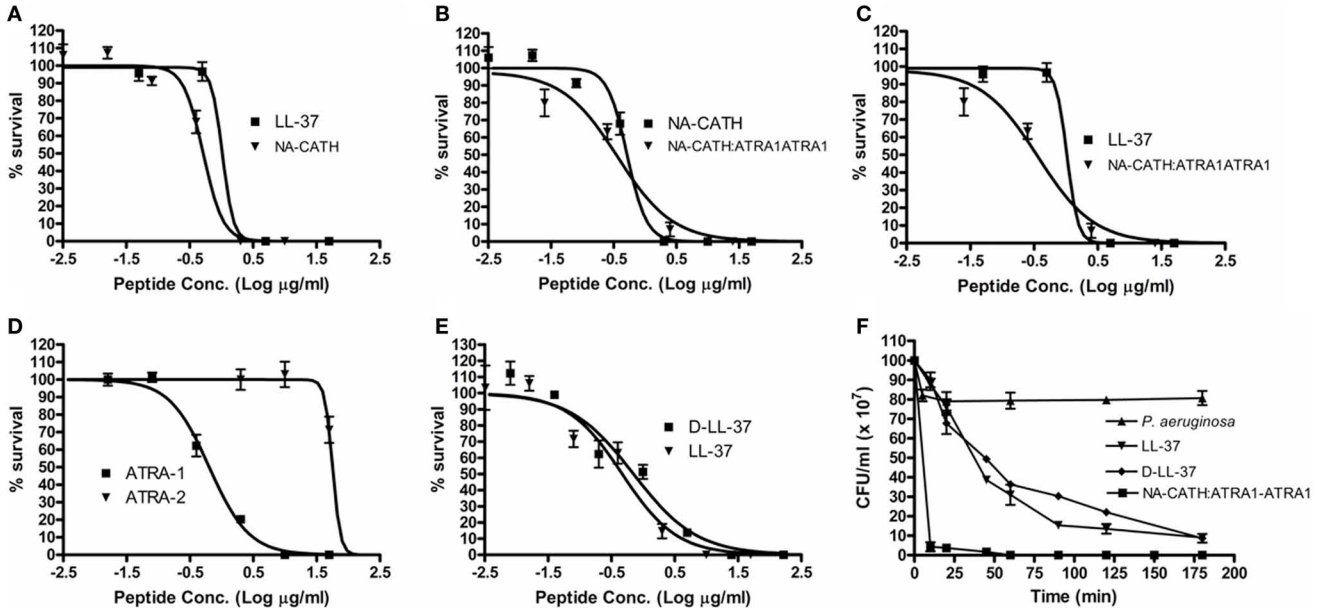

FIGURE 1 | Effectiveness of anti-microbial peptides against *P. aeruginosa*. Percent (%) survival was calculated by counting CFUs, after 3 h incubations with various peptide concentrations in 10 mM sodium phosphate (pH 7.4). **(A)** The EC50s were found to be 0.52 μg/ml for NA-CATH and 0.74 μg/ml for LL-37. **(B)** EC50s were found to be 0.37 μg/ml for NA-CATH:ATRA1-ATRA1 and 0.52 μg/ml for NA-CATH. **(C)** EC50s were found to be 0.37 μg/ml for NA-CATH:ATRA1-ATRA1 and 0.47 μg/ml for LL-37. **(D)** EC50s were found to be 0.64 μg/ml for ATRA-1 and 62.8 μg/ml for ATRA-2. **(E)** EC50s were found to be 0.72 μg/ml for D-LL-37 and 0.47 μg/ml for LL-37. **(F)** The peptides were incubated in 10 mM sodium phosphate with *P. aeruginosa* at the EC50 (0.47 μg/ml for LL-37, 0.72 μg/ml for D-LL-37 and 0.37 μg/ml for NA-CATH:ATRA1-ATRA1). Killing kinetics assays were plated in triplicate time points over 3 h.

MOTILITY ASSAYS

Twitching motility of *P. aeruginosa* (ATCC 19429) was measured after 16, 20, 24, and 48 h of incubation at 37°C on LB plates containing 1% (wt/vol) agar and LB broth (Overhage et al., 2008). *P. aeruginosa* cells were spot inoculated using a needle, and zone diameters were determined by measuring at least three times across the zone. Assays were carried out with 4 μg/ml of either D-LL-37 or L-LL-37, and controls. ($n = 30$).

EXAMINATION OF D- AND L-LL-37 SENSITIVITY TO TRYPSIN

The proteolytic stability of the enantiomers, L- and D-LL-37, was assessed in the presence of trypsin. The peptides (18 μg) were dissolved in 90 μl of water. Then, either water or 0.05% trypsin (10 μl) was added to the peptide solutions, and incubated at 37°C for 1 h. After incubation, 10 μl aliquots were taken into an 18% Tris-Glycine gel. After running the gel, the gel was transferred to a clean tray and a silver stain was performed.

GALLERIA MELLONELLA EXPERIMENT

The *Galleria mellonella* (wax moth caterpillars) were obtained from Vanderhorst Wholesale (Saint Marys, OH, USA). Eight to twelve caterpillars of equal size/weight were randomly assigned to each group. A 1-ml tuberculin syringe was used to inject 10 μl of 1×10^3 CFU/ml of *P. aeruginosa* into each caterpillar's central cavity (hemocoel). The caterpillars were then immediately injected with 10 μl of either PBS, 5 μg of ciprofloxacin, 10 μg D- or L-LL-37, or 10 μg of NA-CATH:ATRA1-ATRA1 in the dorsal side of the caterpillar. Caterpillar control groups included: injected with only PBS, ciprofloxacin, or AMPs. The insects were then observed daily for their survival status.

ANALYSIS OF *rhlA* AND *rhlB* GENE EXPRESSION IN THE PRESENCE OF D- AND L-LL-37 BY qRT-PCR

Quantitative real time RT-PCR analysis was performed in a MyiQ Single Color Real-Time PCR Detection System (BioRad Laboratories). 1×10^3 CFUs of *P. aeruginosa* (ATCC 19429) were incubated in 2 ml of TSB at 37°C for 24 h. In duplicate, from two independent experiments, RNA from biofilm formed in an untreated control, D-LL-37 (1 μg/ml) and LL-37 (1 μg/ml) was isolated (RNAeasy Mini Kit, Qiagen) and reverse transcribed (Super Script™ III Reverse Transcriptase, Invitrogen). The protocol was performed as previously described (Amer et al., 2010).

The primer sequence for *rhlA* 5′-TCTGTTGGTATCGGTTTG CAAGGG-3′ forward and 5′-ACAGCACCACGTTGAAATGTTCG G-3′ reverse; the primer sequence for rhlB 5′-CATCGCTCACGAG AAGTACG-3′, forward and 5′-GTTGAACTTGGGGTGTACCG-3′ were obtained from Invitrogen. The relative levels of transcription was normalized by correcting for rpoD transcription levels.

STATISTICAL ANALYSIS

Anti-microbial assay measurements were performed in triplicates, biofilm assays done at least in duplicate with $n = 6$ each time, and representative experiments are shown. Standard deviations of the mean of each set are represented on each graph. Where the error bars cannot be seen, the error is very small. CI (95%) is presented for EC50 determinations demonstrating the statistical overlap of

the data. Statistically significant differences were assessing using Student's *t* tests.

RESULTS

ANTI-MICROBIAL PERFORMANCE

Effect of chirality: D- vs L-LL-37 against P. aeruginosa

A common concern against the use of AMPs as potential therapeutics is their potential sensitivity to host or bacterial proteases (Braff et al., 2005b). In order to generate a protease-resistant peptide mimetic of the human cathelicidin (Wade et al., 1990), we synthesized an all-D-amino acid version of LL-37. The anti-microbial EC50 for D-LL-37 against *P. aeruginosa* (ATCC 19429) was determined to be 0.72 μg/ml, compared to 0.47 μg/ml for wild-type LL-37 (**Table 2**; **Figure 1E**); there is not a significant difference in anti-microbial activity between them by examining the 95% CIs. Additionally, D- and L-LL-37 display similar killing kinetics over the 3h duration of a killing kinetics assay against *P. aeruginosa* (ATCC 19429) **Figure 1F**.

Small synthetic peptides demonstrate anti-microbial activity against P. aeruginosa

Pseudomonas aeruginosa (ATCC 19429) was also subjected to treatment with two short, synthetic peptides (**Table 1**), ATRA-1 and ATRA-2. These two ATRA peptides differ by two residues at the 3rd (F/A) and 10th (L/P) position. This has been shown to affect the anti-microbial activity of those peptides against *Francisella novicida, Escherichia coli* (Amer et al., 2010), *Aggregatibacter actinomycetemcomitans* (de Latour et al., 2010), and *Staphylococcus aureus* (Dean et al., 2011). The EC50 values of ATRA-1 and ATRA-2 were determined to be statistically different ($p < 0.05$, Student's *t* test) at 0.64 and 62.8 μg/ml, respectively (**Table 2**; **Figure 1D**). These two peptides have the same net charge of +8, highly similar sequence and both are 11 amino acid residues in length.

LL-37 and NA-CATH-derived peptide are anti-microbial against P. aeruginosa

The anti-microbial effectiveness of NA-CATH was tested against *P. aeruginosa* (ATCC 19429), and the performance of this peptide was compared to that of the well-studied cathelicidin LL-37. The EC50 for NA-CATH was found to be 0.52 μg/ml (**Figure 1A**). The peptide NA-CATH:ATRA1-ATRA1 incorporates modifications to the NA-CATH sequence in which the second ATRA motif has been changed to match the sequence of the first ATRA motif (**Table 2**) thus creating a perfect repeat. This synthetic cathelicidin had an EC50 value that was determined to be 0.37 μg/ml, not statistically more effective against *P. aeruginosa* (ATCC 19429) than the NA-CATH parent peptide (**Figure 1B**), or LL-37 (**Figure 1C**). We previously demonstrated that the alterations made to NA-CATH to create NA-CATH:ATRA1-ATRA1 resulted in a statistically significant ($p < 0.05$, Student's *t* test) improvement in anti-microbial activity against *S. aureus* (Dean et al., 2011).

In agreement with reported potencies (Gordon et al., 2005), we found that the EC50 for LL-37 is 0.47 μg/ml. This is within the range of the level of LL-37 reported in human plasma (1.18 μg/ml; Sorensen et al., 1997), suggesting that this is a physiologically relevant potency of LL-37.

INHIBITION OF ATTACHMENT AND BIOFILM FORMATION, AND DEGRADATION OF ESTABLISHED *P. AERUGINOSA* BIOFILM AT SUB-ANTI-MICROBIAL CONCENTRATIONS

A concern of the utility of AMPs as potential therapeutics is the sensitivity of the anti-microbial activity to salt. Multiple studies have shown that LL-37 demonstrates reduced anti-microbial action in environments with high ionic concentrations (Travis et al., 2000; Cox et al., 2003) such as in physiologic salt concentration (123–150 mM NaCl). However, LL-37 can inhibit biofilm formation by *P. aeruginosa* PAO1 (Overhage et al., 2008), *S. epidermidis* (Hell et al., 2010), *F. novicida* (Amer et al., 2010), and *S. aureus* in media with high concentrations of salt. LL-37 is reported to be capable of degrading pre-formed biofilms of *S. aureus* and *P. aeruginosa* (Altman et al., 2006; Overhage et al., 2008).

In this study, we demonstrate similar salt-independent anti-biofilm activity for D-LL-37 against *P. aeruginosa*. We incubated various concentrations of NA-CATH, NA-CATH:ATRA1-ATRA1, LL-37, D-LL-37, mCRAMP, and scrambled LL-37 with *P. aeruginosa* in biofilm experiments in TSB (relatively high salt; 86 mM NaCl) for 24 h. Both mCRAMP, a murine-homolog of LL-37 (67% identical to LL-37 in amino acid sequence), and scrambled LL-37 (identical amino acids in random order) functioned as negative controls for this experiment; the former was previously known to have no inhibitory effect (Overhage et al., 2008). The scrambled version of LL-37, having the same charge and net amino acid composition as LL-37, but lacking significant helical character, showed no inhibition of biofilm formation at any concentration

Table 2 | EC50s of AMPs against *P. aeruginosa*.

Anti-microbial peptide	Molecular weight (g/mol)	EC50 (μg/ml)	95% CI	EC50 (μM)
NA-CATH	5885.5	0.52	0.39–0.69	0.09
NA-CATH:ATRA1-ATRA1	5977.6	0.37	0.21–0.63	0.06
ATRA-1	2409.06	0.64	0.52–0.79	0.27
ATRA-2	2316.96	62.8	27.7–949	27.1
LL-37	5177.42	0.47	0.28–0.78	0.09
D-LL-37	5177.42	0.72	0.37–1.36	0.14

This table indicates the EC50 of the peptides against P. aeruginosa in an anti-microbial assay. The molecular weight reported here for each peptide reflects the TFA salts of the peptides. These molecular weights were then used to convert the EC50 in μg/ml to μM, to enable comparisons on a molecule-by-molecule basis.

tested (**Figure 2B**), thus demonstrating sequence specificity of the anti-biofilm effect.

Figures 2A–C shows that levels of bacterial growth (OD600 nm at 24 h) were not decreased even at the peptide concentrations equal to that of its calculated EC50 in 10 mM sodium phosphate. We could not calculate the MIC because there was no inhibition of growth at any concentration tested, thus MIC > 1 μg/ml. When the biofilm production was determined in the presence of varying amounts of peptide, there was significant inhibition of biofilm formation by L- and D-LL-37; all other peptides were

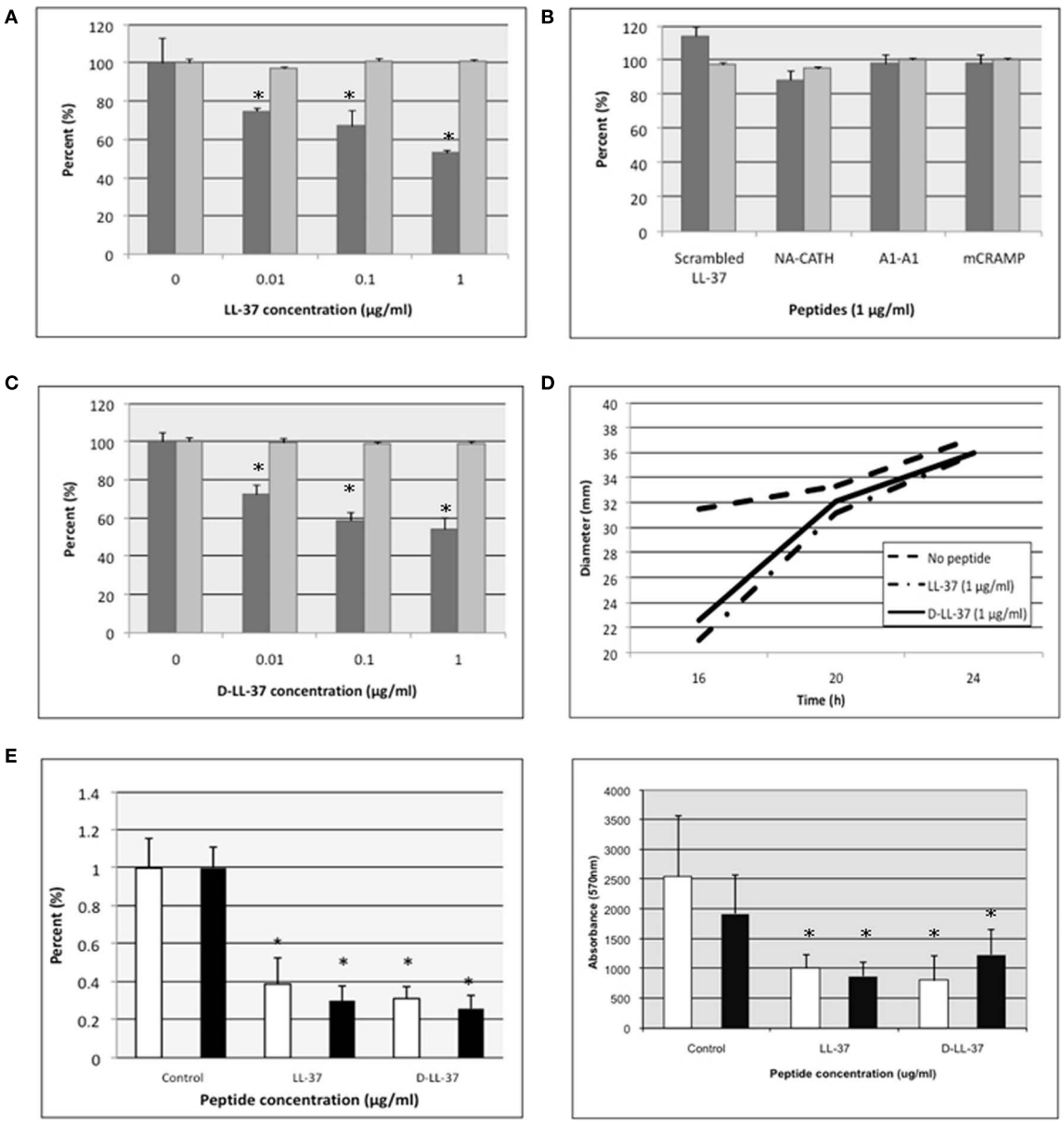

FIGURE 2 | D- and L-LL-37 inhibit biofilm formation and attachment, stimulate twitching motility, and degrade biofilm of *P. aeruginosa*. (A–C) Inhibition of *P. aeruginosa* biofilm formation was demonstrated for D- and L-LL-37 **(A,C)**, while scrambled LL-37, NA-CATH, NA-CATH:ATRA1-ATRA1 (indicated by A1-A1) and mCRAMP all showed a lack of anti-biofilm activity **(B)**. Growth (absorbance at 600 nm) is indicated by light gray bars with "0 peptide" control set to 100%. Biofilm detection on a polystyrene 96-well plate at 37°C after 24 h of growth in TSB was detected as the absorbance of crystal violet stain (570 nm). Percent biofilm production is indicated by dark gray bars ($n = 6$), relative to "0 peptide" control. Each experiment is a representative of at least two independent trials. Error bars indicate the standard deviation from the mean. The asterisk (*) indicates statistically different than the positive control ($p < 0.01$). **(D)** D- and L-LL-37 increased the rate of twitching motility in *P. aeruginosa*. Motility was measured after 16, 20, and 24 h of incubation at 37°C on plates containing 1% (wt/vol) agar and LB broth. Plates were inoculated using a needle, and diameters were recorded. Assays were carried out with 4 μg/ml of either D-LL-37 or L-LL-37, and controls. ($n = 30$). **(E)** Attachment assays were performed in the presence of 1 μg/ml of D- or L-LL-37; ATCC 19429 is indicated by white columns, ATCC 27853 is represented by black columns. **(F)** Pre-formed biofilm assays were performed in the presence of 1 μg/ml D- or L-LL-37; ATCC 19429 is indicated by white columns, ATCC 27853 is represented by black columns.

ineffective. Thus, LL-37 was found to inhibit biofilm formation up to ∼50% of control at 1 μg/ml (**Figure 2C**). D-LL-37 was also found to be an active anti-biofilm peptide, with maximal biofilm inhibition observed at 1 μg/ml, inhibiting ∼50% of biofilm formation (**Figure 2B**).

In addition to showing that D- and L-LL-37 can inhibit the formation of *P. aeruginosa* (ATCC 19429), we determined that D- and L-LL-37 significantly reduces attachment at 1 μg/ml (**Figure 2E**) after 1 h for *P. aeruginosa* (ATCC 19429 and ATCC 27853). Suggesting a possible mechanism for biofilm inhibition. The amount of inhibition of attachment (∼15–22% for LL-37, and ∼39% for D-LL-37) may not account for the full ability of the peptides to inhibit biofilm formation at the same concentration, but may contribute to this effect.

In order to determine the ability of D- and L-LL-37 to degrade pre-formed biofilm of *P. aeruginosa* (ATCC 19429 and ATCC 27853), the bacteria were allowed to establish biofilm for 24 h, then the wells were treated with either D- or L-LL-37 (1 μg/ml). After incubation with peptide, biofilm production was quantified with the crystal violet stain method (Durham-Colleran et al.), and significant degradation of established *P. aeruginosa* biofilm was observed (**Figure 2F**).

D- AND L-LL-37 STIMULATES TWITCHING MOTILITY

Twitching motility is required for the formation of *Pseudomonas* biofilm (O'Toole and Kolter, 1998). To assess the ability of D-LL-37 and L-LL-37 to stimulate twitching motility of *P. aeruginosa* (ATCC 19429), we tested the bacteria in the presence of these peptides. Both D- and L-LL-37 stimulated the rate of twitching motility (**Figure 2D**) at low concentrations of peptide (4 μg/ml). This significant ($p < 0.01$, Student's t test) increase in the rate of twitching on surface motility was 1.7 and 1.9 mm/h on LB (1% agarose) plates, for D- and L-LL-37, respectively. *P. aeruginosa* in the absence of peptide, was determined to have a twitching motility rate of 0.7 mm/h. LL-37 has been shown to have an insignificant effect on the swimming and swarming motility of *P. aeruginosa* PAO1 (Overhage et al., 2008); neither D- nor L-LL-37 appeared to effect swimming and swarming motility of *P. aeruginosa* (ATCC 19429) in this study (data not shown).

RHAMNOSYLTRANSFERASES ARE DOWNREGULATED IN THE PRESENCE OF D- AND L-LL-37

qRT-PCR was used to determine the total amount of *rhlA* and *rhlB* mRNA following treatment by of 1 μg/ml of either D- or L-LL-37. We determined that both peptides significantly downregulated the expression of these genes involved in the Rhl quorum sensing system in *P. aeruginosa* (ATCC 19429; **Table 3**). D-LL-37 had a −7.2 and −3.0 fold change in regulation of *rhlA* and *rhlB*, respectively, relative to the control.

CD SPECTRAL ANALYSIS OF THE SECONDARY STRUCTURE OF D- AND L-LL-37

Circular dichroism spectra of the peptides were obtained for D- and L-LL-37. Pronounced dichroic minima at 222 and 208 nm are traits of helical peptides. Cathelcidins often exhibit little helical behavior at low concentrations in aqueous buffer (Johansson et al., 1998), assuming their most helical structure when in association with a biological membrane or a hydrophobic membrane mimic such as TFE, a strongly helix-promoting environment. The stable helical character when interacting with bacterial membranes may contribute to their potent anti-microbial activity (Park et al., 2004).

The spectra for L- and D-LL-37 in 10 mM sodium phosphate (pH 7.4) exhibit helical character, which is consistent with the results reported by Johansson et al. (1998). As was expected, the spectrum for D-LL-37 is the mirror image of that of the L-enantiomer (**Figure 3A**). Under these conditions, the conformation of L-LL-37 is estimated to have ∼28% alpha-helical content and that of D-LL-37 has ∼19% helical content. The spectra for both D- and L-LL-37 in 50% TFE (a membrane mimic) in 10 mM sodium phosphate (pH 7.4) show significantly greater helical character, ∼48% and ∼58%, respectively. The helical content of these peptides was estimated based on their respective ellipticities at 208 nm using the method reported by Greenfield and Fasman (1969).

D-LL-37 IS RESISTANT TO TRYPSIN

Figure 3B shows the stability of the enantiomeric pair of peptides, D- and L-LL-37, in the presence of 0.05% trypsin, after incubation for 1 h at 37°C. The L-form peptide, LL-37 at 200 μg/ml, was 100% digested by the protease after the 1-h incubation. D-LL-37 at 200 μg/ml showed no degradation in presence of trypsin with equal band intensities as quantified by densitometry (NIH ImageJ, data not shown).

GALLERIA MELLONELLA INFECTION BY *P. AERUGINOSA* WITH AMP AND ANTIBIOTIC TREATMENTS

The wax moth caterpillar *Galleria mellonella* infected with *P. aeruginosa* was used as a model *in vivo* system (Jander et al., 2000) to study the effectiveness of treatment with selected AMPs.

Table 3 | *Pseudomonas aeruginosa* genes downregulated in the presence of D- or L-LL-37.

Treatment	Identification	Protein	Designation	Fold change	*p*-value
L-LL-37 (1 μg/ml)	PA3478	Rhamnosyltransferase chain B	*rhlB*	−8.4	2.73E-02
L-LL-37 (1 μg/ml)	PA3479	Rhamnosyltransferase chain A	*rhlA*	−4.5	6.36E-03
D-LL-37 (1 μg/ml)	PA3478	Rhamnosyltransferase chain B	*rhlB*	−7.2	6.50E-04
D-LL-37 (1 μg/ml)	PA3479	Rhamnosyltransferase chain A	*rhlA*	−3.0	4.11E-06

This table indicates the fold change (relative to the control) in regulation of P. aeruginosa genes rhlA and rhlB in the presence of 1 μg/ml of either D- or L-LL-37 in biofilm. The relative levels of transcription was normalized by correcting for rpoD transcription levels.

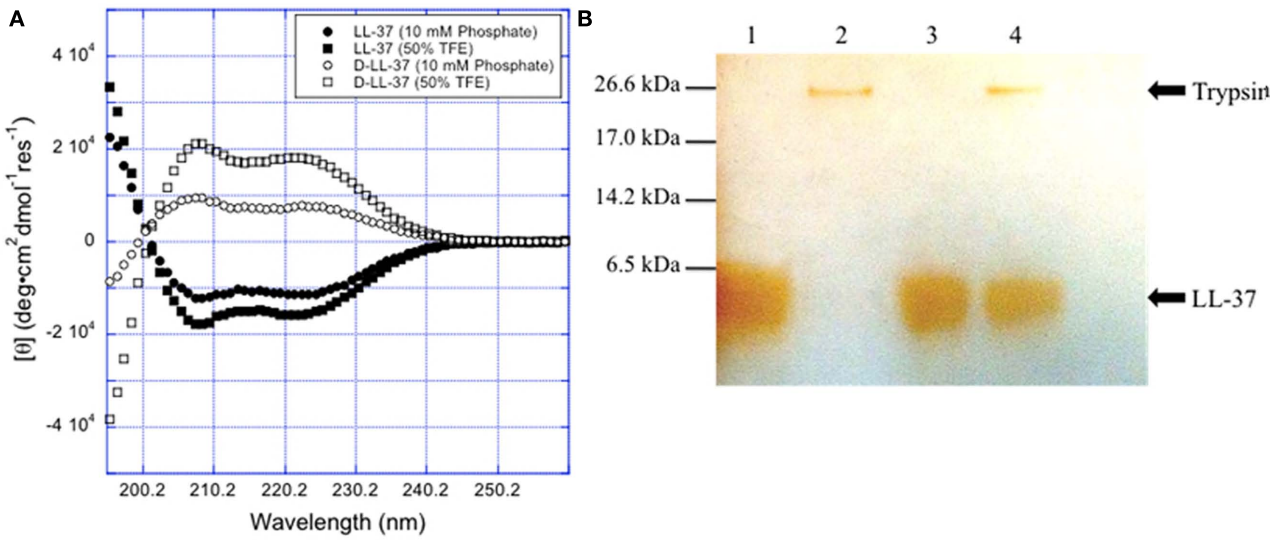

FIGURE 3 | Chirality affects D-LL-37 susceptibility to proteases. (A) The spectra for L- and D-LL-37 (125 μM) exhibit significant helical character in pH = 7.4 10 mM sodium phosphate. As expected, the spectrum for D-LL-37 (●) is the mirror image of that of the L-peptide (○). The spectra for both D- and L-LL-37 (≤ and, ′ respectively) become more intense when the peptides are in 50% TFE in pH 7.4 10 mM phosphate, consistent with the peptides exhibiting more helical character. These results are consistent with what has been reported in the literature for these peptides. **(B)** D-LL-37 demonstrated resistance degradation by trypsin. Peptides (18 μg) were dissolved in water (90 μg) with either water or 0.05% trypsin (10 μl) and incubated (37°C, 1 h). 10 μl aliquots were then taken, and a silver stain was performed. Lane 1, LL-37; Lane 2, LL-37 with trypsin; Lane 3, D-LL-37; Lane 4, D-LL-37 with trypsin.

G. mellonella were infected with either 1×10^3 CFU bacteria of *P. aeruginosa* (ATCC 19429) and immediately treated with a single dosing of 10 μl PBS (no treatment control), 5 μg ciprofloxacin, 10 μg LL-37, 10 μg D-LL-37, or 10 μg NA-CATH:ATRA1-ATRA1 (data not shown; **Figure 4**). Multiple controls were utilized (PBS, and injections of non-infected *G. mellonella* with the treatment used in the experiment) in order to measure the effects that the injection or the anti-microbials have on the host system. These negative controls had similar effects; no death was observed (data not shown). In the positive contol, no *P. aeruginosa*-infected *G. mellonella* survived beyond 24 h. The ciprofloxacin (60%) and D-LL-37 (60%) treated groups demonstrated similarly prolonged survival (p-value < 0.01) and were not statistically different from each other overall. D-LL-37 was shown to be significantly more effective than LL-37 (p-value < 0.01) over the duration the experiment. NA-CATH:ATRA1-ATRA1 treatment did not improve survival, with all *G. mellonella* dead by 24 h (not shown). There was no significant difference in the survival rate between D- and L-LL-37.

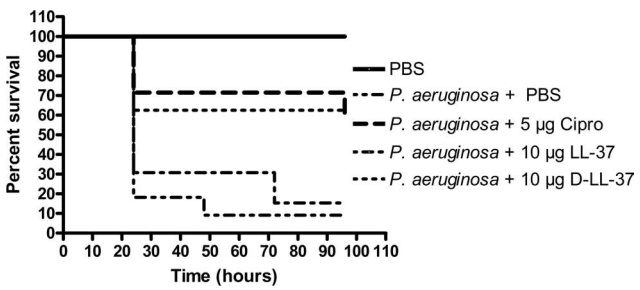

FIGURE 4 | Treatment of *P. aeruginosa*-infected *G. mellonella*. D- and L-LL-37 significantly prolonged the survival of *G. mellonella* infected with 1 × 10³ CFU *P. aeruginosa*. Non-infected control groups consisted of a PBS injection, 5 μg Ciprofloxacin injection, 10 μg LL-37 injection, or a 10 μg D-LL-37 injection. All non-infected groups fair similarly to the PBS only group (data not shown). The non-infected PBS only group experienced the highest survival rate and was significantly different from all other groups (p-value < 0.002). The infected group without treatment failed to survive beyond 24 h. A single dose of 5 μg ciprofloxacin, or 10 μg of either D- or L-LL-37 was found effective when compared to the infected control group (p-value < 0.01), but not different from each other overall. At 48 h D-LL-37 was found to be more effective than L-LL-37 (p-value < 0.04) NA-CATH:ATRA1-ATRA1 (10 (μg), also tested, was determined to be ineffective at prolonging survival, as this group failed to survive past 24 h (data not shown).

DISCUSSION

In this study, we present novel peptides that address some of the challenges that have prevented the widespread use of AMPs. Among these concerns are the noted sensitivity of their anti-microbial activity to high salt concentrations (Goldman et al., 1997; Johansson et al., 1998) and the sensitivity of AMPs to bacterial and host secretions of proteases (Braff et al., 2005b). We describe the anti-microbial and anti-biofilm activity of these peptides.

We were able to demonstrate that LL-37 and its D-enantiomer, D-LL-37, NA-CATH, and NA-CATH-derived peptides have strong anti-microbial activity against the Gram-negative opportunistic pathogen, *Pseudomonas aeruginosa.* The effective peptides' EC50 values, when converted from μg/ml to μM to reflect the number of molecules of peptide and to accommodate the different molecular weights of the peptides, ranged from 0.06 to 0.27 μM. The lowest EC50 belonged to NA-CATH:ATRA1-ATRA1, consistent with our prior observations for other bacteria (Dean et al., 2011).

Based on earlier work (Amer et al., 2010; de Latour et al., 2010), we determined that when using SDS is as a model system for studying the structural properties of AMPs and their interaction with negatively charged membranes (Tack et al., 2002; Wang, 2008), that the degree of helical propensity had a significant correlation with anti-microbial activity. However, NA-CATH:ATRA1-ATRA1 and NA-CATH were ineffective in inhibiting biofilm formation despite being highly effective AMPs, and highly helical.

We were able to determine that both L- and D-LL-37 were able to inhibit the development of biofilms in two ways: through stimulatory activity in twitching motility assays, as well as anti-biofilm activity in anti-biofilm assays. In confirmation with a previous study (Overhage et al., 2008), we demonstrated that LL-37 promotes twitching in *P. aeruginosa*. Twitching is a form of surface motility that is regulated by type IV pili (Whitchurch et al., 1991). LL-37 accomplished this increase in twitching by up-regulating genes related to this form of motility (Overhage et al., 2008), which in turn causes the bacteria to move instead of forming biofilm. This action has been shown to significantly lower the net mass of *P. aeruginosa* biofilm (Singh et al., 2002; Pioreanu et al., 2007). We have also demonstrated the equivalently stimulatory activity of D-LL-37 at increasing the rate of *Pseudomonas* surface motility. Thus, we believe that the ability of the peptides to stimulate twitching motility and thus inhibit biofilm formation in *Pseudomonas* is independent of peptide chirality.

Considering that the formation of biofilm protects bacteria during infections, such as in chronic wound and hospital-related pneumonia (Costerton et al., 1999; Prince, 2002; Dieter, 2004; Kaneko et al., 2007), the inhibition of biofilm formation by these AMPs is a critically important quality as a potential therapeutic, possibly more so than their anti-microbial activity. In our anti-biofilm assays, we were able to demonstrate that both L-LL-37 and D-LL-37 are capable of inhibiting *P. aeruginosa* biofilm formation to similar extents (∼50%, **Figures 2A,C**), this result compares well to other studies (Overhage et al., 2008). In addition, D-LL-37 downregulated *rhlA* and *rhlB* gene expression to a similar extent as reported for L-LL-37 (Overhage et al., 2008), suggesting that they may share a conserved mechanism of action.

We have previously shown that LL-37, D-LL-37, NA-CATH:ATRA1-ATRA1, NA-CATH, and ATRA at concentration of 100 μg/ml elicited no significant hemolysis against red blood cells (de Latour et al., 2010; Dean et al., 2011), suggesting that these peptides have very low host-cell cytotoxic effects (Ryadnov et al., 2002).

To address the effectiveness of AMPs to thwart *in vivo* infection, we studied the abilities of select peptides in comparison to treatment with an established antibiotic in the wax moth caterpillar model system *Galleria mellonella*. *G. mellonella* has been shown to be a good model for the determination of the effects of virulence during infection by *P. aeruginosa* when compared to the mouse model (Jander et al., 2000). In this experiment, we determined that *Pseudomonas*-infected *G. mellonella,* when treated with ciprofloxacin, L-LL-37, and D-LL-37, have a significantly prolonged survival time when compared to the no-treatment control group. Further, D-LL-37 displays significant effectiveness when compared to the L-LL-37 and ciprofloxacin-treatment groups at 48 h (**Figure 4**). One interesting result is in the NA-CATH:ATRA1-ATRA1-treatment group, when all of the caterpillars were dead at 24 h, despite the peptide exhibiting strong *in vitro* anti-microbial activity. While NA-CATH:ATRA1-ATRA1 was determined to be the most effective AMP in our sodium phosphate study, it did not display anti-biofilm activity against *P. aeruginosa*. From this result, we suggest that the inability of NA-CATH:ATRA1-ATRA1 to prolong survival of *G. mellonella* could be attributed to the lack of anti-biofilm activity, thus exemplifying the importance of this anti-biofilm property for the potential AMP's future therapeutic applications.

Finally, we addressed the sensitivity of AMPs to proteolytic degradation. It has been shown for mammalian hosts and several bacteria, including *P. aeruginosa* and *S. aureus,* that they express proteases capable of degrading and inactivating LL-37 (Sieprawska-Lupa et al., 2004). In addition, other peptides that are capable of preventing the formation of biofilm, such as lactoferrin (Singh et al., 2002), are also degraded and inactivated in the presence of bacterial and host proteases. In our study, we have determined that the protease trypsin does not degrade D-LL-37 (**Figure 3**) while L-LL-37 is completely degraded and therefore experiences a loss of function. Protease resistance and the CD spectra are the two main aspects in which the chirality of the peptide appears to be critical. In all other aspects measured in this study, they are equivalent. This property of protease resistance may allow D-LL-37 peptide to remain active when applied to a wound despite protease production and therefore may be able to inhibit biofilm formation *in vivo*. We will test this hypothesis in future studies.

Novel treatments for chronic wounds, pneumonia, and medical implant-associated infections are critically needed. These infections are often characterized by polymicrobial infections by biofilm-forming bacteria, including *P. aeruginosa* (James et al., 2008, Murray, 2008). Desired characteristics of a novel therapeutic for these wounds would include a broad-spectrum, anti-biofilm treatment that is capable of withstanding the host environment, including protease secretions. We previously demonstrated the effectiveness of D-LL-37 to inhibit biofilm formation of *S. aureus*, another pathogen commonly found in infected wounds (Dean et al., 2011). In this work, we explore the ability of an enantiomer of a naturally occurring AMP to inhibit biofilm formation and treat infection by *P. aeruginosa*. D-LL-37 represents a potential therapeutic candidate by being a protease-resistant peptide that is effective in inhibiting biofilm formation, increasing the rate of twitching motility, and possesses potentially wound-healing properties toward the host (Pioreanu et al., 2007), while increasing the survival time of infected wax-moth caterpillars. For example, such a peptide may have potential to be developed as topical treatments for biofilm-forming bacteria in skin wounds, such as *P. aeruginosa*. It may be more appropriate to refer to these peptides as anti-biofilm peptides rather than AMPs, reflecting the current understanding of the role of biofilms in infection. Incorporation of anti-biofilm peptides or their synthetic derivatives in therapeutic topical applications may improve outcomes for infections ranging from chronic wounds, burns, implanted medical devices and pneumonia.

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REFERENCES

Altman, H., Steinberg, D., Porat, Y., Mor, A., Fridman, D., Friedman, M., and Bachrach, G. (2006). In vitro assessment of antimicrobial peptides as potential agents against several oral bacteria. *J. Antimicrob. Chemother.* 58, 198–201.

Amer, L. S., Bishop, B. M., and van Hoek, M. L. (2010). Antimicrobial and antibiofilm activity of cathelicidins and short, synthetic peptides against Francisella. *Biochem. Biophys. Res. Commun.* 396, 246–251.

Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E. P., and Høiby N. (2004). *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. *Antimicrob. Agents Chemother.* 48, 1175–1187.

Bals, R., Wang, X., Zasloff, M., and Wilson, J. M. (1998). The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9541–9546.

Beckloff, N., Laube, D., Castro, T., Furgang, D., Park, S., Perlin, D., Clements, D., Tang, H., Scott, R. W., Tew, G. N., and Diamond, G. (2007). Activity of an antimicrobial peptide mimetic against planktonic and biofilm cultures of oral pathogens. *Antimicrob. Agents Chemother.* 51, 4125–4132.

Bone, R. C. (1993). Gram-negative sepsis: a dilemma of modern medicine. *Clin. Microbiol. Rev.* 6, 57–68.

Braff, M. H., Hawkins, M. A., Di Nardo, A., Lopez-Garcia, B., Howell, M. D., Wong, C., Lin, K., Streib, J. E., Dorschner, R., Leung, D. Y. M., and Gallo, R. L. (2005a). Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities. *J. Immunol.* 174, 4271–4278.

Braff, M. H., Zaiou, M., Fierer, J., Nizet, V., and Gallo, R. L. (2005b). Keratinocyte production of cathelicidin provides direct activity against bacterial skin pathogens. *Infect. Immun.* 73, 6771–6781.

Bucki, R., Pastore, J. J., Randhawa, P., Vegners, R., Weiner, D. J., and Janmey, P. A. (2004). Antibacterial activities of rhodamine B-conjugated gelsolin-derived peptides compared to those of the antimicrobial peptides cathelicidin LL37, magainin II, and melittin. *Antimicrob. Agents Chemother.* 48, 1526–1533.

Chennupati, S. K., Chiu, A. G., Tamashiro, E., Banks, C. A., Cohen, M. B., Bleier, B. S., Kofonow, J. M., Tam, E., and Cohen, N. A. (2009). Effects of an LL-37-derived antimicrobial peptide in an animal model of biofilm *Pseudomonas sinusitis*. *Am. J. Rhinol. Allergy* 23, 46–51.

Costerton, J. W., Stewart, P. S., and Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318–1322.

Cox, D. L., Sun, Y., Liu, H., Lehrer, R. I., and Shafer, W. M. (2003). Susceptibility of *Treponema pallidum* to host-derived antimicrobial peptides. *Peptides* 24, 1741–1746.

Dean, S. N., Bishop, B. M., and van Hoek, M. L. (2011). Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against *Staphyloccus aureus*. *BMC Microbiol.* 11, 114. PMID: 21605457. [Epub ahead of print].

de Latour, F. A., Amer, L. S., Papanstasiou, E. A., Bishop, B. M., and van Hoek, M. L. (2010). Antimicrobial activity of the *Naja atra* cathelicidin and related small peptides. *Biochem. Biophys. Res. Commun.* 396, 825–830.

Dieter, R. S. (2004). Coronary artery stent infection. *Catheter. Cardiovasc. Interv.* 62, 281.

Donlan, R. M., and Costerton J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167–193.

Drenkard, E., and Ausubel F. M. (2002). Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 416, 740–743.

Durham-Colleran, M. W., Verhoeven, A. B., and van Hoek, M. L. (2010). *Francisella novicida* forms in vitro biofilms mediated by an orphan response regulator. *Microb. Ecol.* 59, 457–465.

Ganz, T., and Weiss J. (1997). Antimicrobial peptides of phagocytes and epithelia. *Semin. Hematol.* 34, 343–354.

Gennaro, R., Scocchi, M., Merluzzi, L., and Zanetti, M. (1998). Biological characterization of a novel mammalian antimicrobial peptide. *Biochim. Biophys. Acta* 1425, 361–368.

Goldman, M. J., Anderson, G. M., Stolzenberg, E. D., Kari, U. P., Zasloff, M., and Wilson, J. M. (1997). Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* 88, 553–560.

Gordon, Y. J., Huang, L. C., Romanowski, E. G., Yates, K. A., Proske, R. J., and McDermott AM. (2005). Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Curr. Eye Res.* 30, 385–394.

Greenfield, N., and Fasman G. D. (1969). Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* 8, 4108–4116.

Han, S., Bishop, B. M., and van Hoek, M. L. (2008). Antimicrobial activity of human beta-defensins and induction by Francisella. *Biochem. Biophys. Res. Commun.* 371, 670–674.

Hancock, R. E., and Speert D. P. (2000). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resist. Updat.* 3, 247–255.

Hell, E., Giske, C. G., Nelson, A., Römling, U., and Marchini, G. (2010). Human cathelicidin peptide LL37 inhibits both attachment capability and biofilm formation of *Staphylococcus epidermidis*. *Lett. Appl. Microbiol.* 50, 211–215.

Heyland, D. K., Cook, D. J., Griffith, L., Keenan, S. P., and Brun-Buisson, C. (1999). The attributable morbidity and mortality of ventilator-associated pneumonia in the critically ill patient. The Canadian Critical Trials Group. *Am. J. Respir. Crit. Care Med.* 159(Pt 1), 1249–1256.

Hoiby, N., Krogh Johansen, H., Moser, C., Song, Z., Ciofu, O., and Kharazmi, A. (2001). *Pseudomonas aeruginosa* and the in vitro and in vivo biofilm mode of growth. *Microbes Infect.* 3, 23–35.

James, G. A., Swogger, E., Wolcott, R., Pulcini, E., Secor, P., Sestrich, J., Costerton, J. W., and Stewart, P. S. (2008). Biofilms in chronic wounds. *Wound Repair Regen.* 16, 37–44.

Jander, G., Rahme, L. G., and Ausubel, F. M. (2000). Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J. Bacteriol.* 182, 3843–3845.

Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D., and Agerberth, B. (1998). Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J. Biol. Chem.* 273, 3718–3724.

Kaneko, Y., Thoendel, M., Olakanmi, O., Britigan, B. E., and Singh, P. K. (2007). The transition metal gallium disrupts *Pseudomonas aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. *J. Clin. Invest.* 117, 877–888.

Lee, K. H., Shin, S. Y., Hong, J. E., Yang, S. T., Kim, J. I., Hahm, K. S., and Kim, Y. (2003). Solution structure of termite-derived antimicrobial peptide, spinigerin, as determined in SDS micelle by NMR spectroscopy. *Biochem. Biophys. Res. Commun.* 309, 591–597.

Leszczynska, K., Namiot, A., Cruz, K., Byfield, F. J., Won, E., Mendez, G., Sokolowski, W., Savage, P. B., Bucki, R., and Janmey, P. A. (2011). Potential of ceragenin CSA-13 and its mixture with pluronic F-127 as treatment of topical bacterial infections. *J. Appl. Microbiol.* 110, 229–238.

Lopez-Leban, F., Kiran, M. D., Wolcott, R., and Balaban, N. (2010). Molecular mechanisms of RIP, an effective inhibitor of chronic infections. *Int. J. Artif. Organs* 33, 582–589.

Maher, S., and McClean S. (2006). Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro. *Biochem. Pharmacol.* 71, 1289–1298.

May, T., Ito, A., and Okabe, S. (2009). Induction of multidrug resistance mechanism in *Escherichia coli* biofilms by interplay between tetracycline and ampicillin resistance genes. *Antimicrob. Agents Chemother.* 53, 4628–4639.

Moser, C., Weiner, D. J., Lysenko, E., Bals, R., Weiser, J. N., and Wilson, J. M. (2002). beta-Defensin 1 contributes to pulmonary innate immunity in mice. *Infect. Immun.* 70, 3068–3072.

Murray, C. K. (2008). Infectious disease complications of combat-related injuries. *Crit. Care Med.* 36(7 Suppl), S358–S364.

Nizet, V., Ohtake, T., Lauth, X., Trowbridge, J., Rudisill, J., Dorschner, R. A., Pestonjamasp, V., Piraino, J., Huttner, K., and Gallo, R. L. (2001). Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414, 454–457.

Oldak, E., and Trafny E. A. (2005). Secretion of proteases by *Pseudomonas aeruginosa* biofilms exposed to ciprofloxacin. *Antimicrob. Agents Chemother.* 49, 3281–3288.

O'Toole, G. A., and Kolter R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* 30, 295–304.

Ouhara, K., Komatsuzawa, H., Kawai, T., Nishi, H., Fujiwara, T., Fujiue, Y., Kuwabara, M., Sayama, K., Hashimoto, K., and Sugai, M. (2008). Increased resistance to cationic antimicrobial peptide LL-37 in methicillin-resistant strains of *Staphylococcus aureus. J. Antimicrob. Chemother.* 61, 1266–1269.

Overhage, J., Campisano, A., Bains, M., Torfs, E. C., Rehm, B. H., and Hancock, R. E. (2008). Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect. Immun.* 76, 4176–4182.

Papanastasiou, E. A., Hua, Q., Sandouk, A., Son, U. H., Christenson, A. J., Van Hoek, M. L., and Bishop, B. M. (2009). Role of acetylation and charge in antimicrobial peptides based on human beta-defensin-3. *APMIS* 117, 492–499.

Park, I. Y., Cho, J. H., Kim, K. S., Kim, Y. B., Kim, M. S., and Kim, S. C. (2004). Helix stability confers salt resistance upon helical antimicrobial peptides. *J. Biol. Chem.* 279, 13896–13901.

Picioreanu, C., Kreft, J. U., Klausen, M., Haagensen, J. A., Tolker-Nielsen, T., and Molin, S. (2007). Microbial motility involvement in biofilm structure formation – a 3D modelling study. *Water Sci. Technol.* 55, 337–343.

Pollard, J., Wright, J., Feng, Y., Geng, D., Genberg, C., and Savag, P. B. (2009). Activities of ceragenin CSA-13 against established biofilms in an in vitro model of catheter decolonization. *AntiInfect Agents Med. Chem.* 8, 290–294.

Prince, A. S. (2002). Biofilms, antimicrobial resistance, and airway infection. *N. Engl. J. Med.* 347, 1110–1111.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J., and Ziebuhr, W. (2000). Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis. Antimicrob. Agents Chemother.* 44, 3357–3363.

Rello, J., Ausina, V., Ricart, M., Castella, J., and Prats, G. (1993). Impact of previous antimicrobial therapy on the etiology and outcome of ventilator-associated pneumonia. *Chest* 104, 1230–1235.

Ressner, R. A., Murray, C. K., Griffith, M. E., Rasnake, M. S., Hospenthal, D. R., and Wolf, S. E. (2008). Outcomes of bacteremia in burn patients involved in combat operations overseas. *J. Am. Coll. Surg.* 206, 439–444.

Richards, M. J., Edwards, J. R., Culver, D. H., and Gaynes, R. P. (1999). Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit. Care Med.* 27, 887–892.

Ryadnov, M. G., Degtyareva, O. V., Kashparov, I. A., and Mitin, Y. V. (2002). A new synthetic all-D-peptide with high bacterial and low mammalian cytotoxicity. *Peptides* 23, 1869–1871.

Saiman, L., Tabibi, S., Starner, T. D., San Gabriel, P., Winokur, P. L., Jia, H. P., McCray, P. B. Jr., and Tack, B. F. (2001). Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis. *Antimicrob. Agents Chemother.* 45, 2838–2844.

Sieprawska-Lupa, M., Mydel, P., Krawczyk, K., Wójcik, K., Puklo, M., Lupa, B., Suder, P., Silberring, J., Reed, M., Pohl, J., Shafer, W., McAleese, F., Foster, T., Travis, J., and Potempa, J. (2004). Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrob. Agents Chemother.* 48, 4673–4679.

Singh, P. K., Parsek, M. R., Greenberg, E. P., and Welsh, M. J. (2002). A component of innate immunity prevents bacterial biofilm development. *Nature* 417, 552–555.

Sorensen, O., Cowland, J. B., Askaa, J., and Borregaard, N. (1997). An ELISA for hCAP-18, the cathelicidin present in human neutrophils and plasma. *J. Immunol. Methods* 206, 53–59.

Stewart, P. S., Rayner, J., Roe, F., and Rees, W. M. (2001). Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. *J. Appl. Microbiol.* 91, 525–532.

Tack, B. F., Sawai, M. V., Kearney, W. R., Robertson, A. D., Sherman, M. A., Wang, W., Hong, T., Boo, L. M., Wu, H., Waring, A. J., and Lehrer, R. I. (2002). SMAP-29 has two LPS-binding sites and a central hinge. *Eur. J. Biochem.* 269, 1181–1189.

Travis, S. M., Anderson, N. N., Forsyth, W. R., Espiritu, C., Conway, B. D., Greenberg, E. P., McCray, P. B. Jr., Lehrer, R. I., Welsh, M. J., and Tack, B. F. (2000). Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect. Immun.* 68, 2748–2755.

Turner, J., Cho, Y., Dinh, N. N., Waring, A. J., and Lehrer, R. I. (1998). Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob. Agents Chemother.* 42, 2206–2214.

Van Delden, C., and Iglewski B. H. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerging Infect. Dis.* 4, 551–560.

Wade, D., Boman, A., Wåhlin, B., Drain, C. M., Andreu, D., Boman, H. G., and Merrifield, R. B. (1990). All-D amino acid-containing channel-forming antibiotic peptides. *Proc. Natl. Acad. Sci. U.S.A.* 87, 4761–4765.

Wang, G. (2008). Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles. *J. Biol. Chem.* 283, 32637–32643.

Wang, Y., Hong, J., Liu, X., Yang, H., Liu, R., Wu, J., Wang, A., Lin, D., and Lai, R. (2008). Snake cathelicidin from *Bungarus fasciatus* is a potent peptide antibiotics. *PLoS ONE* 3, e3217. doi: 10.1371/journal.pone.0003217

Whatley, W. S., Chandra, R. K., and MacDonald, C. B. (2006). Systemic absorption of gentamicin nasal irrigations. *Am. J. Rhinol.* 20, 251–254.

Whitchurch, C. B., Hobbs, M., Livingston, S. P., Krishnapillai, V., and Mattick, J. S. (1991). Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene* 101, 33–44.

Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., López-Boado, Y. S., Stratman, J. L., Hultgren, S. J., Matrisian, L. M., and Parks, W. C. (1999). Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 286, 113–117.

Yasin, B., Wang, W., Pang, M., Cheshenko, N., Hong, T., Waring, A. J., Herold, B. C., Wagar, E. A., and Lehrer, R. I. (2004). Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *J. Virol.* 78, 5147–5156.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* 415, 389–395.

Zhao, H., Gan, T. X., Liu, X. D., Jin, Y., Lee, W. H., Shen, J. H., and Zhang, Y. (2008). Identification and characterization of novel reptile cathelicidins from elapid snakes. *Peptides* 29, 1685–1691.

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Protein secretion systems in *Pseudomonas aeruginosa*: an essay on diversity, evolution, and function

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Protein secretion systems are molecular nanomachines used by Gram-negative bacteria to thrive within their environment. They are used to release enzymes that hydrolyze complex carbon sources into usable compounds, or to release proteins that capture essential ions such as iron. They are also used to colonize and survive within eukaryotic hosts, causing acute or chronic infections, subverting the host cell response and escaping the immune system. In this article, the opportunistic human pathogen *Pseudomonas aeruginosa* is used as a model to review the diversity of secretion systems that bacteria have evolved to achieve these goals. This diversity may result from a progressive transformation of cell envelope complexes that initially may not have been dedicated to secretion. The striking similarities between secretion systems and type IV pili, flagella, bacteriophage tail, or efflux pumps is a nice illustration of this evolution. Differences are also needed since various secretion configurations call for diversity. For example, some proteins are released in the extracellular medium while others are directly injected into the cytosol of eukaryotic cells. Some proteins are folded before being released and transit into the periplasm. Other proteins cross the whole cell envelope at once in an unfolded state. However, the secretion system requires conserved basic elements or features. For example, there is a need for an energy source or for an outer membrane channel. The structure of this review is thus quite unconventional. Instead of listing secretion types one after each other, it presents a melting pot of concepts indicating that secretion types are in constant evolution and use basic principles. In other words, emergence of new secretion systems could be predicted the way Mendeleïev had anticipated characteristics of yet unknown elements.

Keywords: cell envelope, nanomachine, macromolecular complex, channel, targeting

As for any other living cells, a hydrophobic membrane that guarantees their insulation against toxic substances present in the environment surrounds bacteria. Yet it is crucial for bacteria to establish a communication with the external milieu and paths and channels should be created across these membranes to allow the passage of molecules. Most bacteria need to release enzymes such as proteases or lipases to transform complex macromolecules in nutrients usable by the cell. Pathogenic bacteria developed strategies to efficiently infect their hosts. They deliver virulence factors into host cells which then act to minimize efficiency of host defense, subvert host cell signaling to their own benefit and/or utilize the host as a niche for replication (Cossart and Sansonetti, 2004; Merrell and Falkow, 2004). In most cases, the virulence factors produced by the pathogen need to be transported not only across the bacterial cell envelope but also across the host cell plasma membrane. In order to achieve these processes bacteria have evolved a wide range of protein secretion systems (Desvaux et al., 2009; Bleves et al., 2010). Studying protein secretion and identifying new secretion systems rely on evidences which demonstrate that a protein produced by a bacterium can reach the extracellular compartment in a specific manner (Filloux and Hardie, 1998). This means that appearance of exoproteins in the extracellular milieu should be independent of any cell leakage, cell lysis or even release of outer membrane vesicles.

There are many ways for describing and discussing protein secretion systems. To avoid a shopping list-style presentation, the review is formatted on the basis of two criteria. The first one was to choose *Pseudomonas aeruginosa* as a model organism. The versatility of this microorganism and the large size of its genome provide a rich playground for protein secretion specialists (Bleves et al., 2010). The second criterion was not to list protein secretion systems one after each other but rather consider basic molecular concepts.

PROTEIN SECRETION SYSTEMS IN GRAM-NEGATIVE BACTERIA

In Gram-negative bacteria, such as *P. aeruginosa*, the cell envelope is composed of two hydrophobic membranes. The inner and outer membranes are separated by a hydrophilic space, the periplasm. The secreted proteins, enzymes, or toxins, do have to travel through the hydrophobic environment of the membranes. These proteins are usually large hydrophilic molecules and therefore need to be accommodated into aqueous channels or other type of conduits that are spanning the cell envelope. These paths to the external medium are built *via* the assembly of more or less

sophisticated macromolecular complexes which we may call secretion machines. The composition and nature of these complexes may vary, but they are broadly conserved across Gram-negative bacteria (Filloux et al., 1990). Nowadays, six different types have been identified which are recognizable by the characteristics of proteins/components forming the secretion machine. These are called type I to type VI secretion systems (T1SS–T6SS; Desvaux et al., 2009; Durand et al., 2009). The secretion systems could also be split into two categories. Those that transport proteins at once across the bacterial cell envelope, thus directly from the cytoplasm to the cell surface, are known as one-step secretion mechanism. Alternatively, the secreted proteins could transit through the periplasm before crossing the outer membrane. These systems are known as two-step secretion mechanism.

TWO-STEP SECRETION MECHANISM

A two-step mechanism involves a stopover of the secreted proteins into the periplasm. It is likely that the periplasmic stay is very brief and the protein is trapped there only when the secretion machine is faulty. In principle, the secreted protein reaches the periplasm independently of the secretion system. Instead, it uses a general export pathway that is also involved in the transport of periplasmic and outer membrane proteins. The most essential general export pathway is known as the Sec pathway and is made with the Sec proteins, among which SecA (ATPase) and SecYEG (translocon; Papanikou et al., 2007). The proteins using the Sec pathway have a N-terminal signal peptide and are released in the periplasm after cleavage of this peptide (Choo et al., 2009). Another pathway for protein translocation across the inner membrane, though not essential, was later on identified for the transport of folded protein. This system called Tat, for Twin Arginine Translocation, involves three membrane components, TatABC (Sargent, 2007). The Tat-dependent proteins have a cleavable signal peptide that is recognizable from the Sec signal by its length and the presence of tandem arginine residues.

The type II secretion system

Pseudomonas aeruginosa studies contributed to the pioneering work on the discovery of the type II secretion system (T2SS). Mutations in genes, called *xcp*, resulted in the periplasmic accumulation of otherwise secreted enzymes such as elastase (LasB; Bever and Iglewski, 1988), lipase (LipA; Wohlfarth et al., 1992), alkaline phosphatase (PhoA; Filloux et al., 1988), or phospholipase C (PlcH; Wretlind and Pavlovskis, 1984; Filloux et al., 1987, 1989; Ostroff and Vasil, 1987). The *xcp* genes encode proteins with similarity to the Pul proteins that were shown to be involved in the secretion of pullulanase in *Klebsiella* species (d'Enfert et al., 1987; Filloux et al., 1990; Bally et al., 1992; Akrim et al., 1993). This observation pointed out that each bacterium do not have its own specific secretion system. Instead secretion systems are conserved across Gram-negative bacteria (Filloux et al., 1990). However, the T2SS remains specific and heterologous secretion rarely occurs (de Groot et al., 1991, 1996; Michel et al., 1995).

The Xcp system is encoded in a cluster containing 11 genes, *xcpP–Z* (Filloux, 2004). A twelfth gene is essential to Xcp function, namely *xcpA*, which is located elsewhere on the chromosome (Bally et al., 1991, 1992). In fact XcpA is also known as PilD (Nunn and Lory, 1991) and is also involved in type IV piliation. Data mining in the genome sequence of the PAO1 strain (Stover et al., 2000) showed that it exists a second T2SS gene cluster which is called *hxc* (homologous to *xcp*; Ball et al., 2002). The genetic organization of the *hxc* and *xcp* clusters is different but the *hxc* cluster contains the 11 *xcp* homologs, *hxcP-Z*. The Hxc system also requires the function of the XcpA/PilD protein.

There are as much as 14 characterized Xcp substrates whose genes are scattered all over the chromosome (**Table 1**). In contrast, there is only one Hxc-dependent substrate, namely LapA, known as low-molecular weight alkaline phosphatase (Ball et al., 2002). In this case, the *lapA* gene is clustered with the *hxc* genes indicating that the Hxc system may exclusively be assigned to LapA secretion.

Most Xcp and Hxc substrates carry a cleavable N-terminal signal peptide and are routed to the periplasm in a Sec-dependent manner. Once in the periplasm the proteins adopt a 3D conformation that is a prerequisite for recognition by the T2SS machinery. For example, in the case of the Xcp-dependent elastase (LasB), it was shown that the N-terminal propeptide acts as an intra-molecular chaperone to promote the folding of the LasB catalytic domain (Kessler and Safrin, 1994; McIver et al., 1995, 2004; Braun et al., 1996). In the absence of a functional propeptide, LasB does not fold, is not secreted and accumulates in the periplasm. The lack of secretion is proposed to result from the lack of a conformational signal thus preventing specific recognition by the Xcp machinery (Voulhoux et al., 2000). What exactly is the nature of the secretion signal in Xcp- or Hxc-dependent proteins is unknown. Only in a few cases T2SS secretion motifs have been highlighted, e. g. with the pullulanase from *Klebsiella* species in which a series of motifs have been proposed to be essential for recognition by the Pul system (Francetic and Pugsley, 2005). Another issue about targeting of substrates to the secretion machine is the identification of the T2SS component involved in the specific recognition. In *P. aeruginosa*, there is no study indicating which Xcp component could directly bind to a T2SS substrate. However, it was shown that exchange of *xcp* genes between two related *Pseudomonas* species, *P. aeruginosa* and *Pseudomonas alcaligenes*, results in the assembly of a hybrid T2SS machine which remains functional (de Groot et al., 2001). Whereas this is true for most *xcp* genes, it is not for the *xcpP* and *xcpQ* genes. From this observation two possibilities were considered, i.e., (i) the XcpP and XcpQ component have complementary interface and their interaction is species-specific; (ii) XcpP and/or XcpQ directly interacts with the secreted substrate in a species-specific manner. XcpP is a bitopic inner membrane protein with a large domain protruding in the periplasm and which interacts with the XcpQ outer membrane protein called secretin (Bleves et al., 1996; Gerard-Vincent et al., 2002; Robert et al., 2005b). It is proposed that the interaction between these two components is crucial to control the gating of the secretory channel formed by XcpQ (Tommassen et al., 1992). Whether XcpP and/or XcpQ directly interact with a T2SS substrate is not documented in *Pseudomonas* species. Nevertheless, a specific interaction between a T2SS substrate and the secretin is described in at least two cases. OutD of *Erwinia chrysanthemi* binds specifically to the PelB pectate lyase (Shevchik et al., 1997) and an interaction has been demonstrated between the cholera toxin and the secretin

Table 1 | Characterized secretion systems and secreted proteins in *P. aeruginosa*.

Secretion type	Secretion system	Outer membrane channel	ATPase	Secreted protein	Function of secreted protein	Reference
T1SS (Apr)	AprDEF	AprF (PA1248)	AprD (PA1246)	AprA (PA1249)	Alkaline protease	Guzzo et al. (1991a)
				AprX (PA1245)	Unknown	Duong et al. (2001)
T1SS (Has)	HasDEF	HasF (PA3404)	HasD (PA3406)	HasAp (PA3403)	Heme acquisition protein	Letoffe et al. (1998)
T2SS (Xcp)	XcpP–Z, XcpA	XcpQ (PA3105)	XcpR (PA3103)	CbpD (PA0852)	Chitin binding protein	Folders et al. (2000)
				LasA (PA1871)	Protease–elastase	Braun et al. (1998)
				LasB (PA3724)	Protease–elastase	Braun et al. (1998)
				LipA (PA2862)	Lipase	Jaeger et al. (1994)
				LipC (PA4813)	Lipase	Martinez et al. (1999)
				LoxA (PA1169)	Lipoxygenase	Vance et al. (2004)
				PaAP (PA2939)	Aminopeptidase	Braun et al. (1998)
				PhoA (PA3296)	Alkaline phosphatase	Filloux et al. (1988)
				PlcB (PA0026)	Phospholipase	Barker et al. (2004)
				PlcH (PA0844)	Hemolytic phospholipase	Voulhoux et al. (2001)
				PlcN (PA3319)	Phospholipase	Voulhoux et al. (2001)
				PmpA (PA0572)	Putative protease	Bleves et al. (2010)
				PrpL (PA4175)	Protease	Fox et al. (2008)
				ToxA (PA1948)	Exotoxin A-ADP ribosyltransferase	Lu et al. (1993)
T2SS (Hxc)	HxcP–HxcZ,	HxcQ (PA0685)	HxcR (PA0686)	LapA (PA0688)	Low-molecular weight alkaline phosphatase	Ball et al. (2002)
T3SS	PscB–L, PscN–U PopN, PoBD Pcr1–4, PcrD PcrRGVH	PscC (PA1716)	PscN (PA1697)	ExoS (PA3841)	GTPase activating protein (GAP) domain and adenosine diphosphate ribosyltransferase domain (ADPRT)	Yahr et al. (1997)
				ExoT (PA0044)	GTPase activating protein (GAP) domain and adenosine diphosphate ribosyltransferase domain (ADPRT)	Yahr et al. (1997)
				ExoU (PA14_51530)	Patatin-like, phospholipase	He et al. (2004)
				ExoY (PA2191)	Adenylate cyclase	Yahr et al. (1997)
T5aSS		EstA C-terminus		EstA (PA5112)	Lipase	van den Berg (2010)
T5bSS (TPS)	CdrB	CdrB (PA4624)	NA	CdrA (PA4625)	Adhesin	Borlee et al. (2010)
T5bSS	LepB	LepB (PA541)	NA	LepA (PA4540)	Protease	Kida et al. (2008)
P-usher	CupB3	CupB3 (PA4084)	NA	CupB5 (PA4082)	Putative adhesin	Ruer et al. (2008)
T5dSS		PlpD C-terminus	NA	PlpD (PA3339)	Patatin-like, lipolytic enzyme	Salacha et al. (2010)

(Continued)

Table 1 | Characterized secretion systems and secreted proteins in *P. aeruginosa*.

Secretion type	Secretion system	Outer membrane channel	ATPase	Secreted protein	Function of secreted protein	Reference
T6SS	H1-T6SS (Hsi-I) HsiA1-H1, HsiJ1 IcmF1, DotU1, Lip1 ClpV1, Hcp1, VgrG1a	NA	ClpV1 (PA0090)	Tse1 (PA1844)	Unknown	Hood et al. (2010)
				Tse2 (PA2702)	Bacterial toxin	Hood et al. (2010)
				Tse3 (PA3484)	Unknown	Hood et al. (2010)
Unknown	Unknown	NA	NA	ChiC (PA2300)	Chitinase	Folders et al. (2001)

NA, not applicable.

EpsD of *Vibrio cholerae* (Reichow et al., 2010). Interestingly, XphA and XqhA are two other XcpP and XcpQ homologs, respectively. The *xphA/xqhA* genetic locus does not contain any other *xcp*-like genes. However, it was shown that XphA and XqhA could replace the XcpP and XcpQ protein in the T2SS/Xcp system. The hybrid Xcp machine containing XphA/XqhA is functional and achieves the secretion of all Xcp substrates but the aminopeptidase PaAP (Michel et al., 2007). This is another indication of a specific interaction between the XcpP/XcpQ-like subcomplex and T2SS substrates.

From previous studies it is clear that T2SS substrates need to be folded in the periplasm for recognition by the secretion machine. This is in contrast with what is observed in other secretion systems such as with the T3SS (Feldman et al., 2002). Whereas Sec-dependent substrates are translocated across the inner membrane in an unfolded conformation, the Tat-dependent substrates are transported through the membrane in a folded conformation. The majority of the Tat-dependent substrates are periplasmic proteins that need to acquire a co-factor in the cytoplasm in order to fulfill their function. This is for example the case of the multicopper enzyme nitrous oxide reductase in *Pseudomonas stutzeri* (Heikkila et al., 2001). However, it was shown that *P. aeruginosa* T2SS substrate, such as the phospholipases PlcH and PlcN are transported to the periplasm in a Tat-dependent manner (Voulhoux et al., 2001). This observation clearly shows that in the two-step secretion mechanism, such as with the T2SS, the two successive steps of translocation across the membranes are disconnected. In the first step of translocation across the inner membrane the T2SS substrates can use either the Sec or Tat machinery. However, in the second step both Tat and Sec-dependent substrates converge to the T2SS, Xcp, or Hxc in the case of *P. aeruginosa*, for translocation across the outer membrane and release in the extracellular medium.

The type V secretion system

The type V secretion system is the most simple of all. It was originally described for the IgA protease in *Neisseria gonorrhoeae* (Pohlner et al., 1987). Basically, all the secretion information is contained within one single polypeptide. The protein has a cleavable signal peptide for targeting to the periplasm by the Sec system and a C-terminal domain that forms a β-barrel structure into the outer membrane. The passenger, or protease domain, could travel through the channel formed by theβ-barrel. This is still under debate since other studies indicate that the β-barrel of the autotransporter hemoglobin protease might not be fully folded and inserted in the outer membrane while the passenger domain is already accessible from the surface (Sauri et al., 2009). An alternative transport pathway in this case may involve the Bam/Omp85 complex. Once exposed to the cell surface the protease is released from the β-barrel *via* autocatalytic cleavage. Because there is no need for accessory proteins to help the secretion process, the mechanism was called autotransporter and coined Type Va secretion system (T5aSS; Dautin and Bernstein, 2007; Yen et al., 2008). The *P. aeruginosa* PAO1 genome contains three genes encoding proteins with a typical autotransporter C-terminal β-barrel attached to a N-terminal passenger domain. Two, PA3535 ad PA0328, have not been functionally characterized but are predicted to carry serine protease and metallopeptidase activity, respectively. In contrast, EstA has been extensively characterized. It has lipolytic activity (Wilhelm et al., 1999) and its 3D structure has been solved (**Figure 1**; van den Berg, 2010). The passenger domain is a member of the GDSL family of lipases (Akoh et al., 2004). The structure shows that the size of the barrel is too narrow to accommodate a folded protein and therefore translocation across the outer membrane should occur in an unfolded state. It is suggested that the EstA passenger may fold sequentially starting from the C-terminus. The vectorial passenger folding provides the driving force for the translocation. The catalytic triad is located on the apical surface of the passenger protein. Finally, there is a central α-helix that connects the extracellular passenger to the periplasmic side of the β-barrel that further suggests that the passenger is traveling through the channel. In this case the so-called secreted protein remains anchored at the bacterial surface and is not released into the supernatant.

Slightly more sophisticated as compared to autotransporters are the two partner secretion systems, coined TPS or T5bSS (Jacob-Dubuisson et al., 2001; Mazar and Cotter, 2007). In this case the β-barrel domain and the passenger domain are two distinct polypeptides, TpsB and TspsA, respectively. The β-barrel structure in T5aSS involved 12 β-strands (Oomen et al., 2004), whereas it is 16 β-strands that are found in T5bSS (Clantin et al., 2007). One

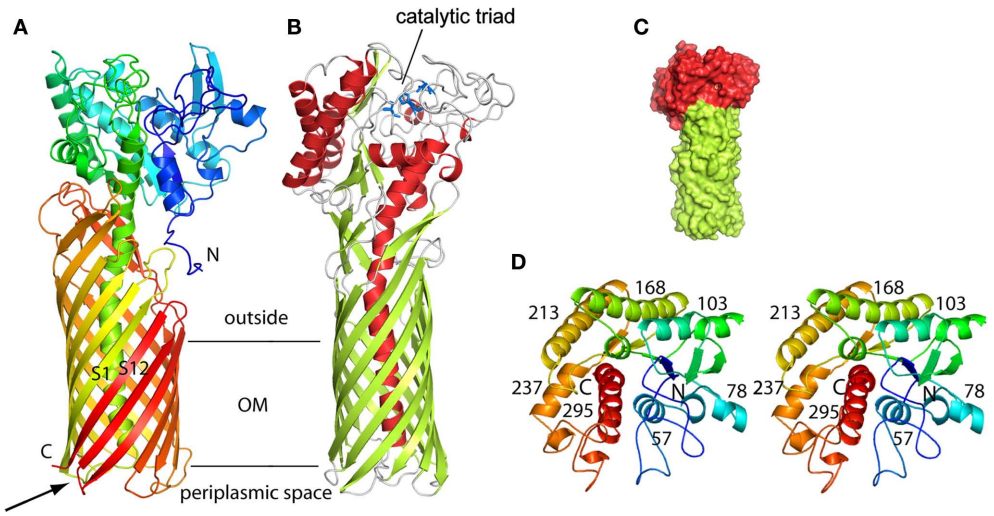

FIGURE 1 | Structure of the *P. aeruginosa* EstA autotransporter (T5aSS), reproduced from van den Berg (2010). (A) Backbone representation viewed from the side, with the protein colored by a gradient from blue at the N-terminus to red at the C-terminus. **(B)** Backbone view 90° rotated within the plane of the membrane relative to **(A)**, with helices colored red, β-strands colored green, and loops colored gray. The catalytic triad residues are shown as blue stick models. Horizontal lines indicate the approximate location of the outer membrane core. **(C)** Surface view of EstA from the side with the β-barrel domain colored green and the passenger domain colored red. **(D)** Stereo view of the EstA passenger from the extracellular side, colored as a rainbow from dark blue at the N-terminus to dark red at the C-terminus. The numbers are those for the central residue of the α-helix.

extensively characterized TPS system is involved in the secretion of the filamentous hemagglutinin (FHA) in *Bordetella pertussis* (Hodak et al., 2006). In addition to a classical Sec-dependent signal peptide, the mature FHA passenger displays a N-terminal secretion motif known as TPS motif that is required for targeting/interaction with the β-barrel domain formed by the TpsB component, in this case FhaC. The interaction is specific and occurs between the TPS motif and a polypeptide-transport-associated (POTRA) domain found at the N-terminus of the β-barrel (Clantin et al., 2007). The POTRA domains are connected to the barrel but protrude into the periplasm where they can interact with the passenger protein. Mining the PAO1 genome identified at least five putative TPSs, Tps1: PA2462–PA2463; Tps2: PA0040–PA0041; Tps3: PA4624–PA4625; Tps4: PA4540–PA4541 and Tps5: PA0690–PA0692. Among these, only few have been functionally characterized. In the case of LepA/LepB (Tps4-PA4541/PA4540; Kida et al., 2008) the passenger protein, LepA, contains a RGD motif that may contribute to eukaryotic cell attachment. However, the most striking feature is the identification of a trypsin-like serine protease motif and the demonstration that LepA has proteolytic activity. In the case of CdrA/CdrB (Tps3-PA4625/PA4624), CdrA is a putative c-diGMP regulated adhesin with a β-helix structural motif (Borlee et al., 2010). The CdrA adhesin was shown to promote *P. aeruginosa* biofilm formation and bacterial auto-aggregation in liquid culture. Finally, from the PAO1 genome, a sixth putative TPS could be considered, PA2542/PA2543, since PA2542 displays some similarities with the C-terminal domain of TpsA1 and PA2543 is predicted to be an outer membrane protein.

In addition to the 5–6 pairs of TpsA–TpsB, which are canonical examples of the T5bSS, *P. aeruginosa* contains an additional TpsA-encoding gene which is located within a gene cluster encoding *cup* fimbrial genes (Vallet et al., 2001). The secretion of this protein, CupB5 (**Figure 5B**), does not involve a cognate TpsB partner. Instead, it involves an outer membrane protein, CupB3, which is a typical usher protein involved in the assembly of fimbriae at the bacterial cell surface (Ruer et al., 2008). Furthermore, the CupB3 usher contains an N-terminal extension that shares similarities with POTRA domains found in TpsB proteins (Clantin et al., 2007). It is proposed that CupB3 is a chimera between an usher and a TpsB protein and that the POTRA domain is required for proper secretion of the putative CupB5 adhesin, but dispensable for the assembly of the CupB1 fimbriae (Ruer et al., 2008). CupB3 was called a P-usher for POTRA-containing Usher.

As indicated earlier, three autotransporters (T5aSS) have been identified in *P. aeruginosa*. Recently, a fourth autotransporter was described, PlpD (Salacha et al., 2010). PlpD is a member of the lipolytic enzyme family of patatin-like proteins (PLPs). Patatins are major proteins in potato tubers. They display lipid acyl hydrolase activity and have been proposed to be protective against plant pathogens (Banerji and Flieger, 2004). Interestingly, one of the *P. aeruginosa* T3SS effectors, ExoU, is also classified as a patatin-like protein and carries phospholipase A_2 activity (Sato and Frank, 2004; Lee et al., 2007). PlpD is considered an autotransporter since it has a two-domain organization. This includes the passenger lipase domain and a domain predicted to form the β-barrel in the outer membrane. It is predicted that the β-barrel of PlpD has 16 and not 12 strands which suggests that the protein does not belong to the T5aSS family but is closer from the T5bSS. Sequence analysis also revealed that a POTRA like domain could be identified within the PlpD protein, also a characteristic of TpsB and T5bSS family. PlpD thus represents a novel secretion type that mixes T5aSS and T5bSS and was given the name T5dSS (Salacha et al., 2010). Of note, T5cSS was already coined for a particular category of autotransporter whose β-barrel is formed by the assembly of a trimer

(Kajava and Steven, 2006; Meng et al., 2006). Such trimeric assembly is somehow similar to what was described for the TolC protein (Koronakis et al., 2000).

ONE-STEP SECRETION MECHANISM

By contrast with the two-step mechanism, the one-step secretion mechanism is based on the concept that no pool of secreted protein should be identified in the periplasm. In other words, when the secretion system is defective the secreted protein is blocked in the cytosol and possibly degraded. A continuous channel crossing the bacterial cell envelope, channel consisting of one or a combination of proteins, could make the transition from the bacterial cytosol to the extracellular environment.

The type I secretion system

The *Escherichia coli* α-hemolysin (HlyA) represents the T1SS archetype (Mackman et al., 1985). The T1SS consists of three components of which an ATP-binding cassette (ABC) protein (HlyB), an outer membrane protein (TolC) and an inner membrane protein (HlyD) largely protruding in the periplasm (Membrane fusion protein, MFP; Holland et al., 2005). HlyB and HlyD are plasmid-encoded together with HlyA, whereas TolC is chromosome-encoded. Studies conducted with *P. aeruginosa* have also pioneered the field with the alkaline protease, AprA (Guzzo et al., 1990, 1991a,b; Baumann et al., 1993). AprA has long remained the only type I-dependent substrate in *P. aeruginosa* until AprX, a protein with unknown function, was shown to use the same secretion system (Duong et al., 2001). The genes encoding the secretion system, AprDEF that are homologous to HlyBC and TolC, respectively, and the genes encoding AprA and AprX, are clustered together (**Figure 2**). This observation suggests that the Apr system is specific for these two substrates. This is in contrast with the Xcp system that is used to secrete proteins encoded from genes scattered all over the chromosome. Another type I secretion system was discovered later which is involved in iron uptake (Letoffe et al., 1998). This system called HasDEF (**Figure 2**) is very similar to the *Serratia marcescens* system (Ghigo et al., 1997; Arnoux et al., 1999; Wandersman and Delepelaire, 2004) and involves a heme-binding protein, HasAp. The protein is secreted in a T1SS-dependent manner, binds heme from hemoglobin and is then taken up *via* the cell surface

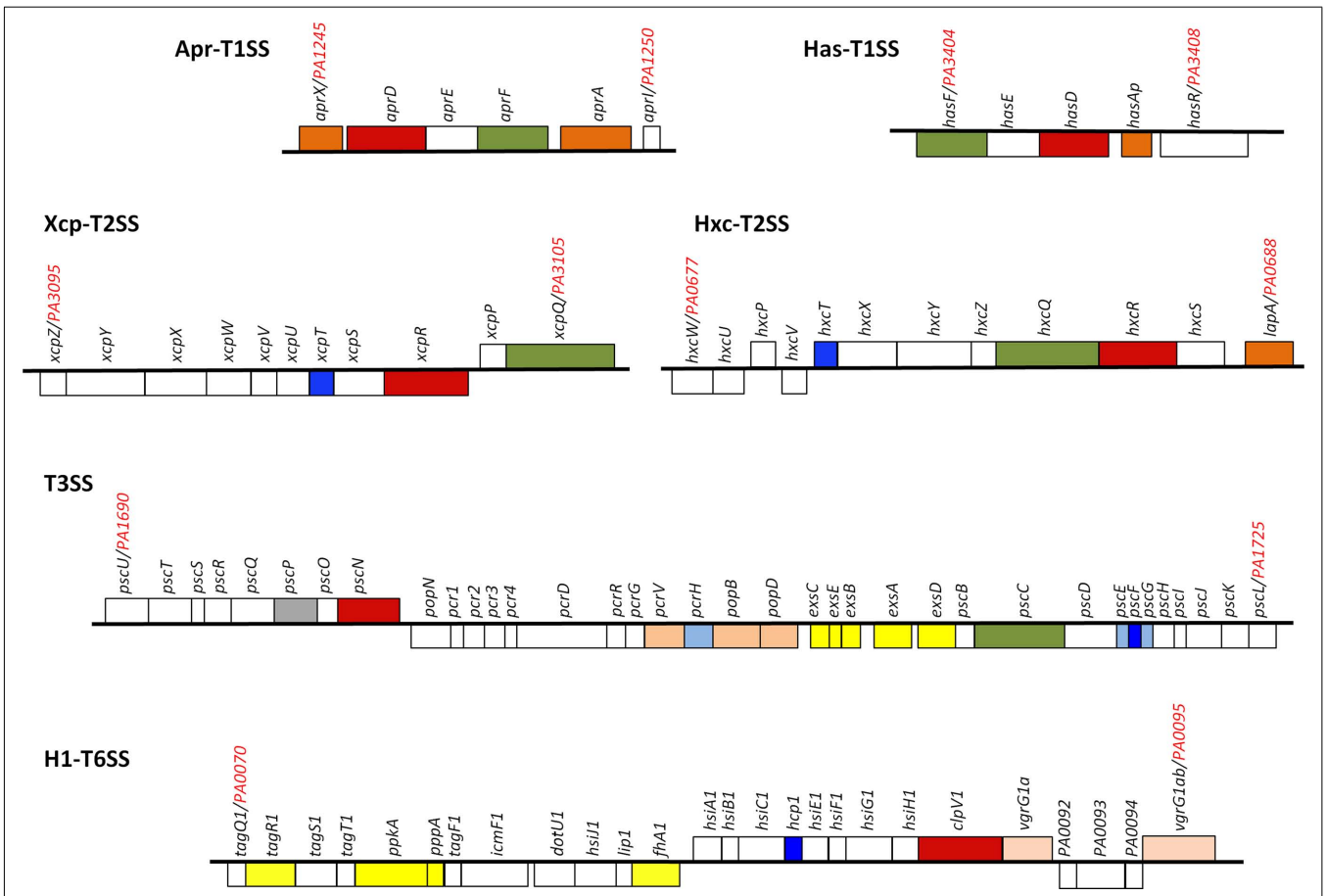

FIGURE 2 | *Pseudomonas aeruginosa* secretion gene clusters. Within the clusters shown, the gene encoding the ATPase is colored red, the gene encoding the outer membrane channel is colored green. The genes encoding secreted proteins are colored dark orange. Genes encoding components of the T2SS pseudopilus, the T3SS needle and the T6SS tail tube are colored dark blue. The genes in light orange encode either translocators of the T3SS or puncturing device of the T6SS. Shown in light blue are T3SS chaperones and in gray the T3SS ruler. Shown in yellow are genes encoding proteins involved in regulation. For more details see text.

receptor HasR (Letoffe et al., 1999). The NMR structure of HasAp has been solved and gave insight on the kinetic parameter for heme-binding and uptake (Jepkorir et al., 2010; Yukl et al., 2010). Another *P. aeruginosa* T1SS could be identified by genome mining. The system is coded by PA1875–PA1877 while a gene adjacent to the secretion cluster is encoding a large protein (PA1874) reminiscent of proteins secreted by the T5bSS (van Ulsen et al., 2008). This organization recalls what was reported with *Pseudomonas putida* in which LapA, a large adhesin involved in biofilm formation, is transported to the cell surface by a T1SS made with the LapBCD components (Hinsa et al., 2003). A fourth complete T1SS is found encoded in the PAO1 genome, which is PA4142–PA4144. No functional data are available regarding this system but PA4142 and PA4143 are homologous to CvaA and CvaB, a T1SS involved in the secretion of colicin V (ColV) in *E. coli* (Zhang et al., 1995; Zhong et al., 1996). ColV is a 109 amino-acids-long toxin and it is striking to observe that the gene located downstream the putative *P. aeruginosa* T1SS cluster, PA4141, encodes a small protein of 99 amino acids.

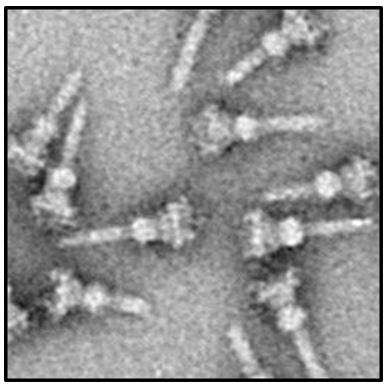

FIGURE 3 | A transmission electron-microscope image of isolated T3SS needle complexes from *S. typhimurium* reproduced from Schraidt et al. (2010).

The type III secretion system

The T3SS is a fascinating secretion system. In *Yersinia enterocolitica*, it was proposed to secrete proteins called *Yersinia* outer membrane protein (Yop; Michiels et al., 1990). These Yop proteins were recovered with outer membrane proteins during fractionation procedures. This unusual localization was due to aggregation of the proteins once released in the extracellular medium. In these experiments, extracellular secretion was obtained upon artificial induction of the T3SS using Ca^{2+} chelation. It is now established that T3SS activity is cell contact-dependent and therefore the T3SS substrates are injected directly from the bacterial cytoplasm into the eukaryotic cells cytosol (Cornelis, 2010).

In *P. aeruginosa* the T3SS was discovered in 1996 (Yahr et al., 1996) and since 36 genes have been associated with T3SS function (Frank, 1997; Brutinel et al., 2008). The genes encoding elements of the secretion machine and genes encoding regulatory proteins are clustered but organized in several operons (**Figure 2**). In contrast, the genes encoding the effector proteins are scattered all over the chromosome (**Table 1**). The secretion machine is made of over 20 different proteins that in *P. aeruginosa* are called the Psc, Pop, or Pcr components. The T3SS machine resembles a needle whose first observation in *Salmonella* Typhimurium (**Figure 3**; Kubori et al., 1998) came as a real flash of inspiration for the scientific community. It materialized the idea of injection that led to name the T3SS nanomachine, injectisome. High-resolution images are now available for *S.* Typhimurium and *Shigella flexneri* injectisomes (Hodgkinson et al., 2009; Schraidt and Marlovits, 2011). Whereas the needle-like structure is central to the injection process (Blocker et al., 2008), a set of three proteins, PopBD and PcrV in *P. aeruginosa*, are crucial to establish a pore into the membrane of eukaryotic cells (Dacheux et al., 2001; Schoehn et al., 2003; Goure et al., 2004). In fact these proteins are the first proteins to be transported through the needle but they are not injected into the cytosol. Instead, they remain inserted into the membrane to form a pore 2.8–6.0 nm in diameter that is called the translocon. Genuine effectors are subsequently transported through the needle and injected in the eukaryotic cell cytosol by passing through the pore formed by the translocon. PopB and PopD are the proteins that insert into the eukaryotic cell membrane. The role of PcrV is unclear but it seems to form a scaffold at the tip of the needle and antibodies against PcrV inhibit injection of T3SS effectors (Sawa et al., 1999; Goure et al., 2005; Broz et al., 2007).

In *P. aeruginosa*, the T3SS is a major virulence weapon that contributes to cytotoxicity and acute infections (Hauser et al., 2002; Hauser, 2009). Cytotoxicity is associated with the activity of effector proteins that are secreted in a T3SS-dependent manner. In *P. aeruginosa* only four effectors, named ExoS, ExoT, ExoU, and ExoY, have been identified. ExoS and ExoT have a GTPase activating protein (GAP) domain and an adenosine diphosphate ribosyl transferase domain (ADPRT; Fleiszig et al., 1997). ExoU is the most potent cytotoxin, it has a phospholipase activity and can be classified within the PLP family (Sato and Frank, 2004). ExoY has an adenylate cyclase activity. The presence of the genes encoding these effectors may vary from one isolate to the other but it is generally observed that *exoS* and *exoU* genes are mutually exclusive (Finck-Barbancon et al., 1997; Berthelot et al., 2003; Wolfgang et al., 2003; Kulasekara et al., 2006). For example when looking at the sequence of available *P. aeruginosa* genomes, *exoS* is found only in PAO1 and LESB58. In contrast, *exoU* is found exclusively in PA14 and is located within a well-characterized pathogenicity island, PAPI-I (Qiu et al., 2006). Finally, *exoT* and *exoY* are found in all three strains, PAO1, PA14, and LESB58. The fourth sequenced *P. aeruginosa* strain, PA7(Roy et al., 2010), is a non-respiratory human isolate that is highly resistant to antibiotics. Strikingly, not only this strain lacks genes encoding all four effectors but it also lacks all the genes encoding the components of the T3SS machinery. The effector proteins are transported through the needle in an unfolded manner and are targeted to the T3SS by a cognate chaperone which they bind to prior secretion (Parsot et al., 2003). The chaperone for ExoS and ExoT is called SpsC, whereas the chaperone for ExoU is SpcU (Finck-Barbancon et al., 1998; Shen et al., 2008). The *spsC* gene is clustered with the *exoS* gene. Interestingly, in the PA14 strain the *exoS* gene is lacking whereas the *spsC* gene is kept, likely for ExoT secretion. No cognate chaperone has been characterized to

date for ExoY (Yahr et al., 1998). The PopBD translocon proteins that are also transported by the needle-like structure have an identified cognate chaperone, namely PcrH (Broms et al., 2003).

THE TYPE VI SECRETION SYSTEM

It is yet unclear whether the T6SS is definitely a one-step mechanism since proteins secreted by the T6SS have not been clearly identified, or their fate incompletely characterized. In *P. aeruginosa* there are three different gene clusters encoding a set of 15–20 genes involved in T6SS function (Filloux et al., 2008). These clusters were initially identified through the characterization of a gene pair encoding proteins similar to IcmF and DotU (Das and Chaudhuri, 2003). These proteins are required in several bacteria for the function of the T4SS (Sexton and Vogel, 2002). Genomic analyses revealed that in many cases the pair *icmF/dotU* is clustered with a conserved set of genes unrelated to T4SS genes and the name T6SS was coined. The three *P. aeruginosa* T6SS clusters were initially called HSI, for Hcp Secretion Island (Mougous et al., 2006). Later on, the three clusters were renamed H1-T6SS to H3-T6SS (Hood et al., 2010). Though the three clusters displayed a set of core genes, there is a level of variability in their composition and genetic organization. The most complex and best studied is the H1-T6SS cluster (**Figure 2**) since it is highly expressed in a strain mutated for a gene encoding the RetS kinase sensor (Mougous et al., 2006; Goodman et al., 2009).

Historically, the T6SS substrates were considered to be proteins named Hcp and VgrG (Pukatzki et al., 2006). On the *P. aeruginosa* PAO1 genome, a BLAST search identified 10 *vgrG* genes and 5 *hcp* genes (Hachani et al., 2011). Some of these genes are clustered with T6SS genes but others are scattered around the chromosome. One *hcp* gene, *hcp1*, and two *vgrG* genes, *vgrG1a* and *vgrG1b*, are associated with the H1-T6SS cluster (**Figure 2**). Hcp1 and VgrG1a are secreted in a T6SS-dependent manner (Mougous et al., 2006; Hood et al., 2010; Hachani et al., 2011). However, the structure of Hcp1(Mougous et al., 2006), and later VgrG1a (Leiman et al., 2009; Hachani et al., 2011), showed that these proteins form multimeric complexes and are likely to be part of the secretion machine rather than being genuine effectors (**Figure 4**). The occurrence of these proteins in the extracellular medium could be incidental. They may constitute a cell surface exposed portion of the T6SS machine that may be sheared off during sub-cellular fractionation procedures. Recently, three genuine effectors for the *P. aeruginosa* H1-T6SS machine have been identified and given the name Tse1–3, for type six exported (Hood et al., 2010). Importantly, it was shown that the Tse2 protein is a toxin able to kill competing bacteria. The *tse2* gene is clustered with a gene called *tsi2*. The *tsi2* gene encodes a protein that protects the producing strain from the effect of the Tse2 toxin.

MOLECULAR NEEDS FOR THE FUNCTION OF SECRETION MACHINES

Most secretion machines are relatively complex and each secretion type is identified by the characteristics of the constituents involved. However, there are a number of features and rules that are hallmarks of all secretion systems. For example, these machines require a component providing energy to the process, usually an ATPase. It also requires an outer membrane protein that is the

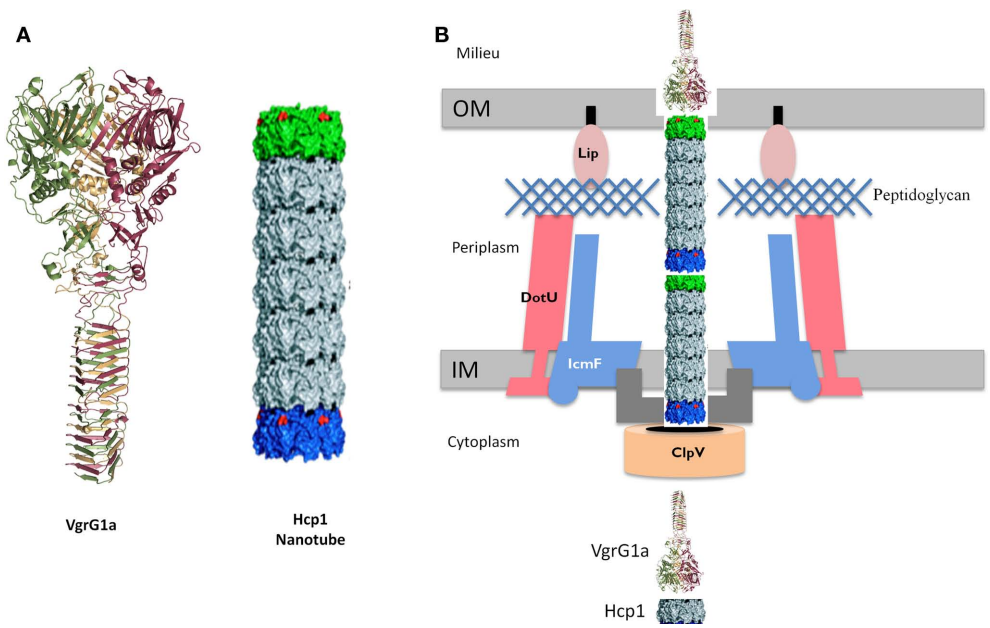

FIGURE 4 | Schematic model for the T6SS. (A) Structural-based homology prediction for the VgrG1a protein of *P. aeruginosa* reproduced from Hachani et al. (2011). The Hcp1 nanotube reproduced from Ballister et al. (2008). **(B)** Schematic representation of the T6SS, in which VgrG1a (puncturing device) and Hcp1 nanotube have been included as depicted in **(A)**. Few other T6SS components are shown and the system punctures the bacterial cell envelope from inside to outside. See text for more details.

ultimate channel before the secreted protein can access the external milieu. Other components of the system are involved in the scaffolding and/or regulation of the macromolecular complex or in the specific recognition of secreted substrates.

ENERGIZING THE SECRETION PROCESS

Translocation of macromolecules across membranes is an active process that is powered by energy sources. The Sec machinery which is universally used by bacteria, requires an ATPase, SecA, which pushes substrates across the cytoplasmic membrane (Sardis and Economou, 2010). In contrast the Tat system that is involved in the translocation of folded proteins has been shown to use the proton motive force to move polypeptides across the cytoplasmic membrane (Palmer and Berks, 2003; Bageshwar and Musser, 2007). Most protein secretion systems use ATPases to promote movement of polypeptides across the whole bacterial cell envelope (Saier, 2006).

T1SS, T2SS, T3SS, and T6SS all contain at least one component that has a typical Walker motif characteristic of proteins able to bind and hydrolyze ATP. In *P. aeruginosa*, XcpR and HxcR are part of the T2SS and belong to the family of traffic ATPases (Planet et al., 2001). These proteins have a highly conserved Walker A, a less conserved Walker B and their own signature which is a highly conserved aspartate-rich region, or aspartate box, located between the Walker A and B (Possot and Pugsley, 1994). Additional conserved motifs in this family of ATPases are a histidine box with no assigned function and a tetracysteine motif whose role might be to coordinate divalent cations. The ATPase AprD (Duong et al., 1992), involved in the secretion of AprA, is a member of the ABC-transporter family (Higgins, 2001) which is found in all T1SS (Saier, 2006). This family of ATPases, in addition to the Walker motifs, displays a unique signature sequence (LSGGQ) in each nucleotide binding domain (NBD; Loo et al., 2002). In the case of T3SS a hydrophilic component homologous to F1/V1-ATPases (Pozidis et al., 2003) is involved in the secretion process, namely PscN in *P. aeruginosa*. Finally, for the T6SS a AAA ATPase, called ClpV1, ClpV2, or ClpV3 in *P. aeruginosa*, is involved. The AAA motif is a conserved sequence of about 250 residues that includes the Walker sequences and other domains unique to AAA proteins (Patel and Latterich, 1998). It is remarkable that each secretion system described previously involves an ATPase of a distinct family. This is not always the case since in the T4SS that does not exist in *P. aeruginosa* the energizing component is a traffic ATPase, such as the T2SS ATPases XcpR and HxcR (Sagulenko et al., 2001).

The 3D structures of some of these ATPases have been solved, although none were obtained from *P. aeruginosa*. These proteins show a hexameric structure and a central channel as with the T2SS traffic ATPase from *Vibrio cholerae*, EpsE, (Robien et al., 2003). The T6SS ATPases are also hexamers and the *V. cholerae* ClpV member has been proposed to modulate the formation of tubules which are made of two other T6SS components. These are VipA and VipB that are homologous to the HsiB and HsiC proteins in *P. aeruginosa* (Bonemann et al., 2009, 2010; Filloux, 2009). The T2SS and T6SS-associated ATPases are not integral membrane proteins. Instead, they could be peripherally associated with the inner face of the cytoplasmic membrane *via* interaction with another specific component. In the case of XcpR the integral membrane protein XcpY keeps XcpR associated with the membrane and with the rest of the T2SS machine (Ball et al., 1999). In the case of *P. aeruginosa* ClpV1, it was shown that the presence of other components of the T6SS is required to make a ClpV1–GFP fusion appeared as discrete foci, whereas ClpV1 distribution becomes diffuse in the absence of IcmF1 or Hcp1(Mougous et al., 2006).

Whereas the T2SS and T6SS-associated ATPases appeared to function as hexamers, the ABC-transporters involved in T1SS-dependent secretion are dimeric (Lin et al., 2009). The ABC-transporters from T1SS are usually integral membrane proteins with a transmembrane domain connected to the NBD domain that extends in the cytoplasm. The NBD domain structure of the canonical HlyB from *E. coli* has been solved and displays the common overall architecture of ABC-transporter NBDs (Schmitt et al., 2003). The direct interaction between the ABC-transporter and the secreted protein is largely documented (Letoffe et al., 1996; Thanabalu et al., 1998; Benabdelhak et al., 2003) which supports the idea that the ABC-transporter directly contributes to the translocation of the secreted protein across the cytoplasmic membrane.

In the case of T3SS, the ATPase is tightly associated but not inserted into the cytoplasmic membrane. It contains a NBD domain related to F1-ATPase. Biochemical and structural studies have been performed with HcrN from *Pseudomonas syringae* showing that the most active form of HcrN is an oligomer (Pozidis et al., 2003).

In the case of T5SS, the translocation of exoproteins across the outer membrane is disconnected from the cytoplasmic membrane. The one and only accessory component of the T5SS machine is an outer membrane protein. The dynamic of T5SS-dependent translocation across this outer membrane pore has been proposed to depend on energy resulting from the folding of the secreted protein (Junker et al., 2009). This hypothesis was further supported by recent structural data obtained with the *P. aeruginosa* EstA (van den Berg, 2010; **Figure 1**).

Pore formation across the outer membrane

Secretion systems are also characterized by the existence of a protein that forms an outer membrane pore. The structural characterization of the *E. coli* TolC protein has been a seminal step forward in the understanding of the T1SS mechanism (Koronakis et al., 2000). This protein forms a trimer with two distinct domains. One domain is a β-barrel which is similar to barrels found in outer membrane proteins such as the porins (Cowan et al., 1992). A main difference is that the barrel forms upon assembly of the trimer whereas with porins the barrel is a monomeric protein. The most striking structural feature of TolC is that it contains a second domain essentially made of helices forming a long channel which spans the periplasm (Koronakis et al., 2000). This periplasmic conduit that connects the outer membrane pore of TolC to the ABC protein (HlyB) within the cytoplasmic membrane is a demonstration that T1SS-dependent substrates may travel through the secretion apparatus and never be exposed to the periplasm. It thus validates the idea of a one-step mechanism. The TolC protein in *E. coli* is not only involved in T1SS but also in the formation of efflux pumps, such as with ArcAB (Symmons et al., 2009). In *P. aeruginosa* several efflux pump systems have

been identified of which the MexAB-OprM is extensively characterized. The structure of the outer membrane protein OprM is available (Lambert et al., 2005; Phan et al., 2010; Trepout et al., 2010) and suggests a conserved TolC-like structural organization. This family of proteins also includes AprF (Duong et al., 1992) and HasF in the *P. aeruginosa* T1SS. The TolC protein in *P. aeruginosa* has not been functionally characterized but is suggested to be PA4974.

The assembly of a β-barrel structure for the formation of a pore in the outer membrane is also the adopted strategy in the case of T5SS. The structure of the EstA autotransporter provides a typical illustration of this organization (**Figure 1**) although the number of β-strands varies when comparing the T5aSS (12 β-strands; Oomen et al., 2004) and the T5bSS (16 β-strands; Clantin et al., 2007). The structure of the *P. aeruginosa* T5bSS CdrB could be modeled using the Phyre software (Kelley and Sternberg, 2009) and displays the typical β-barrel fold with POTRA domains at the N-terminus (**Figure 5A**).

Another fascinating protein, which has been the object of a large number of studies is the secretin found in T2SS and T3SS. The XcpQ protein was one of the first to reveal the donut shape-like structure adopted by these proteins which suggests a homo-multimeric complex with a central channel (Koster et al., 1997; Bitter et al., 1998). Other secretions have since been characterized but none of them could be taken into crystallographic studies (Collins et al., 2003; Chami et al., 2005) except for their periplasmic domain (Korotkov et al., 2009; Spreter et al., 2009). Nevertheless, quality images are obtained from Cryo-EM analysis and 3D reconstruction as with *V. cholerae* secretin GspD (Reichow et al., 2010; Korotkov et al., 2011). The global view is a cylinder formed by about 12–15 subunits. The cylinder extends in a periplasm *via* a domain called vestibule. The secretin channel is open on the side facing the outer membrane but gated from the periplasmic end (Korotkov et al., 2011). In *P. aeruginosa*, the dimension of the central channel formed by XcpQ is 95 Å and is compatible with the transport of folded protein (Bitter et al., 1998). The XcpQ secretin has two domains. The C-terminal domain is inserted in the outer membrane and forms the channel whereas the N-terminus protrudes in the periplasm and forms the vestibule (Brok et al., 1999; Korotkov et al., 2011). Up to now, it has not been possible to understand whether the secretin inserts in the membrane through the formation of a β-barrel or whether it adopts a different mechanism. Recent studies have highlighted that some outer membrane proteins can adopt a unique fold which is a barrel formed by amphipathic α-helices (Dong et al., 2006; Chandran et al., 2009; Kowalska et al., 2010).

None of the T6SS components can be considered as an outer membrane pore. The only putative outer membrane protein is a lipoprotein which is anchored at the inner face of the outer membrane (Aschtgen et al., 2008). It is unlikely to form a pore and the T6SS might involve an unprecedented mechanism for translocation across the outer membrane.

Function of accessory proteins

In addition to ATPases and pore-forming outer membrane proteins, secretion systems involve accessory proteins whose function is far from being elucidated. Some of these proteins could be important to collate the machinery. In the case of T2SS for example, the integral inner membrane protein XcpY is essential for anchoring the ATPase XcpR to the inner face of the cytoplasmic membrane (Ball et al., 1999). In the absence of XcpY, XcpR remains cytosolic. XcpZ, another inner membrane protein, is important for the stability of the XcpY protein and thus for the maintenance of the XcpYR complex (Michel et al., 1998).

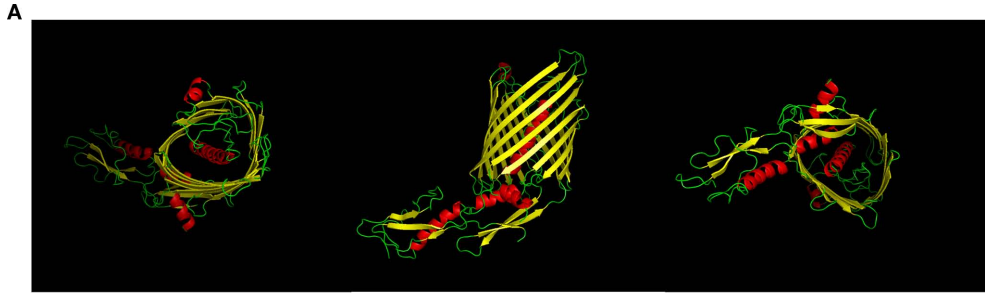

FIGURE 5 | The Two partner secretion systems (T5bSSs). (A) Structural prediction and modeling of *P. aeruginosa* CdrB, the carrier domain, performed using Phyre (http://www.sbg.bio.ic.ac.uk/phyre). Figures were made using PyMOL (http://www.pymol.org). From left to right, top (facing extracellular milieu), side, and bottom (facing periplasm) view. **(B)** Amino-acid sequence alignment of the "TPS" domains of several TpsAs passenger including FHA (*B. pertussis*), HMW1 (*H. influenzae*), TpsA1–5 and CupB5 (*P. aeruginosa*). The NPNL motif in FHA and TpsA1–2 (red) or NxxGx motif in all TpsAs (blue) is indicated with bold characters. The alignment was performed using clustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2).

The secretin component has also been proposed to require accessory component for proper targeting and insertion in the outer membrane. This component is more currently known as pilotin (Hardie et al., 1996; Shevchik and Condemine, 1998). It is a small lipoprotein able to bind at the C-terminus of the secretin (Daefler et al., 1997). This has been shown for several bacterial species, including *Klebsiella* and *Erwinia*. In *P. aeruginosa* no pilotins have been identified for XcpQ (T2SS) or PscC (T3SS). Recently, it was shown that the secretin HxcQ does not require a pilotin but is self-piloted by a lipid modification at its N-terminus (Viarre et al., 2009). Finally, the inner membrane component XcpP has been shown to interact both with the secretin XcpQ and the XcpYRZ complex (Bleves et al., 1999; Gerard-Vincent et al., 2002; Robert et al., 2005a,b). A proposed function for XcpP is the gate keeping of the secretin channel.

In the T1SS, there is only one additional component that is known as the MFP or periplasmic adaptor. Several structural data are available for MFP from efflux pump systems such as AcrA in *E. coli* (Mikolosko et al., 2006) or MexA in *P. Aeruginosa* (Higgins et al., 2004; Trepout et al., 2010). These structures suggest that the periplasmic adaptor is central for the assembly of the whole complex and the interaction between the adaptor and the protruding coiled coil domains of the outer membrane protein TolC may be crucial for controlling the opening of the channel (Lobedanz et al., 2007; Symmons et al., 2009; Pei et al., 2011). The overall structure of a drug efflux pump has been obtained (Symmons et al., 2009) and is likely to apply to T1SS macromolecular complexes.

Protein targeting

Pseudomonas aeruginosa secretes a wide range of proteins and possesses most of the secretion types at the exception of the T4SS. Therefore the bacterial cell needs to select the suitable substrates for each of these machineries using a specific recognition process and avoiding all invalid pairing which may jeopardize the function of the secretion system (Filloux, 2010). Targeting to the Sec or Tat translocon has been largely documented and requires a cleavable N-terminal signal peptide displaying recognizable features (Vonheijne, 1985; Palmer et al., 2010).

In the case of T2SS, the N-terminal signal peptide found on *P. aeruginosa* exoproteins, e. g. elastase, exotoxin A, or lipase, is sufficient for translocation across the inner membrane in a Sec-dependent manner. In the case of phospholipases, PlcH and PlcN are translocated in a Tat-dependent manner (Voulhoux et al., 2001) whereas PlcB is translocated in a Sec-dependent manner (Barker et al., 2004). In all cases, this first step of translocation is not sufficient to target exoproteins to the T2SS. Additional secretion motifs are needed which provide a strong specificity to the secretion process. For example, there is no cross-exchange of substrates between the two *P. aeruginosa* T2SS, Xcp, and Hxc. The Hxc-dependent LapA is neither recognized nor secreted by the Xcp system (Ball et al., 2002). Despite many studies on the T2SS in *P. aeruginosa* and other Gram-negative bacteria, it has no been possible to identify an obvious secretion motif in T2SS substrates. Instead, it is suggested that the motif is conformational and results from the assembly of a patch of residues within the folded protein. Such conclusion has been drawn from studies on elastase or exotoxin A that showed that folding is a prerequisite to secretion and improperly folded proteins are trapped in the periplasm (Braun et al., 1996; Voulhoux et al., 2000).

In the case of T5aSS, no motif other than the signal peptide is needed. The passenger protein is connected to the translocation channel, as described for the *P. aeruginosa*, EstA (**Figure 1**), and thus no need for targeting. In the case of T5bSS the passenger protein (TpsA) is distinct from the outer membrane translocation channel (TpsB). The recognition process relies on two motifs, one located in the TpsA protein and called TPS motif, and one in the transporter TpsB called POTRA domain (Mazar and Cotter, 2007). This is well demonstrated with the couple FHA/FhaC in *B. pertussis* (Clantin et al., 2007) and sequence analysis of the T5bSSs in *P. aeruginosa* shows that these features are rather well conserved (**Figure 5**).

The T1SS does not require an initial step of translocation across the cytoplasmic membrane *via* the Sec or Tat system. Consequently, direct targeting of the exoprotein to the T1SS machine occurs. The secretion motif lies in the C-terminus of the secreted protein and is not cleaved. For example, the secretion signal of hemolysin HlyAlies within the last 53 residues. This region includes a 18-amino-acid amphiphilic α-helix, a cluster of charged residues and a weakly hydrophobic terminal sequence (Koronakis et al., 1989). Furthermore, the NBD of HlyB and a C-terminal 23 kDa fragment of HlyA have been shown to interact with each other in a specific manner (Benabdelhak et al., 2003). In the case of *P. aeruginosa* AprA, the 50 C-terminal residues constitute an autonomous secretion signal which is specific for AprD recognition but fails to recognize the HlyB ABC-transporter (Duong et al., 1996). Recent studies suggest that additional secretion signals could participate to the earlier steps of recognition, whereas the C-terminal signal might be essential at later stages for the release of the substrate from the T1SS machine (Masi and Wandersman, 2010). In some T1SS-dependent protein, such as *P. aeruginosa* AprA, a glycine rich repeat domain contributes to yield a roll conformation (Baumann et al., 1993). The secretion signal could be used to promote the secretion of unrelated passenger protein as shown with the C terminal domain of a *Pseudomonas fluorescens* lipase (TliA; Chung et al., 2009).

In the case of T3SS, many studies converge to the idea that the targeting signal is located at the N-terminus of the exoprotein. The targeting signal could be bipartite with one region located in the very first amino acids, i.e., 1–15 in the case of *P. aeruginosa* ExoS (Hauser, 2009). Furthermore, for the effectors that require a cognate chaperone, the chaperone-binding site is also located at the N-terminus, i.e., 15–51 in the case of ExoS (Deng and Barbieri, 2008; Hauser, 2009). The structural organization of the signal is likely to be the same with ExoT and ExoU whereas for ExoY no chaperone seems to be required. Strikingly, from other T3SS studies the nature of the signal has been proposed to be located within the secondary structure of the mRNA rather than being directly dependent on the amino-acid sequence (Anderson and Schneewind, 1997). This is a complex issue and both concepts have not been reconciled yet.

Studies on the T6SS do not provide any information on how proteins are targeted to the secretion machine, but very little, if any, investigations have addressed this issue.

RELATIONSHIP BETWEEN SECRETION SYSTEMS AND OTHER BIOLOGICAL PROCESSES

A remarkable feature about secretion systems is that several of them derived from other membrane bound systems that have been adapted for a different purpose. Past studies revealed that T2SS is a copy–paste when compared with the system required for assembly of type IV pili (Hobbs and Mattick, 1993; Filloux, 2004). Whereas type IV pili are polymeric structures attached at the cell surface (Mattick, 2002), the T2SS assembles an abortive pilus, called pseudopilus (Sauvonnet et al., 2000; Durand et al., 2003). Pseudopilus elongation/retraction within the periplasm works as a piston expelling secreted proteins through an outer membrane channel. The T3SS shows exquisite resemblance with the flagellar basal body (Blocker et al., 2003; Journet et al., 2003). The latter is required for secretion and polymerization of the flagellin subunit into a flagellum, an important device for bacterial mobility (Macnab, 2003), whereas the T3SS needle is used for the injection of T3SS effectors within the cytosol of the host cells (Cornelis, 2006). Interestingly, the T4SS, though lacking in *P. aeruginosa*, was originally identified in *A. tumefaciens* as a translocating machine targeting bacterial T-DNA into the nucleus of plant host cells (Christie et al., 1989). This machinery is analogous to the system assembling F-pili that are required for DNA exchange in bacterial conjugation (Winans et al., 1996; Cascales and Christie, 2003). The T4SS is now recognized in many bacteria as a genuine protein secretion system, injecting proteins such as *Legionella pneumophila* RalF, or *Helicobacter pylori* CagA, into the host cell cytosol (Backert et al., 2000; Nagai et al., 2005). Finally, and this is a major discovery in the field of protein secretion over the last decade, the T6SS shares extensive similarity with the bacteriophage tail which is used to inject the phage DNA by puncturing the bacterial cell envelope (Leiman et al., 2009).

THE T2SS PSEUDOPILUS

The *P. aeruginosa* Xcp secretion is a multiprotein complex comprising an inner membrane platform, XcpSPYZ, a traffic ATPase, XcpR, and a secretin, XcpQ which is the outer membrane pore through which exoproteins are ultimately secreted (Filloux, 2004). Another structure has been characterized which is called pseudopilus after its similarity with type IV pilus (T4P). Type IV pili are long fimbrial structures present at the cell surface of various Gram-negative bacteria (Mattick, 2002). Their formation initiates at the cytoplasmic membrane while polymerization of the subunit PilA results in pilus extrusion through the outer membrane pore PilQ (secretin family; Wolfgang et al., 2000). Type IV pili are involved in adhesion, aggregation, or host cell invasion, whereas pilus retraction promotes type IV pili-dependent mobility on solid surfaces.

In *P. aeruginosa*, five Xcp proteins (XcpT–X) are classified as type IV pilin-like protein and called pseudopilins (Bally et al., 1992; Bleves et al., 1998). Like PilA, all are substrates for the prepilin peptidase, XcpA/PilD, which has a dual function and contributes both to type IV piliation and type II secretion (Bally et al., 1992; Nunn and Lory, 1992). XcpA cleaves a short consensus leader peptide located at the N-terminus and preceding a hydrophobic region. Because of this similarity, it was proposed that pseudopilins could form a pilus-like structure or pseudopilus (Tommassen et al., 1992; Hobbs and Mattick, 1993). Attempts to visualize an Xcp-dependent pseudopilus were for long unsuccessful, possibly because such structure may be shorter than a type IV pilus and/or only transiently assembled during the secretion process. However, when overproduced, the most abundant pseudopilin (Nunn and Lory, 1993) XcpT is readily assembled into a long pilus (Durand et al., 2003; **Figure 6**). The 3D structure of XcpT has been resolved and an alphabeta-loop region could be highlighted as a specific motif for identifying major pseudopilins (Alphonse et al., 2010). The long pilus structure may be called hyperpseudopilus or HPP, to emphasize the difference with a physiologically relevant T2SS pseudopilus (Campos et al., 2010, 2011). The extension of the HPP beyond the cell surface is likely to go through the secretin XcpQ. Indeed, formation of the HPP locks the channel and prevents secretion of T2SS substrates which remain inside the bacterial periplasm (Durand et al., 2003).

Among the five pseudopilins, only XcpT is able to form a HPP structure when overproduced, suggesting subtle functions for the other pseudopilins. The unique ability of type IV pili (T4P) to retract and to drive motility relies on the activity of at least two traffic ATPases (Chiang et al., 2008), PilB for elongation (Turner et al., 1993) and PilT for retraction (Misic et al., 2010). In the T2SS, only one ATPase is involved, XcpR (Turner et al., 1993), and thus other mechanism may control the length of the pseudopilus and prevent abnormal elongation such as in the HPP. It has been shown that the pseudopilin XcpX controls the assembly of XcpT into HPP (Durand et al., 2005). Indeed, when the number of XcpX

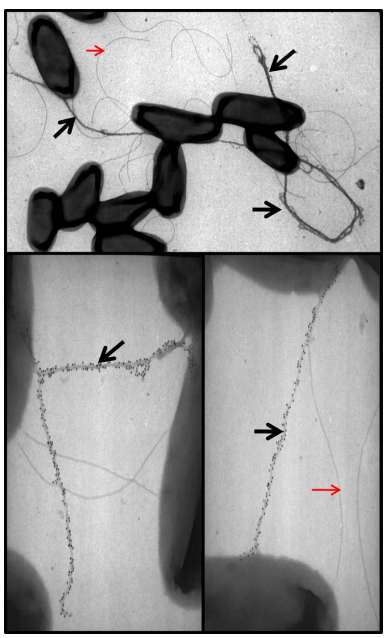

FIGURE 6 | XcpT pseudopilin overexpression results in pseudopilus formation. Shown are the results of TEM analysis of *P. aeruginosa* PAO1 overexpressing *xcpT* from pMTWT as described in Durand et al. (2003). The pseudopili are indicated with a black arrow and are labeled with gold particles coupled to antibodies against XcpT. Red arrows indicate unlabeled flagella for comparison.

subunits increases within the cell the length of the HPP decreases. Conversely, in the absence of XcpX the pseudopilus length is even-longer. The impact of XcpX on XcpT polymerization could be part of the molecular mechanism underlying the dynamic control of pseudopilus elongation and retraction. Several recent studies suggest that the other three minor pilins, XcpU–W, together with XcpX, form a quaternary structure present at the tip of the pilus (Korotkov and Hol, 2008; Douzi et al., 2009). It is worth noting that the globular domain found in the XcpX bulk structure may prevent access of the elongating pseudopilus to the surface by blocking the secretin channel (Korotkov and Hol, 2008). A hypothetical model thus suggests that upon contact between the pseudopilus tip and the secretin, the pseudopilus disassembles/retracts and cycles of such event may push T2SS substrates through the secretin channel in a piston-like mechanism. Recent work indicates that in the Hxc system HxcT does not form a HPP. This characteristic could be assigned to the lack of conserved residues in HxcT that are otherwise found in XcpT-like proteins (Durand et al., 2011). This striking difference may distinguish two subtypes of T2SS.

In conclusion, T2S and T4P systems are similar nanomachines involved in distinct functions but likely constructed from a common ancestor. It is interesting to point out that the T2S/T4P dichotomy may break down in some cases where one system can have both functions, such as with the *V. cholerae* T4P involved in the secretion of a soluble colonization factor, TcpF (Kirn et al., 2003). A major difference is that pili should be long whereas pseudopili should be restricted in length within the space of the periplasm. "To grow or not to grow," this is the issue between type II secretion and type IV piliation. From an evolution perspective, it is amusing to recall Lamarck's thoughts about the length of a giraffe neck. Did the giraffe neck grow as the result of a habit to feed on the highest branch trees or long-necked giraffe were selected because they had an obvious advantage to get food and survive during dearth in places where the soil is nearly always arid and barren.

THE T3SS NEEDLE

The visualization of the needle-like structure in *S.* Typhimurium illustrated the idea of an injectisome (Kubori et al., 1998; **Figure 2**). In *P. aeruginosa* the component of the needle is called PscF which assembles into small needles 60–80 nm in length and 6–7 nm in width (Pastor et al., 2005). PscF multimerization process is dependent on two other Psc components, known as co-chaperones, PscE and PscG (Quinaud et al., 2005). The formation of the needle/filament is prevented when PscF interacts with PscE and PscG which stabilize the monomeric form of PscF (Ple et al., 2010). The needle is likely to protrude at the cell surface by growing through the secretin channel formed by the PscC secretin. As discussed for the pseudopilus, the length of the needle might be tightly controlled by other T3SS components. Studies in *Y. enterocolitica* showed that the C-terminal domain of YscP is the molecular ruler that determines the T3SS needle length (Journet et al., 2003; Mota et al., 2005). PscP has a similar function since its C-terminus could functionally replace the YscP C-terminus (Agrain et al., 2005).

The lower part of the injectisome, lying beneath the needle, structurally resembles the basal body of the flagellum (Schraidt et al., 2010). This observation suggests a common evolutionary origin between the two systems especially because flagellar assembly systems secrete other proteins than flagellin in the extracellular medium, as shown in some rare occasions (Young et al., 1999). The basal body of the T3SS or flagellar assembly system is made by the export apparatus, at the basis of which lies the ATPase, and constitutes the MS ring in the inner membrane (Chevance and Hughes, 2008). Another series of rings are found in the periplasm (P ring) and the outer membrane (L ring) that form the channel through which the flagellin or needle component may travel before assembly. A major difference between flagella and T3SS is the presence of a secretin in the T3SS instead of the L ring in the case of flagellum, but the overall structural of the T3SS nanomachine is now well described (Worrall et al., 2011).

THE T6SS TAIL TUBE

The lack of an outer membrane channel in the T6SS suggests an alternative delivery strategy. Perhaps local puncturing of the cell envelope avoids cell lysis whilst allowing transient assembly of the secretion machine? Interestingly, several studies report similarities between T6SS proteins and components of the T4 bacteriophage (Leiman et al., 2009). Bacteriophages are bacterial viruses which inject their DNA into the bacterial cytosol (Rossmann et al., 2004). Once in the cytosol the phage DNA is replicated, the phage gene products (gp) are synthesized and the bacteria can be used as a phage factory. The injection of the DNA requires a so-called tail tube at the tip of which sits a tail spike or puncturing device which perforates the bacterial cell envelope (Leiman et al., 2010). The tube is made by polymerization of the phage protein gp19, whereas the puncturing device results from the trimeric assembly of two phage proteins, gp27 and gp5.

The discovery that components of the T6SS are structurally similar to gp19, gp5, and gp27 has been a major breakthrough and put the scientific community on a trail to understand how the T6SS machine may work. Briefly, the structure of the Hcp1 protein from the *P. aeruginosa* H1-T6SS was solved in 2006 (Mougous et al., 2006). Hcp1 forms a hexameric ring with an external diameter of 90 Å and a central channel of 40 Å. The Hcp1 hexameric rings can form nanotubes (**Figure 4**) by stacking on top of each other (Ballister et al., 2008). The dimensions of the Hcp nanotube fit with those of the T4 phage tail tube that is composed of the gp19 protein. Thus, despite a low level of amino-acid identity Hcp1 oligomers resemble bacteriophage tail tubes.

The gp5 and gp27 T4 phage components form a trimeric structure reported as the bacteriophage tail spike (Kanamaru et al., 2002). The gp27 homo-trimer forms a cylinder, and is connected to another homo-trimeric complex formed by gp5. The gp5 N-terminal domain forms an oligonucleotide/oligosaccharide-binding site (OB) involved in binding to the bacterial peptidoglycan. The central region contains a lysozyme domain. Upon oligomerization, the C-terminal domains associate into a triple-stranded β-helix. This helix is considered as the needle part of the phage tail that is responsible for puncturing the bacterial outer membrane. Once the membrane is disrupted the OB and lysozyme domains have access to the peptidoglycan, disrupt it and make way to the tail tube and phage DNA injection (Rossmann et al., 2004). Strikingly, VgrG proteins, essential T6SS components, consist of a fusion between the T4 phage proteins gp27 and gp5 (**Figure 4**),

with the exception of the gp5 lysozyme domain that is lacking in VgrGs (Pukatzki et al., 2007; Leiman et al., 2009).

In summary, VgrGs are similar to the tail-spike puncturing device of the T4 phage and might create a channel across the bacterial cell envelope. The VgrG puncturing device is pushed forward across the bacterial cell envelope by a growing nanotube formed by the Hcp component (**Figure 4**). In other words, it could be that the T6SS is a translocation machine operating from the inside to the outside of the bacterial cell and mirrors the phage translocation machine which operates from the outside to the inside of the bacterial cell.

Before the T6SS mechanism can be understood, several questions remain to be answered. Firstly, what are the proteins secreted by the T6SS? Initially VgrG and Hcp were considered as the secreted proteins (Mougous et al., 2006; Pukatzki et al., 2006). However, in light of their homology with the T4 phage tail, it is now believed that these components are reaching the cell surface as part of the secretion machine but are not genuine T6SS effectors. In some cases, the VgrG proteins have a C-terminal extension, such as the actin cross-linking domain of the *V. cholerae* VgrG1(Pukatzki et al., 2007; Ma et al., 2009), or VIP-2 domain of the *Aeromonas hydrophila* VgrG1(Suarez et al., 2010). In this case the effector domain is transported through the cell envelope together with VgrG1 that acts both as a puncturing device and as a carrier for a secreted passenger protein. In *P. aeruginosa*, out of the 10 VgrG that could be identified from the PAO1 genome (Hachani et al., 2011), only the VgrG2b protein seem to have a C-terminal extension with similarity to metalloproteases (Pukatzki et al., 2007). In other cases, genuine secreted proteins have been identified, as the *P. aeruginosa* Tse2 toxin (Hood et al., 2010). One may suggest that Tse2 binds a VgrG protein in a non-covalent manner and is co-transported (Hachani et al., 2011). Alternatively, Tse2 could use the Hcp tube to travel across the cell envelope.

The second important question is: what is the function of the other T6SS components and how much further could the similarity with the phage structure be extended? The answer can be included in the question since it is likely that T6SS components are structurally similar to other proteins from the T4 phage. Indeed, the T4 phage rigid tube (gp19 or Hcp) is surrounded by a contractile sheath and terminated by a multiprotein complex called the baseplate (Kostyuchenko et al., 2003; Yap et al., 2010). The baseplate contains a structure called a wedge (composed of at least seven proteins: gp11, gp10, gp7, gp8, gp6, gp53, and gp25) that is joined around a cylindrical structure that is called the "hub," which includes the puncturing device, gp5/gp27. Importantly, a gp25-like protein has been identified among the T6SS components and may open the list of a long series

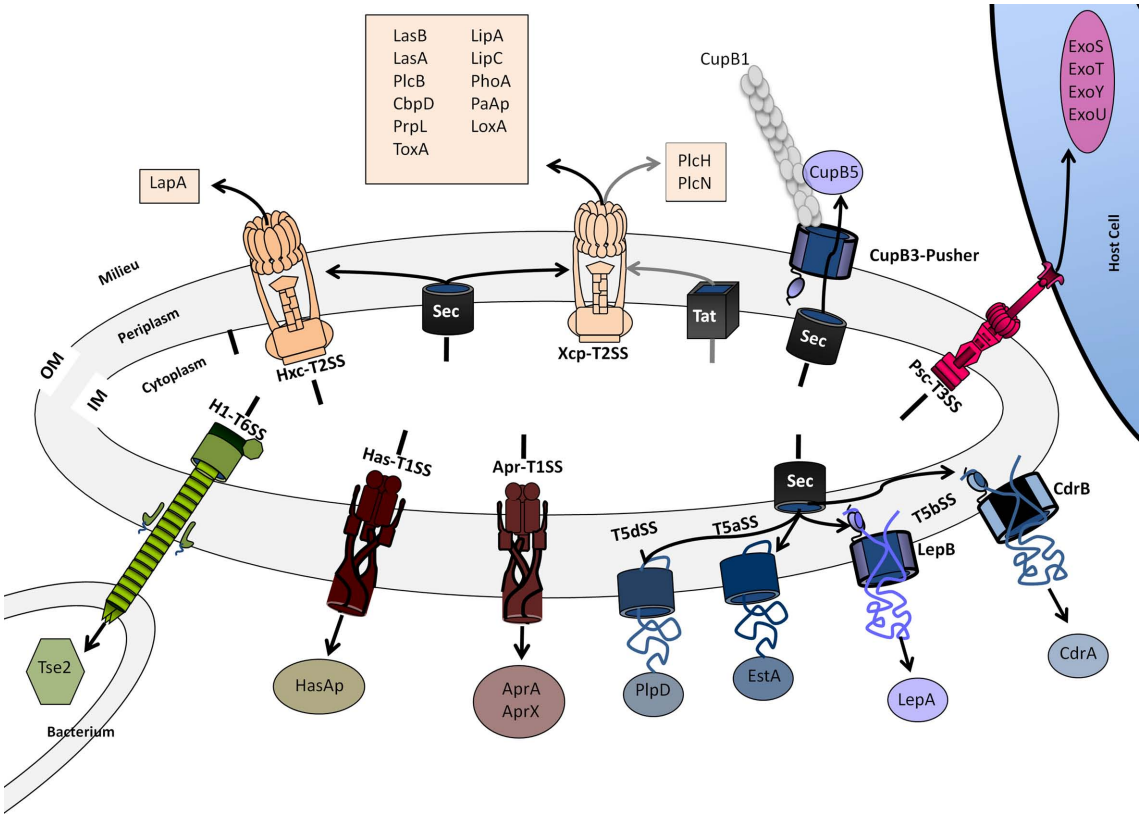

FIGURE 7 | Secretion systems in *P. aeruginosa*. The systems shown are discussed in the main text. For characteristics of exoproteins see also **Table 1**. The design of the secretion systems is as previously presented in Bleves et al. (2010). Color code is: brown (T1SS), light Orange (T2SS), red (T3SS), blue/purple (T5SS), green (T6SS).

of similarity between T6SS and gp proteins (Leiman et al., 2009).

CHAPERONE–USHER PATHWAY: THE LOST KID

Secretion in Gram-negative bacteria involves translocation of proteins across the cell envelope and ultimately across the outer membrane that results in proteins being released in the extracellular medium. Somehow it excluded proteins that remain anchored in the outer membrane. Consequently, assembly of pili was not initially considered as a genuine secretion process. However, in light of what is observed with autotransporters that for most of them remains attached to the membrane, the question may deserve to be readdressed. Coining the autotransporter as a secretion system was adequate considering the IgA protease (Pohlner et al., 1987) since autoproteolytic cleavage released the protease in the medium. These questions are now debatable and reviews readdressing this issue are flourishing (Desvaux et al., 2006, 2009).

In this respect, the chaperone–usher pathway has been for years forgotten in secretion stories, though it is likely the pathway which is best characterized in terms of molecular mechanisms and structural details (Waksman and Hultgren, 2009). The system involves an outer membrane protein, called usher, which oligomerizes and forms a channel. The fimbrial subunits are targeted to the usher by periplasmic chaperones. These chaperones bind the fimbrial subunits emerging from the Sec system and prevent their misfolding in the periplasm. Finally, the fimbrial subunits reach the extracellular medium but they remain bound together to form the filament while the filament remains cell-anchored. Nevertheless, considering the usher as a secretion channel for fimbrial subunits makes sense in light of the description we made of the CupB3 P-usher (Ruer et al., 2008).

In *P. aeruginosa* the pili best described are the type IV pili. However, since their discovery in 2001(Vallet et al., 2001) the importance of chaperone–usher pathways (Cup) in surface attachment and biofilm formation has continuously increased. Several *P. aeruginosa cup* gene clusters have been functionally studied. On the PAO1 genome, four clusters, *cupA*, *cupB*, *cupC*, and *cupE* are identified (Vallet et al., 2001; Giraud et al., 2011). In all cases the cluster includes a gene encoding an usher and one or two genes encoding a chaperone. The *cupA-C* gene clusters encode typical fimbrial subunits whereas the *cupE* gene cluster encodes fimbrial subunits with different characteristics and domains which were first identified in the fimbrial subunits of the chaperone–usher system in *Acinetobacter baumanii* (Tomaras et al., 2003). Both CupB6 and CupE6, display the characteristic two-domain organization of fimbrial adhesins, and CupA4 might also be an adhesin. No gene encoding a putative fimbrial adhesin has been associated with the *cupC* gene cluster. From one *P. aeruginosa* isolate to another the composition in *cup* genes may vary. Remarkably, in the PA14 strain there is an additional *cup* gene cluster, *cupD* (Mikkelsen et al., 2009; Nicastro et al., 2009), which is located on the pathogenicity island PAPI-1(He et al., 2004) and which is very similar in composition and structure to the *cupA* gene cluster. Of note the *exoU* gene is also located on PAPI-1(Kulasekara et al., 2006).

CONCLUDING REMARKS

The diversity of secretion systems is fascinating and likely results from an evolutionary process which has optimized various biological functions into nanomachines whose role is to give access to the extracellular medium for a large variety of proteins.

The diversity may exist within one single organism such as *P. aeruginosa* that possesses all the secretion systems known to date but the T4SS (**Figure 7**). The lack of T4SS in *P. aeruginosa* may be interpreted by the fact that a similar function is exerted by the T3SS, i.e., injecting proteins in eukaryotic host cells.

The specificity of a single system may not be a luxury but a fine-tuned secretion mechanism adapted to the characteristics and features of a subset of exoproteins. Some systems may have evolved after duplication while others have been acquired *via* horizontal gene transfer. In some cases the determination of the architecture reached exquisite details such as with the T3SS. However, in other cases the assembly and overall architecture is now emerging, like with the T2SS. The visualization of these impressive macromolecular complexes always raises the question on how the cell envelope, and particularly the peptidoglycan, are reorganized to make them fit. One obvious answer is the use of specific transglycosylases, or the assembly of the nanomachine at the pole (Senf et al., 2008), region where the peptidoglycan mesh is loose.

In conclusion, though much need to be discovered to understand secretion systems, basic rules are well established and should allow a better understanding of every individual system at the molecular level.

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REFERENCES

Agrain, C., Callebaut, I., Journet, L., Sorg, I., Paroz, C., Mota, L. J., and Cornelis, G. R. (2005). Characterization of a Type III secretion substrate specificity switch (T3S4) domain in YscP from *Yersinia enterocolitica*. *Mol. Microbiol.* 56, 54–67.

Akoh, C. C., Lee, G. C., Liaw, Y. C., Huang, T. H., and Shaw, J. F. (2004). GDSL family of serine esterases/lipases. *Prog. Lipid Res.* 43, 534–552.

Akrim, M., Bally, M., Ball, G., Tommassen, J., Teerink, H., Filloux, A., and Lazdunski, A. (1993). Xcp-mediated protein secretion in *Pseudomonas aeruginosa*: identification of two additional genes and evidence for regulation of xcp gene expression. *Mol. Microbiol.* 10, 431–443.

Alphonse, S., Durand, E., Douzi, B., Waegele, B., Darbon, H., Filloux, A., Voulhoux, R., and Bernard, C. (2010). Structure of the *Pseudomonas aeruginosa* XcpT pseudopilin, a major component of the type II secretion system. *J. Struct. Biol.* 169, 75–80.

Anderson, D. M., and Schneewind, O. (1997). A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* 278, 1140–1143.

Arnoux, P., Haser, R., Izadi, N., Lecroisey, A., Delepierre, M., Wandersman, C., and Czjzek, M. (1999). The crystal structure of HasA, a hemophore secreted by *Serratia marcescens*. *Nat. Struct. Biol.* 6, 516–520.

Arts, J., van Boxtel, R., Filloux, A., Tommassen, J., and Koster, M. (2007). Export of the pseudopilin XcpT of the *Pseudomonas aeruginosa* type II secretion system via the signal recognition particle-Sec pathway. *J. Bacteriol.* 189, 2069–2076.

Aschtgen, M. S., Bernard, C. S., De Bentzmann, S., Lloubes, R., and Cascales, E. (2008). SciN is an outer

membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia coli*. *J. Bacteriol.* 190, 7523–7531.

Backert, S., Ziska, E., Brinkmann, V., Zimny-Arndt, U., Fauconnier, A., Jungblut, P. R., Naumann, M., and Meyer, T. F. (2000). Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell. Microbiol.* 2, 155–164.

Bageshwar, U. K., and Musser, S. M. (2007). Two electrical potential-dependent steps are required for transport by the *Escherichia coli* Tat machinery. *J. Cell Biol.* 179, 87–99.

Ball, G., Chapon-Herve, V., Bleves, S., Michel, G., and Bally, M. (1999). Assembly of XcpR in the cytoplasmic membrane is required for extracellular protein secretion in *Pseudomonas aeruginosa*. *J. Bacteriol.* 181, 382–388.

Ball, G., Durand, E., Lazdunski, A., and Filloux, A. (2002). A novel type II secretion system in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 43, 475–485.

Ballister, E. R., Lai, A. H., Zuckermann, R. N., Cheng, Y., and Mougous, J. D. (2008). In vitro self-assembly of tailorable nanotubes from a simple protein building block. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3733–3738.

Bally, M., Ball, G., Badere, A., and Lazdunski, A. (1991). Protein secretion in *Pseudomonas aeruginosa*: the xcpA gene encodes an integral inner membrane protein homologous to *Klebsiella pneumoniae* secretion function protein PulO. *J. Bacteriol.* 173, 479–486.

Bally, M., Filloux, A., Akrim, M., Ball, G., Lazdunski, A., and Tommassen, J. (1992). Protein secretion in *Pseudomonas aeruginosa*: characterization of seven xcp genes and processing of secretory apparatus components by prepilin peptidase. *Mol. Microbiol.* 6, 1121–1131.

Banerji, S., and Flieger, A. (2004). Patatin-like proteins: a new family of lipolytic enzymes present in bacteria? *Microbiology* 150, 522–525.

Barker, A. P., Vasil, A. I., Filloux, A., Ball, G., Wilderman, P. J., and Vasil, M. L. (2004). A novel extracellular phospholipase C of *Pseudomonas aeruginosa* is required for phospholipid chemotaxis. *Mol. Microbiol.* 53, 1089–1098.

Baumann, U., Wu, S., Flaherty, K. M., and McKay, D. B. (1993). Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel beta roll motif. *EMBO J.* 12, 3357–3364.

Benabdelhak, H., Kiontke, S., Horn, C., Ernst, R., Blight, M. A., Holland, I. B., and Schmitt, L. (2003). A specific interaction between the NBD of the ABC-transporter HlyB and a C-terminal fragment of its transport substrate haemolysin A. *J. Mol. Biol.* 327, 1169–1179.

Berthelot, P., Attree, I., Plesiat, P., Chabert, J., de Bentzmann, S., Pozzetto, B., and Grattard, F. (2003). Genotypic and phenotypic analysis of type III secretion system in a cohort of *Pseudomonas aeruginosa* bacteremia isolates: evidence for a possible association between O serotypes and exo genes. *J. Infect. Dis.* 188, 512–518.

Bever, R. A., and Iglewski, B. H. (1988). Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. *J. Bacteriol.* 170, 4309–4314.

Bitter, W., Koster, M., Latijnhouwers, M., de Cock, H., and Tommassen, J. (1998). Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 27, 209–219.

Bleves, S., Gerard-Vincent, M., Lazdunski, A., and Filloux, A. (1999). Structure-function analysis of XcpP, a component involved in general secretory pathway-dependent protein secretion in *Pseudomonas aeruginosa*. *J. Bacteriol.* 181, 4012–4019.

Bleves, S., Lazdunski, A., and Filloux, A. (1996). Membrane topology of three Xcp proteins involved in exoprotein transport by *Pseudomonas aeruginosa*. *J. Bacteriol.* 178, 4297–4300.

Bleves, S., Viarre, V., Salacha, R., Michel, G. P., Filloux, A., and Voulhoux, R. (2010). Protein secretion systems in *Pseudomonas aeruginosa*: a wealth of pathogenic weapons. *Int. J. Med. Microbiol.* 300, 534–543.

Bleves, S., Voulhoux, R., Michel, G., Lazdunski, A., Tommassen, J., and Filloux, A. (1998). The secretion apparatus of *Pseudomonas aeruginosa*: identification of a fifth pseudopilin, XcpX (GspK family). *Mol. Microbiol.* 27, 31–40.

Blocker, A., Komoriya, K., and Aizawa, S. (2003). Type III secretion systems and bacterial flagella: insights into their function from structural similarities. *Proc. Natl. Acad. Sci. U.S.A.* 100, 3027–3030.

Blocker, A. J., Deane, J. E., Veenendaal, A. K., Roversi, P., Hodgkinson, J. L., Johnson, S., and Lea, S. M. (2008). What's the point of the type III secretion system needle? *Proc. Natl. Acad. Sci. U.S.A.* 105, 6507–6513.

Bonemann, G., Pietrosiuk, A., Diemand, A., Zentgraf, H., and Mogk, A. (2009). Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. *EMBO J.* 28, 315–325.

Bonemann, G., Pietrosiuk, A., and Mogk, A. (2010). Tubules and donuts: a type VI secretion story. *Mol. Microbiol.* 76, 815–821.

Borlee, B. R., Goldman, A. D., Murakami, K., Samudrala, R., Wozniak, D. J., and Parsek, M. R. (2010). *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Mol. Microbiol.* 75, 827–842.

Braun, P., de Groot, A., Bitter, W., and Tommassen, J. (1998). Secretion of elastinolytic enzymes and their propeptides by *Pseudomonas aeruginosa*. *J. Bacteriol.* 180, 3467–3469.

Braun, P., Tommassen, J., and Filloux, A. (1996). Role of the propeptide in folding and secretion of elastase of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 19, 297–306.

Brok, R., Van Gelder, P., Winterhalter, M., Ziese, U., Koster, A. J., de Cock, H., Koster, M., Tommassen, J., and Bitter, W. (1999). The C-terminal domain of the *Pseudomonas* secretin XcpQ forms oligomeric rings with pore activity. *J. Mol. Biol.* 294, 1169–1179.

Broms, J. E., Forslund, A. L., Forsberg, A., and Francis, M. S. (2003). PcrH of *Pseudomonas aeruginosa* is essential for secretion and assembly of the type III translocon. *J. Infect. Dis.* 188, 1909–1921.

Broz, P., Mueller, C. A., Muller, S. A., Philippsen, A., Sorg, I., Engel, A., and Cornelis, G. R. (2007). Function and molecular architecture of the *Yersinia* injectisome tip complex. *Mol. Microbiol.* 65, 1311–1320.

Brutinel, E. D., Vakulskas, C. A., Brady, K. M., and Yahr, T. L. (2008). Characterization of ExsA and of ExsA-dependent promoters required for expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol. Microbiol.* 68, 657–671.

Campos, M., Francetic, O., and Nilges, M. (2011). Modeling pilus structures from sparse data. *J. Struct. Biol.* 173, 436–444.

Campos, M., Nilges, M., Cisneros, D. A., and Francetic, O. (2010). Detailed structural and assembly model of the type II secretion pilus from sparse data. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13081–13086.

Cascales, E., and Christie, P. J. (2003). The versatile bacterial type IV secretion systems. *Nat. Rev. Microbiol.* 1, 137–149.

Chami, M., Guilvout, I., Gregorini, M., Remigy, H. W., Muller, S. A., Valerio, M., Engel, A., Pugsley, A. P., and Bayan, N. (2005). Structural insights into the secretin PulD and its trypsin-resistant core. *J. Biol. Chem.* 280, 37732–37741.

Chandran, V., Fronzes, R., Duquerroy, S., Cronin, N., Navaza, J., and Waksman, G. (2009). Structure of the outer membrane complex of a type IV secretion system. *Nature* 462, 1011–1015.

Chevance, F. F., and Hughes, K. T. (2008). Coordinating assembly of a bacterial macromolecular machine. *Nat. Rev. Microbiol.* 6, 455–465.

Chiang, P., Sampaleanu, L. M., Ayers, M., Pahuta, M., Howell, P. L., and Burrows, L. L. (2008). Functional role of conserved residues in the characteristic secretion NTPase motifs of the *Pseudomonas aeruginosa* type IV pilus motor proteins PilB, PilT and PilU. *Microbiology* 154, 114–126.

Choo, K. H., Tan, T. W., and Ranganathan, S. (2009). A comprehensive assessment of N-terminal signal peptides prediction methods. *BMC Bioinformatics* 10(Suppl. 15), S2. doi: 10.1186/1471-2105-10-S15-S2

Christie, P. J., Ward, J. E. Jr., Gordon, M. P., and Nester, E. W. (1989). A gene required for transfer of T-DNA to plants encodes an ATPase with autophosphorylating activity. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9677–9681.

Chung, C. W., You, J., Kim, K., Moon, Y., Kim, H., and Ahn, J. H. (2009). Export of recombinant proteins in *Escherichia coli* using ABC transporter with an attached lipase ABC transporter recognition domain (LARD). *Microb. Cell Fact.* 8, 11.

Clantin, B., Delattre, A. S., Rucktooa, P., Saint, N., Meli, A. C., Locht, C., Jacob-Dubuisson, F., and Villeret, V. (2007). Structure of the membrane protein FhaC: a member of the Omp85-TpsB transporter superfamily. *Science* 317, 957–961.

Collins, R. F., Ford, R. C., Kitmitto, A., Olsen, R. O., Tonjum, T., and Derrick, J. P. (2003). Three-dimensional structure of the *Neisseria meningitidis* secretin PilQ determined from negative-stain transmission electron microscopy. *J. Bacteriol.* 185, 2611–2617.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nat. Rev. Microbiol.* 4, 811–825.

Cornelis, G. R. (2010). The type III secretion injectisome, a complex nanomachine for intracellular "toxin" delivery. *Biol. Chem.* 391, 745–751.

Cossart, P., and Sansonetti, P. J. (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* 304, 242–248.

Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., and Rosenbusch, J. P. (1992). Crystal structures explain functional properties of two *E. coli* porins. *Nature* 358, 727–733.

Dacheux, D., Goure, J., Chabert, J., Usson, Y., and Attree, I. (2001). Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas aeruginosa*-infected macrophages. *Mol. Microbiol.* 40, 76–85.

Daefler, S., Guilvout, I., Hardie, K. R., Pugsley, A. P., and Russel, M. (1997). The C-terminal domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pIVf1 function. *Mol. Microbiol.* 24, 465–475.

Das, S., and Chaudhuri, K. (2003). Identification of a unique IAHP (IcmF associated homologous proteins) cluster in *Vibrio cholerae* and other proteobacteria through in silico analysis. *In silico Biol.* 3, 287–300.

Dautin, N., and Bernstein, H. D. (2007). Protein secretion in gram-negative bacteria via the autotransporter pathway. *Annu. Rev. Microbiol.* 61, 89–112.

de Groot, A., Filloux, A., and Tommassen, J. (1991). Conservation of xcp genes, involved in the two-step protein secretion process, in different *Pseudomonas* species and other gram-negative bacteria. *Mol. Gen. Genet.* 229, 278–284.

de Groot, A., Koster, M., Gerard-Vincent, M., Gerritse, G., Lazdunski, A., Tommassen, J., and Filloux, A. (2001). Exchange of Xcp (Gsp) secretion machineries between *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*: species specificity unrelated to substrate recognition. *J. Bacteriol.* 183, 959–967.

de Groot, A., Krijger, J. J., Filloux, A., and Tommassen, J. (1996). Characterization of type II protein secretion (xcp) genes in the plant growth-stimulating *Pseudomonas putida*, strain WCS358. *Mol. Gen. Genet.* 250, 491–504.

d'Enfert, C., Ryter, A., and Pugsley, A. P. (1987). Cloning and expression in *Escherichia coli* of the *Klebsiella pneumoniae* genes for production, surface localization and secretion of the lipoprotein pullulanase. *EMBO J.* 6, 3531–3538.

Deng, Q., and Barbieri, J. T. (2008). Molecular mechanisms of the cytotoxicity of ADP-ribosylating toxins. *Annu. Rev. Microbiol.* 62, 271–288.

Desvaux, M., Hebraud, M., Henderson, I. R., and Pallen, M. J. (2006). Type III secretion: what's in a name? *Trends Microbiol.* 14, 157–160.

Desvaux, M., Hebraud, M., Talon, R., and Henderson, I. R. (2009). Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. *Trends Microbiol.* 17, 139–145.

Dong, C., Beis, K., Nesper, J., Brunkan-Lamontagne, A. L., Clarke, B. R., Whitfield, C., and Naismith, J. H. (2006). Wza the translocon for *E. coli* capsular polysaccharides defines a new class of membrane protein. *Nature* 444, 226–229.

Douzi, B., Durand, E., Bernard, C., Alphonse, S., Cambillau, C., Filloux, A., Tegoni, M., and Voulhoux, R. (2009). The XcpV/GspI pseudopilin has a central role in the assembly of a quaternary complex within the T2SS pseudopilus. *J. Biol. Chem.* 284, 34580–34589.

Duong, F., Bonnet, E., Geli, V., Lazdunski, A., Murgier, M., and Filloux, A. (2001). The AprX protein of *Pseudomonas aeruginosa*: a new substrate for the Apr type I secretion system. *Gene* 262, 147–153.

Duong, F., Lazdunski, A., Cami, B., and Murgier, M. (1992). Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. *Gene* 121, 47–54.

Duong, F., Lazdunski, A., and Murgier, M. (1996). Protein secretion by heterologous bacterial ABC-transporters: the C-terminus secretion signal of the secreted protein confers high recognition specificity. *Mol. Microbiol.* 21, 459–470.

Durand, E., Alphonse, S., Brochier-Armanet, C., Ball, G., Douzi, B., Filloux, A., Bernard, C., and Voulhoux, R. (2011). The assembly mode of the pseudopilus: a hallmark to distinguish a novel secretion system subtype. *J. Biol. Chem.* 286, 24407–24416.

Durand, E., Bernadac, A., Ball, G., Lazdunski, A., Sturgis, J. N., and Filloux, A. (2003). Type II protein secretion in *Pseudomonas aeruginosa*: the pseudopilus is a multifibrillar and adhesive structure. *J. Bacteriol.* 185, 2749–2758.

Durand, E., Michel, G., Voulhoux, R., Kurner, J., Bernadac, A., and Filloux, A. (2005). XcpX controls biogenesis of the *Pseudomonas aeruginosa* XcpT-containing pseudopilus. *J. Biol. Chem.* 280, 31378–31389.

Durand, E., Verger, D., Rego, A. T., Chandran, V., Meng, G., Fronzes, R., and Waksman, G. (2009). Structural biology of bacterial secretion systems in gram-negative pathogens – potential for new drug targets. *Infect. Disord. Drug Targets* 9, 518–547.

Feldman, M. F., Muller, S., Wuest, E., and Cornelis, G. R. (2002). SycE allows secretion of YopE-DHFR hybrids by the *Yersinia enterocolitica* type III Ysc system. *Mol. Microbiol.* 46, 1183–1197.

Filloux, A. (2004). The underlying mechanisms of type II protein secretion. *Biochim. Biophys. Acta* 1694, 163–179.

Filloux, A. (2009). The type VI secretion system: a tubular story. *EMBO J.* 28, 309–310.

Filloux, A. (2010). Secretion signal and protein targeting in bacteria: a biological puzzle. *J. Bacteriol.* 192, 3847–3849.

Filloux, A., Bally, M., Ball, G., Akrim, M., Tommassen, J., and Lazdunski, A. (1990). Protein secretion in gram-negative bacteria: transport across the outer membrane involves common mechanisms in different bacteria. *EMBO J.* 9, 4323–4329.

Filloux, A., Bally, M., Murgier, M., Wretlind, B., and Lazdunski, A. (1989). Cloning of Xcp-genes located at the 55-min region of the chromosome and involved in protein secretion in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 3, 261–265.

Filloux, A., Bally, M., Soscia, C., Murgier, M., and Lazdunski, A. (1988). Phosphate regulation in *Pseudomonas aeruginosa*: cloning of the alkaline phosphatase gene and identification of phoB- and phoR-like genes. *Mol. Gen. Genet.* 212, 510–513.

Filloux, A., Hachani, A., and Bleves, S. (2008). The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology* 154, 1570–1583.

Filloux, A., and Hardie, K. R. (1998). A systematic approach to the study of protein secretion in Gram-negative bacteria. *Methods Microbiol.* 27, 301–318.

Filloux, A., Murgier, M., Wretlind, B., and Lazdunski, A. (1987). Characterization of 2 *Pseudomonas aeruginosa* mutants with defective secretion of extracellular proteins and comparison with other mutants. *FEMS Microbiol. Lett.* 40, 159–163.

Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J. P., Fleiszig, S. M., Wu, C., Mende-Mueller, L., and Frank, D. W. (1997). ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Mol. Microbiol.* 25, 547–557.

Finck-Barbancon, V., Yahr, T. L., and Frank, D. W. (1998). Identification and characterization of SpcU, a chaperone required for efficient secretion of the ExoU cytotoxin. *J. Bacteriol.* 180, 6224–6231.

Fleiszig, S. M., Wiener-Kronish, J. P., Miyazaki, H., Vallas, V., Mostov, K. E., Kanada, D., Sawa, T., Yen, T. S., and Frank, D. W. (1997). *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. *Infect. Immun.* 65, 579–586.

Folders, J., Algra, J., Roelofs, M. S., van Loon, L. C., Tommassen, J., and Bitter, W. (2001). Characterization of *Pseudomonas aeruginosa* chitinase, a gradually secreted protein. *J. Bacteriol.* 183, 7044–7052.

Folders, J., Tommassen, J., van Loon, L. C., and Bitter, W. (2000). Identification of a chitin-binding protein secreted by *Pseudomonas aeruginosa*. *J. Bacteriol.* 182, 1257–1263.

Fox, A., Haas, D., Reimmann, C., Heeb, S., Filloux, A., and Voulhoux, R. (2008). Emergence of secretion-defective sublines of *Pseudomonas aeruginosa* PAO1 resulting from spontaneous mutations in the vfr global regulatory gene. *Appl. Environ. Microbiol.* 74, 1902–1908.

Francetic, O., and Pugsley, A. P. (2005). Towards the identification of type II secretion signals in a nonacylated variant of pullulanase from *Klebsiella oxytoca*. *J. Bacteriol.* 187, 7045–7055.

Frank, D. W. (1997). The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 26, 621–629.

Gerard-Vincent, M., Robert, V., Ball, G., Bleves, S., Michel, G. P., Lazdunski, A., and Filloux, A. (2002). Identification of XcpP domains that confer functionality and specificity to the *Pseudomonas aeruginosa* type II secretion apparatus. *Mol. Microbiol.* 44, 1651–1665.

Ghigo, J. M., Letoffe, S., and Wandersman, C. (1997). A new type of hemophore-dependent heme acquisition system of *Serratia marcescens* reconstituted in *Escherichia coli*. *J. Bacteriol.* 179, 3572–3579.

Giraud, C., Bernard, C. S., Calderon, V., Yang, L., Filloux, A., Molin, S., Fichant, G., Bordi, C., and de Bentzmann, S. (2011). The PprA-PprB two-component system activates CupE, the first non-archetypal *Pseudomonas aeruginosa* chaperone-usher pathway system assembling fimbriae. *Environ. Microbiol.* 13, 666–683.

Goodman, A. L., Merighi, M., Hyodo, M., Ventre, I., Filloux, A., and Lory, S. (2009). Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev.* 23, 249–259.

Goure, J., Broz, P., Attree, O., Cornelis, G. R., and Attree, I. (2005). Protective anti-V antibodies inhibit *Pseudomonas* and *Yersinia* translocon assembly within host membranes. *J. Infect. Dis.* 192, 218–225.

Goure, J., Pastor, A., Faudry, E., Chabert, J., Dessen, A., and Attree, I. (2004). The V antigen of *Pseudomonas aeruginosa* is required for assembly of the functional PopB/PopD translocation pore in host cell membranes. *Infect. Immun.* 72, 4741–4750.

Guzzo, J., Murgier, M., Filloux, A., and Lazdunski, A. (1990). Cloning of the *Pseudomonas aeruginosa* alkaline protease gene and secretion of the protease into the medium by *Escherichia coli*. *J. Bacteriol.* 172, 942–948.

Guzzo, J., Pages, J. M., Duong, F., Lazdunski, A., and Murgier, M. (1991a). *Pseudomonas aeruginosa* alkaline protease: evidence for secretion genes and study of secretion mechanism. *J. Bacteriol.* 173, 5290–5297.

Guzzo, J., Duong, F., Wandersman, C., Murgier, M., and Lazdunski, A. (1991b). The secretion genes of *Pseudomonas aeruginosa* alkaline protease are functionally related to those of *Erwinia chrysanthemi* proteases and *Escherichia coli* alpha-haemolysin. *Mol. Microbiol.* 5, 447–453.

Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jove, D., and Filloux, A. (2011). Type VI secretion system in *Pseudomonas Aeruginosa*: secretion and multimerization of VgrG proteins. *J. Biol. Chem.* 286, 12317–12327.

Hardie, K. R., Lory, S., and Pugsley, A. P. (1996). Insertion of an outer membrane protein in *Escherichia coli* requires a chaperone-like protein. *EMBO J.* 15, 978–988.

Hauser, A. R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat. Rev. Microbiol.* 7, 654–665.

Hauser, A. R., Cobb, E., Bodi, M., Mariscal, D., Valles, J., Engel, J. N., and Rello, J. (2002). Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. *Crit. Care Med.* 30, 521–528.

He, J., Baldini, R. L., Deziel, E., Saucier, M., Zhang, Q., Liberati, N. T., Lee, D., Urbach, J., Goodman, H. M., and Rahme, L. G. (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2530–2535.

Heikkila, M. P., Honisch, U., Wunsch, P., and Zumft, W. G. (2001). Role of the Tat ransport system in nitrous oxide reductase translocation and cytochrome cd1 biosynthesis in *Pseudomonas stutzeri*. *J. Bacteriol.* 183, 1663–1671.

Higgins, C. F. (2001). ABC transporters: physiology, structure and mechanism – an overview. *Res. Microbiol.* 152, 205–210.

Higgins, M. K., Bokma, E., Koronakis, E., Hughes, C., and Koronakis, V. (2004). Structure of the periplasmic component of a bacterial drug efflux pump. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9994–9999.

Hinsa, S. M., Espinosa-Urgel, M., Ramos, J. L., and O'Toole, G. A. (2003). Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Mol. Microbiol.* 49, 905–918.

Hobbs, M., and Mattick, J. S. (1993). Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. *Mol. Microbiol.* 10, 233–243.

Hodak, H., Clantin, B., Willery, E., Villeret, V., Locht, C., and Jacob-Dubuisson, F. (2006). Secretion signal of the filamentous haemagglutinin, a model two-partner secretion substrate. *Mol. Microbiol.* 61, 368–382.

Hodgkinson, J. L., Horsley, A., Stabat, D., Simon, M., Johnson, S., da Fonseca, P. C., Morris, E. P., Wall, J. S., Lea, S. M., and Blocker, A. J. (2009). Three-dimensional reconstruction of the *Shigella* T3SS transmembrane regions reveals 12-fold symmetry and novel features throughout. *Nat. Struct. Mol. Biol.* 16, 477–485.

Holland, I. B., Schmitt, L., and Young, J. (2005). Type 1 protein secretion in bacteria, the ABC-transporter dependent pathway (review). *Mol. Membr. Biol.* 22, 29–39.

Hood, R. D., Singh, P., Hsu, F., Guvener, T., Carl, M. A., Trinidad, R. R., Silverman, J. M., Ohlson, B. B., Hicks, K. G., Plemel, R. L., Li, M., Schwarz, S., Wang, W. Y., Merz, A. J., Goodlett, D. R., and Mougous, J. D. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe* 7, 25–37.

Jacob-Dubuisson, F., Locht, C., and Antoine, R. (2001). Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. *Mol. Microbiol.* 40, 306–313.

Jaeger, K. E., Ransac, S., Dijkstra, B. W., Colson, C., van Heuvel, M., and Misset, O. (1994). Bacterial lipases. *FEMS Microbiol. Rev.* 15, 29–63.

Jepkorir, G., Rodriguez, J. C., Rui, H., Im, W., Lovell, S., Battaile, K. P., Alontaga, A. Y., Yukl, E. T., Moënne-Loccoz, P., and Rivera, M. (2010). Structural, NMR spectroscopic, and computational investigation of hemin loading in the hemophore HasAp from *Pseudomonas aeruginosa*. *J. Am. Chem. Soc.* 132, 9857–9872.

Journet, L., Agrain, C., Broz, P., and Cornelis, G. R. (2003). The needle length of bacterial injectisomes is determined by a molecular ruler. *Science* 302, 1757–1760.

Junker, M., Besingi, R. N., and Clark, P. L. (2009). Vectorial transport and folding of an autotransporter virulence protein during outer membrane secretion. *Mol. Microbiol.* 71, 1323–1332.

Kajava, A. V., and Steven, A. C. (2006). The turn of the screw: variations of the abundant beta-solenoid motif in passenger domains of Type V secretory proteins. *J. Struct. Biol.* 155, 306–315.

Kanamaru, S., Leiman, P. G., Kostyuchenko, V. A., Chipman, P. R., Mesyanzhinov, V. V., Arisaka, F., and Rossmann, M. G. (2002). Structure of the cell-puncturing device of bacteriophage T4. *Nature* 415, 553–557.

Kelley, L. A., and Sternberg, M. J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.* 4, 363–371.

Kessler, E., and Safrin, M. (1994). The propeptide of *Pseudomonas aeruginosa* elastase acts an elastase inhibitor. *J. Biol. Chem.* 269, 22726–22731.

Kida, Y., Higashimoto, Y., Inoue, H., Shimizu, T., and Kuwano, K. (2008). A novel secreted protease from *Pseudomonas aeruginosa* activates NF-kappaB through protease-activated receptors. *Cell. Microbiol.* 10, 1491–1504.

Kirn, T. J., Bose, N., and Taylor, R. K. (2003). Secretion of a soluble colonization factor by the TCP type 4 pilus biogenesis pathway in *Vibrio cholerae*. *Mol. Microbiol.* 49, 81–92.

Koronakis, V., Koronakis, E., and Hughes, C. (1989). Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes. *EMBO J.* 8, 595–605.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., and Hughes, C. (2000). Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* 405, 914–919.

Korotkov, K. V., Gonen, T., and Hol, W. G. (2011). Secretins: dynamic channels for protein transport across membranes. *Trends Biochem. Sci.* PMID: 21565514. [Epub ahead of print].

Korotkov, K. V., and Hol, W. G. (2008). Structure of the GspK-GspI-GspJ complex from the enterotoxigenic *Escherichia coli* type 2 secretion system. *Nat. Struct. Mol. Biol.* 15, 462–468.

Korotkov, K. V., Pardon, E., Steyaert, J., and Hol, W. G. (2009). Crystal structure of the N-terminal domain of the secretin GspD from ETEC determined with the assistance of a nanobody. *Structure* 17, 255–265.

Koster, M., Bitter, W., de Cock, H., Allaoui, A., Cornelis, G. R., and Tommassen, J. (1997). The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. *Mol. Microbiol.* 26, 789–797.

Kostyuchenko, V. A., Leiman, P. G., Chipman, P. R., Kanamaru, S., van Raaij, M. J., Arisaka, F., Mesyanzhinov, V. V., and Rossmann, M. G. (2003). Three-dimensional structure of bacteriophage T4 baseplate. *Nat. Struct. Biol.* 10, 688–693.

Kowalska, K., Soscia, C., Combe, H., Vasseur, P., Voulhoux, R., and Filloux, A. (2010). The C-terminal amphipathic alpha-helix of *Pseudomonas aeruginosa* PelC outer membrane protein is required for its function. *Biochimie* 92, 33–40.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galán, J. E., and Aizawa, S. I. (1998). Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* 280, 602–605.

Kulasekara, B. R., Kulasekara, H. D., Wolfgang, M. C., Stevens, L., Frank, D. W., and Lory, S. (2006). Acquisition and evolution of the exoU locus in *Pseudomonas aeruginosa*. *J. Bacteriol.* 188, 4037–4050.

Lambert, O., Benabdelhak, H., Chami, M., Jouan, L., Nouaille, E., Ducruix, A., and Brisson, A. (2005). Trimeric structure of OprN and OprM efflux proteins from *Pseudomonas aeruginosa*, by 2D electron crystallography. *J. Struct. Biol.* 150, 50–57.

Lee, V. T., Pukatzki, S., Sato, H., Kikawada, E., Kazimirova, A. A., Huang, J., Li, X., Arm, J. P., Frank, D. W., and Lory, S. (2007). Pseudolipasin A is a specific inhibitor for phospholipase A2 activity of *Pseudomonas aeruginosa* cytotoxin ExoU. *Infect. Immun.* 75, 1089–1098.

Leiman, P. G., Arisaka, F., van Raaij, M. J., Kostyuchenko, V. A., Aksyuk, A. A., Kanamaru, S., and Rossmann, M. G. (2010). Morphogenesis of the T4 tail and tail fibers. *Virol. J.* 7, 355.

Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., Burley, S. K., Almo, S. C., and Mekalanos, J. J. (2009). Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4154–4159.

Letoffe, S., Delepelaire, P., and Wandersman, C. (1996). Protein secretion in gram-negative bacteria: assembly of the three components of ABC protein-mediated exporters is ordered and promoted by substrate binding. *EMBO J.* 15, 5804–5811.

Letoffe, S., Nato, F., Goldberg, M. E., and Wandersman, C. (1999). Interactions of HasA, a bacterial haemophore, with haemoglobin and with its outer membrane receptor HasR. *Mol. Microbiol.* 33, 546–555.

Letoffe, S., Redeker, V., and Wandersman, C. (1998). Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA haemophore. *Mol. Microbiol.* 28, 1223–1234.

Lin, H. T., Bavro, V. N., Barrera, N. P., Frankish, H. M., Velamakanni, S., van Veen, H. W., Robinson, C. V., Borges-Walmsley, M. I., and Walmsley, A. R. (2009). MacB ABC transporter is a dimer whose ATPase activity and macrolide-binding capacity are regulated by the membrane fusion protein MacA. *J. Biol. Chem.* 284, 1145–1154.

Lobedanz, S., Bokma, E., Symmons, M. F., Koronakis, E., Hughes, C., and Koronakis, V. (2007). A periplasmic coiled-coil interface underlying TolC recruitment and the assembly of bacterial drug efflux pumps. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4612–4617.

Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2002). The "LSGGQ" motif in each nucleotide-binding domain of human P-glycoprotein is adjacent to the opposing walker A sequence. *J. Biol. Chem.* 277, 41303–41306.

Lu, H. M., Mizushima, S., and Lory, S. (1993). A periplasmic intermediate in the extracellular secretion pathway of *Pseudomonas aeruginosa* exotoxin A. *J. Bacteriol.* 175, 7463–7467.

Ma, A. T., McAuley, S., Pukatzki, S., and Mekalanos, J. J. (2009). Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell Host Microbe* 5, 234–243.

Mackman, N., Nicaud, J. M., Gray, L., and Holland, I. B. (1985). Identification of polypeptides required for the export of haemolysin 2001 from *E. coli*. *Mol. Gen. Genet.* 201, 529–536.

Macnab, R. M. (2003). How bacteria assemble flagella. *Annu. Rev. Microbiol.* 57, 77–100.

Martinez, A., Ostrovsky, P., and Nunn, D. N. (1999). LipC, a second lipase of *Pseudomonas aeruginosa*, is LipB and Xcp dependent and is transcriptionally regulated by pilus biogenesis components. *Mol. Microbiol.* 34, 317–326.

Masi, M., and Wandersman, C. (2010). Multiple signals direct the assembly and function of a type 1 secretion system. *J. Bacteriol.* 192, 3861–3869.

Mattick, J. S. (2002). Type IV pili and twitching motility. *Annu. Rev. Microbiol.* 56, 289–314.

Mazar, J., and Cotter, P. A. (2007). New insight into the molecular mechanisms of two-partner secretion. *Trends Microbiol.* 15, 508–515.

McIver, K. S., Kessler, E., and Ohman, D. E. (2004). Identification of residues in the *Pseudomonas aeruginosa* elastase propeptide required for chaperone and secretion activities. *Microbiology* 150, 3969–3977.

McIver, K. S., Kessler, E., Olson, J. C., and Ohman, D. E. (1995). The elastase propeptide functions as an intramolecular chaperone required for elastase activity and secretion in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 18, 877–889.

Meng, G., Surana, N. K., St Geme, J. W. III, and Waksman, G. (2006). Structure of the outer membrane translocator domain of the Haemophilus influenzae Hia trimeric autotransporter. *EMBO J.* 25, 2297–2304.

Merrell, D. S., and Falkow, S. (2004). Frontal and stealth attack strategies in microbial pathogenesis. *Nature* 430, 250–256.

Michel, G., Bleves, S., Ball, G., Lazdunski, A., and Filloux, A. (1998). Mutual stabilization of the XcpZ and XcpY components of the secretory apparatus in *Pseudomonas aeruginosa*. *Microbiology* 144(Pt 12), 3379–3386.

Michel, G. P., Durand, E., and Filloux, A. (2007). XphA/XqhA, a novel GspCD subunit for type II secretion in *Pseudomonas aeruginosa*. *J. Bacteriol.* 189, 3776–3783.

Michel, L. O., Sandkvist, M., and Bagdasarian, M. (1995). Specificity of the protein secretory apparatus: secretion of the heat-labile enterotoxin B subunit pentamers by different species of gram- bacteria. *Gene* 152, 41–45.

Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J. M., and Cornelis, G. (1990). Secretion of Yop proteins by Yersiniae. *Infect. Immun.* 58, 2840–2849.

Mikkelsen, H., Ball, G., Giraud, C., and Filloux, A. (2009). Expression of *Pseudomonas aeruginosa* CupD fimbrial genes is antagonistically controlled by RcsB and the EAL-containing PvrR response regulators. *PLoS ONE* 4, e6018. doi: 10.1371/journal.pone.0006018

Mikolosko, J., Bobyk, K., Zgurskaya, H. I., and Ghosh, P. (2006). Conformational flexibility in the multidrug efflux system protein AcrA. *Structure* 14, 577–587.

Misic, A. M., Satyshur, K. A., and Forest, K. T. (2010). *P. aeruginosa* PilT structures with and without nucleotide reveal a dynamic type IV pilus retraction motor. *J. Mol. Biol.* 400, 1011–1021.

Mota, L. J., Journet, L., Sorg, I., Agrain, C., and Cornelis, G. R. (2005). Bacterial injectisomes: needle length does matter. *Science* 307, 1278.

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordoñez, C. L., Lory, S., Walz, T., Joachimiak, A., and Mekalanos, J. J. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312, 1526–1530.

Nagai, H., Cambronne, E. D., Kagan, J. C., Amor, J. C., Kahn, R. A., and Roy, C. R. (2005). A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 826–831.

Nicastro, G. G., Boechat, A. L., Abe, C. M., Kaihami, G. H., and Baldini, R. L. (2009). *Pseudomonas aeruginosa* PA14 cupD transcription is activated by the RcsB response regulator, but repressed by its putative cognate sensor RcsC. *FEMS Microbiol. Lett.* 301, 115–123.

Nunn, D. N., and Lory, S. (1991). Product of the *Pseudomonas aeruginosa* gene pilD is a prepilin leader peptidase. *Proc. Natl. Acad. Sci. U.S.A.* 88, 3281–3285.

Nunn, D. N., and Lory, S. (1992). Components of the protein-excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. *Proc. Natl. Acad. Sci. U.S.A.* 89, 47–51.

Nunn, D. N., and Lory, S. (1993). Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -U, -V, and -W. *J. Bacteriol.* 175, 4375–4382.

Oomen, C. J., van Ulsen, P., van Gelder, P., Feijen, M., Tommassen, J., and Gros, P. (2004). Structure of the translocator domain of a bacterial autotransporter. *EMBO J.* 23, 1257–1266.

Ostroff, R. M., and Vasil, M. L. (1987). Identification of a new phospholipase C activity by analysis of an insertional mutation in the hemolytic phospholipase C structural gene of *Pseudomonas aeruginosa*. *J. Bacteriol.* 169, 4597–4601.

Palmer, T., and Berks, B. C. (2003). Moving folded proteins across the bacterial cell membrane. *Microbiology* 149, 547–556.

Palmer, T., Berks, B. C., and Sargent, F. (2010). Analysis of Tat targeting function and twin-arginine signal peptide activity in *Escherichia coli*. *Methods Mol. Biol.* 619, 191–216.

Papanikou, E., Karamanou, S., and Economou, A. (2007). Bacterial protein secretion through the translocase nanomachine. *Nat. Rev. Microbiol.* 5, 839–851.

Parsot, C., Hamiaux, C., and Page, A. L. (2003). The various and varying roles of specific chaperones in type III secretion systems. *Curr. Opin. Microbiol.* 6, 7–14.

Pastor, A., Chabert, J., Louwagie, M., Garin, J., and Attree, I. (2005). PscF is a major component of the *Pseudomonas aeruginosa* type III secretion needle. *FEMS Microbiol. Lett.* 253, 95–101.

Patel, S., and Latterich, M. (1998). The AAA team: related ATPases with diverse functions. *Trends Cell Biol.* 8, 65–71.

Pei, X. Y., Hinchliffe, P., Symmons, M. F., Koronakis, E., Benz, R., Hughes, C., and Koronakis, V. (2011). Structures of sequential open states in a symmetrical opening transition of the TolC exit duct. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2112–2117.

Phan, G., Benabdelhak, H., Lascombe, M. B., Benas, P., Rety, S., Picard, M., Ducruix, A., Etchebest, C., and Broutin, I. (2010). Structural and dynamical insights into the opening mechanism of *P. aeruginosa* OprM channel. *Structure* 18, 507–517.

Planet, P. J., Kachlany, S. C., DeSalle, R., and Figurski, D. H. (2001). Phylogeny of genes for secretion NTPases: identification of the widespread tadA subfamily and development of a diagnostic key for gene classification. *Proc. Natl. Acad. Sci. U.S.A.* 98, 2503–2508.

Ple, S., Job, V., Dessen, A., and Attree, I. (2010). Cochaperone interactions in export of the type III needle component PscF of *Pseudomonas aeruginosa*. *J. Bacteriol.* 192, 3801–3808.

Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T. F. (1987). Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature* 325, 458–462.

Possot, O., and Pugsley, A. P. (1994). Molecular characterization of PulE, a protein required for pullulanase secretion. *Mol. Microbiol.* 12, 287–299.

Pozidis, C., Chalkiadaki, A., Gomez-Serrano, A., Stahlberg, H., Brown, I., Tampakaki, A. P., Lustig, A., Sianidis, G., Politou, A. S., Engel, A., Panopoulos, N. J., Mansfield, J., Pugsley, A. P., Karamanou, S, and Economou, A. (2003). Type III protein translocase: HrcN is a peripheral ATPase that is activated by oligomerization. *J. Biol. Chem.* 278, 25816–25824.

Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., and Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15508–15513.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F., and Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1528–1533.

Qiu, X., Gurkar, A. U., and Lory, S. (2006). Interstrain transfer of the large pathogenicity island (PAPI-1) of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19830–19835.

Quinaud, M., Chabert, J., Faudry, E., Neumann, E., Lemaire, D., Pastor, A., Elsen, S., Dessen, A., and Attree, I. (2005). The PscE-PscF-PscG complex controls type III secretion needle biogenesis in *Pseudomonas aeruginosa*. *J. Biol. Chem.* 280, 36293–36300.

Reichow, S. L., Korotkov, K. V., Hol, W. G., and Gonen, T. (2010). Structure of the cholera toxin secretion channel in its closed state. *Nat. Struct. Mol. Biol.* 17, 1226–1232.

Robert, V., Filloux, A., and Michel, G. P. (2005a). Subcomplexes from the Xcp secretion system of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* 252, 43–50.

Robert, V., Filloux, A., and Michel, G. P. (2005b). Role of XcpP in the functionality of the *Pseudomonas aeruginosa* secreton. *Res. Microbiol.* 156, 880–886.

Robien, M. A., Krumm, B. E., Sandkvist, M., and Hol, W. G. (2003). Crystal structure of the extracellular protein secretion NTPase EpsE of *Vibrio cholerae*. *J. Mol. Biol.* 333, 657–674.

Rossmann, M. G., Mesyanzhinov, V. V., Arisaka, F., and Leiman, P. G. (2004). The bacteriophage T4 DNA injection machine. *Curr. Opin. Struct. Biol.* 14, 171–180.

Roy, P. H., Tetu, S. G., Larouche, A., Elbourne, L., Tremblay, S., Ren, Q., Dodson, R., Harkins, D., Shay, R., Watkins, K., Mahamoud, Y., and Paulsen, I. T. (2010). Complete genome sequence of the multiresistant taxonomic outlier *Pseudomonas aeruginosa* PA7. *PLoS ONE* 5, e8842. doi: 10.1371/journal.pone.0008842

Ruer, S., Ball, G., Filloux, A., and de Bentzmann, S. (2008). The "P-usher", a novel protein transporter involved in fimbrial assembly and TpsA secretion. *EMBO J.* 27, 2669–2680.

Sagulenko, E., Sagulenko, V., Chen, J., and Christie, P. J. (2001). Role of *Agrobacterium* VirB11 ATPase in T-pilus assembly and substrate selection. *J. Bacteriol.* 183, 5813–5825.

Saier, M. H. Jr. (2006). Protein secretion and membrane insertion systems in gram-negative bacteria. *J. Membr. Biol.* 214, 75–90.

Salacha, R., Kovacic, F., Brochier-Armanet, C., Wilhelm, S., Tommassen, J., Filloux, A., Voulhoux, R., and Bleves, S. (2010). The *Pseudomonas aeruginosa* patatin-like protein PlpD is the archetype of a novel Type V secretion system. *Environ. Microbiol.* 12, 1498–1512.

Sardis, M. F., and Economou, A. (2010). SecA: a tale of two protomers. *Mol. Microbiol.* 76, 1070–1081.

Sargent, F. (2007). The twin-arginine transport system: moving folded proteins across membranes. *Biochem. Soc. Trans.* 35, 835–847.

Sato, H., and Frank, D. W. (2004). ExoU is a potent intracellular phospholipase. *Mol. Microbiol.* 53, 1279–1290.

Sauri, A., Soprova, Z., Wickström, D., de Gier, J. W., Van der Schors, R. C., Smit, A. B., Jong, W. S., and Luirink, J. (2009). The Bam (Omp85) complex is involved in secretion of the autotransporter haemoglobin protease. *Microbiology* 155, 3982–3991.

Sauvonnet, N., Vignon, G., Pugsley, A. P., and Gounon, P. (2000). Pilus formation and protein secretion by the same machinery in *Escherichia coli*. *EMBO J.* 19, 2221–2228.

Sawa, T., Yahr, T. L., Ohara, M., Kurahashi, K., Gropper, M. A., Wiener-Kronish, J. P., and Frank, D. W. (1999). Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury. *Nat. Med.* 5, 392–398.

Schmitt, L., Benabdelhak, H., Blight, M. A., Holland, I. B., and Stubbs, M. T. (2003). Crystal structure of the nucleotide-binding domain of the ABC-transporter haemolysin B: identification of a variable region within ABC helical domains. *J. Mol. Biol.* 330, 333–342.

Schoehn, G., Di Guilmi, A. M., Lemaire, D., Attree, I., Weissenhorn, W., and Dessen, A. (2003). Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in *Pseudomonas*. *EMBO J.* 22, 4957–4967.

Schraidt, O., Lefebre, M. D., Brunner, M. J., Schmied, W. H., Schmidt, A., Radics, J., Mechtler, K., Galán, J. E., and Marlovits, T. C. (2010). Topology and organization of the *Salmonella typhimurium* type III secretion needle complex components. *PLoS Pathog.* 6, e1000824. doi: 10.1371/journal.ppat.1000824

Schraidt, O., and Marlovits, T. C. (2011). Three-dimensional model of *Salmonella*'s needle complex at subnanometer resolution. *Science* 331, 1192–1195.

Senf, F., Tommassen, J., and Koster, M. (2008). Polar secretion of proteins via the Xcp type II secretion system in *Pseudomonas aeruginosa*. *Microbiology* 154, 3025–3032.

Sexton, J. A., and Vogel, J. P. (2002). Type IVB secretion by intracellular pathogens. *Traffic* 3, 178–185.

Shen, D. K., Quenee, L., Bonnet, M., Kuhn, L., Derouazi, M., Lamotte, D., Toussaint. B., and Polack, B. (2008). Orf1/SpcS chaperones ExoS for type three secretion by *Pseudomonas aeruginosa*. *Biomed. Environ. Sci.* 21, 103–109. PMID: 18548848

Shevchik, V. E., and Condemine, G. (1998). Functional characterization of the *Erwinia chrysanthemi* OutS protein, an element of a type II secretion system. *Microbiology* 144 (Pt 11): 3219–3228.

Shevchik, V. E., Robert-Baudouy, J., and Condemine, G. (1997). Specific interaction between OutD, an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway, and secreted proteins. *EMBO J.* 16, 3007–3016.

Spreter, T., Yip, C. K., Sanowar, S., Andre, I., Kimbrough, T. G., Vuckovic, M., Pfuetzner, R. A., Deng, W., Yu, A. C., Finlay, B. B., Baker, D., Miller, S. I., and Strynadka, N. C. (2009). A conserved structural motif mediates formation of the periplasmic rings in the type III secretion system. *Nat. Struct. Mol. Biol.* 16, 468–476.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., and Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406, 959–964.

Suarez, G., Sierra, J. C., Erova, T. E., Sha, J., Horneman, A. J., and Chopra, A. K. (2010). A type VI secretion system effector protein, VgrG1, from *Aeromonas hydrophila* that induces host cell toxicity by ADP ribosylation of actin. *J. Bacteriol.* 192, 155–168.

Symmons, M. F., Bokma, E., Koronakis, E., Hughes, C., and Koronakis, V. (2009). The assembled structure of a complete tripartite bacterial multidrug efflux pump. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7173–7178.

Thanabalu, T., Koronakis, E., Hughes, C., and Koronakis, V. (1998). Substrate-induced assembly of a contiguous channel for protein export from *E. coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. *EMBO J.* 17, 6487–6496.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., and Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology* 149, 3473–3484.

Tommassen, J., Filloux, A., Bally, M., Murgier, M., and Lazdunski, A. (1992). Protein secretion in *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.* 9, 73–90.

Trepout, S., Taveau, J. C., Benabdelhak, H., Granier, T., Ducruix, A., Frangakis, A. S., and Lambert, O. (2010). Structure of reconstituted bacterial membrane efflux pump by cryo-electron tomography. *Biochim. Biophys. Acta* 1798, 1953–1960.

Turner, L. R., Lara, J. C., Nunn, D. N., and Lory, S. (1993). Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 175, 4962–4969.

Vallet, I., Olson, J. W., Lory, S., Lazdunski, A., and Filloux, A. (2001). The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6911–6916.

van den Berg, B. (2010). Crystal structure of a full-length autotransporter. *J. Mol. Biol.* 396, 627–633.

van Ulsen, P., Rutten, L., Feller, M., Tommassen, J., and van der Ende, A. (2008). Two-partner secretion systems of *Neisseria meningitidis* associated with invasive clonal complexes. *Infect. Immun.* 76, 4649–4658.

Vance, R. E., Hong, S., Gronert, K., Serhan, C. N., and Mekalanos, J. J. (2004). The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2135–2139.

Viarre, V., Cascales, E., Ball, G., Michel, G. P., Filloux, A., and Voulhoux, R. (2009). HxcQ liposecretin is self-piloted to the outer membrane by its N-terminal lipid anchor. *J. Biol. Chem.* 284, 33815–33823.

Vonheijne, G. (1985). Signal sequences – the limits of variation. *J. Mol. Biol.* 184, 99–105.

Voulhoux, R., Ball, G., Ize, B., Vasil, M. L., Lazdunski, A., Wu, L. F., and Filloux, A. (2001). Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. *EMBO J.* 20, 6735–6741.

Voulhoux, R., Taupiac, M. P., Czjzek, M., Beaumelle, B., and Filloux, A. (2000). Influence of deletions within domain II of exotoxin A on its extracellular secretion from *Pseudomonas aeruginosa*. *J. Bacteriol.* 182, 4051–4058.

Waksman, G., and Hultgren, S. J. (2009). Structural biology of the chaperone-usher pathway of pilus biogenesis. *Nat. Rev. Microbiol.* 7, 765–774.

Wandersman, C., and Delepelaire, P. (2004). Bacterial iron sources: from siderophores to hemophores. *Annu. Rev. Microbiol.* 58, 611–647.

Wilhelm, S., Tommassen, J., and Jaeger, K. E. (1999). A novel lipolytic enzyme located in the outer membrane of *Pseudomonas aeruginosa*. *J. Bacteriol.* 181, 6977–6986.

Winans, S. C., Burns, D. L., and Christie, P. J. (1996). Adaptation of a conjugal transfer system for the export of pathogenic macromolecules. *Trends Microbiol.* 4, 64–68.

Wohlfarth, S., Hoesche, C., Strunk, C., and Winkler, U. K. (1992). Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1. *J. Gen. Microbiol.* 138, 1325–1335.

Wolfgang, M., van Putten, J. P., Hayes, S. F., Dorward, D., and Koomey, M. (2000). Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *EMBO J.* 19, 6408–6418.

Wolfgang, M. C., Kulasekara, B. R., Liang, X., Boyd, D., Wu, K., Yang, Q., Miyada, C. G., and Lory, S. (2003). Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8484–8489.

Worrall, L. J., Lameignere, E., and Strynadka, N. C. (2011). Structural overview of the bacterial injectisome. *Curr. Opin. Microbiol.* 14, 3–8.

Wretlind, B., and Pavlovskis, O. R. (1984). Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. *J. Bacteriol.* 158, 801–808.

Yahr, T. L., Goranson, J., and Frank, D. W. (1996). Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. *Mol. Microbiol.* 22, 991–1003.

Yahr, T. L., Mende-Mueller, L. M., Friese, M. B., and Frank, D. W. (1997). Identification of type III secreted products of the *Pseudomonas aeruginosa* exoenzyme S regulon. *J. Bacteriol.* 179, 7165–7168.

Yahr, T. L., Vallis, A. J., Hancock, M. K., Barbieri, J. T., and Frank, D. W. (1998). ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. *Proc. Natl. Acad. Sci. U.S.A.* 95, 13899–13904.

Yap, M. L., Mio, K., Leiman, P. G., Kanamaru, S., and Arisaka, F. (2010). The baseplate wedges of bacteriophage T4 spontaneously assemble into hubless baseplate-like structure in vitro. *J. Mol. Biol.* 395, 349–360.

Yen, Y. T., Kostakioti, M., Henderson, I. R., and Stathopoulos, C. (2008). Common themes and variations in serine protease autotransporters. *Trends Microbiol.* 16, 370–379.

Young, G. M., Schmiel, D. H., and Miller, V. L. (1999). A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6456–6461.

Yukl, E. T., Jepkorir, G., Alontaga, A. Y., Pautsch, L., Rodriguez, J. C., Rivera, M., and Moenne-Loccoz, P. (2010). Kinetic and spectroscopic studies of hemin acquisition in the hemophore HasAp from *Pseudomonas aeruginosa*. *Biochemistry* 49, 6646–6654.

Zhang, L. H., Fath, M. J., Mahanty, H. K., Tai, P. C., and Kolter, R. (1995). Genetic analysis of the colicin V secretion pathway. *Genetics* 141, 25–32.

Zhong, X., Kolter, R., and Tai, P. C. (1996). Processing of colicin V-1, a secretable marker protein of a bacterial ATP binding cassette export system, requires membrane integrity, energy, and cytosolic factors. *J. Biol. Chem.* 271, 28057–28063.

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Intrinsic and extrinsic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic pathogen that is particularly problematic in the healthcare setting where it is a frequent cause of pneumonia, bloodstream, and urinary tract infections. An important determinant of *P. aeruginosa* virulence is a type III secretion system (T3SS). T3SS-dependent intoxication is a complex process that minimally requires binding of *P. aeruginosa* to host cells, injection of the cytotoxic effector proteins through the host cell plasma membrane, and induction of T3SS gene expression. The latter process, referred to as contact-dependent expression, involves a well-characterized regulatory cascade that activates T3SS gene expression in response to host cell contact. Although host cell contact is a primary activating signal for T3SS gene expression, the involvement of multiple membrane-bound regulatory systems indicates that additional environmental signals also play a role in controlling expression of the T3SS. These regulatory systems coordinate T3SS gene expression with many other cellular activities including motility, mucoidy, polysaccharide production, and biofilm formation. The signals to which the organism responds are poorly understood but many seem to be coupled to the metabolic state of the cell and integrated within a master circuit that assimilates informational signals from endogenous and exogenous sources. Herein we review progress toward unraveling this complex circuitry, provide analysis of the current knowledge gaps, and highlight potential areas for future studies. Complete understanding of the regulatory networks that control T3SS gene expression will maximize opportunities for the development of strategies to treat *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa*, type III secretion, injectisome, gene regulation, ExsA, cAMP, RsmA, Vfr

INTRODUCTION

Pseudomonas aeruginosa is widely appreciated for its ability to survive under adverse conditions. In its native habitat of soil and water, *P. aeruginosa* is exposed to a myriad of potential growth substrates, competing microorganisms, and outright predators. To cope with these hostile conditions *P. aeruginosa* has a relatively large genome (6.3 Mb) encoding diverse metabolic pathways and defense mechanisms, and has extensive regulatory networks that reprogram gene expression patterns to suit the prevailing environmental conditions (Stover et al., 2000). Many of the defense mechanisms that provide a selective advantage in soil and water also contribute to virulence in the mammalian host. For instance, the *P. aeruginosa* type III secretion system (T3SS) contributes to virulence in ameba, insect, zebrafish, and mammalian infection models and protects *P. aeruginosa* from phagocytosis irrespective of whether the phagocyte is an ameba or a human macrophage (Pukatzki et al., 2002; Miyata et al., 2003; Brannon et al., 2009).

Type III secretion systems contribute to the virulence of many medically relevant Gram-negative organisms and function like a molecular syringe to inject bacterial effector proteins into eukaryotic host cells (Coburn et al., 2007). The injectisome consists of ~25 proteins that form two ring-like structures embedded in the inner and outer membranes, a rod-like structure that spans the periplasmic space, a needle-like structure that extends from the cell surface, and a translocation complex that forms a channel in the host cell membrane (Moraes et al., 2008; Worrall et al., 2011). The effector proteins are transported through the bacterial cell envelope via the hollow rod and needle proteins, and injected through the translocation pore into the host cell. Assembly of the injectisome is dependent upon the T3SS which is located in the inner membrane and is thought to occur in a highly ordered process involving sequential secretion and assembly of the rod, needle, and translocator proteins (Wagner et al., 2010). While the injectisome structure and composition is highly conserved, each pathogen has a unique set of effector proteins suited to its individual lifestyle (Coburn et al., 2007). *P. aeruginosa* uses the injectisome to translocate at least five effector proteins (ExoS, ExoT, ExoU, ExoY, and FliC) into host cells (Hauser, 2009). The translocated effectors facilitate phagocytic avoidance, tissue invasion and systemic spread, and modulate the inflammatory response (Engel and Balachandran, 2009). Regulation of T3SS transcription is intrinsically controlled by the activity of the injectisome through a well-described and self-contained (encoded) regulatory mechanism (Brutinel and Yahr, 2008). T3SS gene expression is also influenced by extrinsic regulatory genes but in most cases the mechanisms involved are poorly understood. In this review we focus on recent progress toward defining mechanisms that regulate T3SS gene expression.

INTRINSIC REGULATION OF T3SS GENE EXPRESSION

ExsA IS THE CENTRAL REGULATOR OF T3SS GENE EXPRESSION

The *P. aeruginosa* T3SS regulon consists of ~40 genes organized within 10 transcriptional units that encode the secretion and translocation machinery, intrinsic regulators of T3SS gene expression

(ExsA, ExsD, ExsC, and ExsE), chaperones, and effectors (Frank, 1997). The central regulator of the T3SS regulon is ExsA, a member of the AraC/XylS family of transcriptional activators (Yahr and Frank, 1994; Hovey and Frank, 1995; Yahr et al., 1995). ExsA autoregulates its own expression through the P_{exsC} promoter and is encoded as the last gene of the *exsCEBA* polycistronic mRNA (Yahr and Frank, 1994). An alignment of the 10 ExsA-dependent promoters has identified a consensus binding site (tAaAAAnwnMyGrCynnnmYTGayAk) with three areas of high sequence conservation: an adenine-rich region, a GxC sequence centered at the -45 position with respect to the transcriptional start site, and a TGxxA sequence located near the -35 position (**Figure 1**; Hovey and Frank, 1995; Brutinel et al., 2008). Mutagenesis studies have confirmed that each of these elements is required for ExsA binding and activation of the P_{exoT} promoter (Brutinel et al., 2008).

Typical of most AraC/XylS family members, ExsA has an amino-terminal domain (NTD) involved in self-association and regulatory functions (discussed further below), and a carboxy-terminal domain (CTD) that contains two helix-turn-helix DNA-binding motifs (Brutinel et al., 2009). In electrophoretic mobility shift assays (EMSA) purified ExsA binds to the P_{exoT}, P_{exsD}, and P_{exsC} promoters with apparent equilibrium constants in the low (1–5) nM range (Brutinel et al., 2008). Two distinct ExsA-promoter complexes are observed: a higher mobility complex representing one ExsA monomer bound to the promoter probe and a lower mobility complex containing two bound ExsA monomers (Brutinel et al., 2008). Mutagenesis and footprinting studies indicate that this binding arrangement represents two adjacent binding sites for monomeric ExsA with binding site 1 overlapping the conserved GxC and TGxxA sequences, and binding site 2 located upstream of site 1 (**Figure 1**). Whereas binding of ExsA to site 1 of the P_{exoT} promoter is required for occupation of site 2, deletion of binding site 2 has no effect on occupation of site 1 (Brutinel et al., 2008). In addition, occupation of binding site 2 is dependent upon monomer–monomer interactions mediated by the amino-terminal domain of ExsA (Brutinel et al., 2009). These combined data are supportive of a monomer assembly model whereby an ExsA monomer bound to site 1 recruits a second ExsA monomer to binding site 2. The apparent lack of nucleotide sequence conservation between ExsA binding sites 1 and 2, however, raises the possibility that monomer–monomer

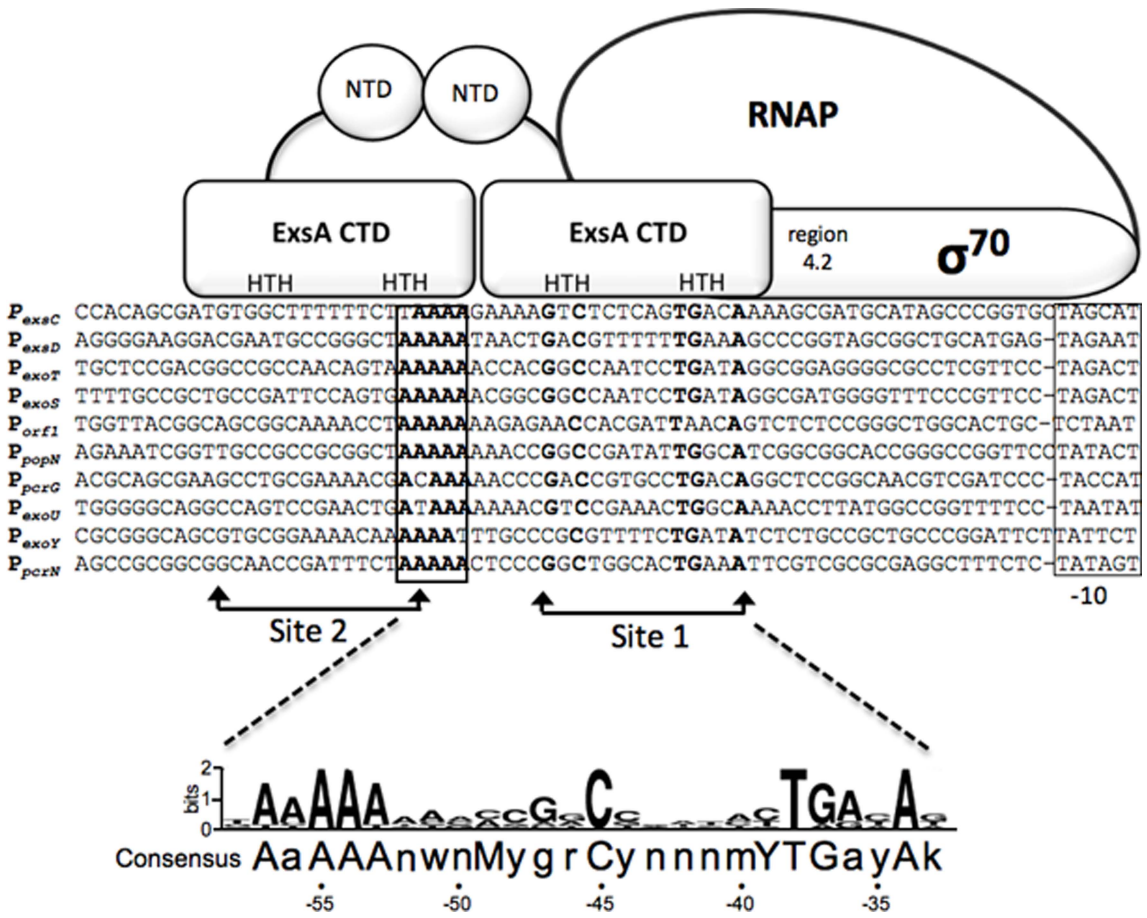

FIGURE 1 | Sequence alignment of ExsA-dependent promoters and working model for promoter recognition and recruitment of σ^{70}-RNAP by ExsA. Nucleotides in bold are highly conserved in all ExsA-dependent promoters and have been shown through mutagenesis and DNA-biding studies to be important for ExsA-dependent activation. The boxed sequences represent the -10 promoter recognition site. Predicted points of interaction for the helix-turn-helix (HTH) DNA-binding motifs of each ExsA monomer are indicated. Studies have shown that ExsA recruits σ^{70}-RNAP through interactions mediated by region 4.2 of σ^{70}. The ExsA consensus binding sequence is indicated at the bottom of the diagram.

interactions are the primary determinant for occupation of site 2. While genetic studies are suggestive of cooperative binding to the P_{exsC}, P_{exoT}, and P_{exsD} promoters, the Hill coefficients derived from EMSA experiments are supportive of cooperative binding only at the P_{exsC} promoter (1.9 ± 0.2; Brutinel et al., 2008). This discrepancy may reflect a fundamental difference in the binding of ExsA to the P_{exsC} promoter and is supported by the observation that ExsA elicits a significant bend (78°) upon binding to the P_{exsC} promoter that correlates with increased promoter activity and cooperative binding interactions (Brutinel et al., 2008).

ExsA-dependent promoters possess -35 and -10 sequence elements similar to those of σ^{70}-dependent promoters with one significant difference (Brutinel et al., 2008; **Figure 1**). Unlike canonical σ^{70}-dependent promoters where the -35 and -10 elements are separated by ~17 bp, the same regions in ExsA-dependent promoters are separated by 21 or 22 bp. *In vitro* transcription and primer extension assays indicate that the promoters are indeed σ^{70}-dependent and that the -10 regions are functional recognition determinants for σ^{70} (Vakulskas et al., 2009). The apparent -35 elements, however, are not required for recognition by σ^{70} but rather serve as determinants for ExsA binding (Vakulskas et al., 2010). ExsA activates transcription by compensating for the lack of functional -35 sites primarily through recruitment of RNA polymerase-σ^{70} holoenzyme to the promoter but also has a small effect on isomerization to an open complex (Vakulskas et al., 2009). Like many AraC/XylS family members, ExsA interacts with region 4.2 of σ^{70} to recruit RNAP-σ^{70} to the promoter (Vakulskas et al., 2010). Somewhat atypical for an AraC/XylS family member, however, is that interactions with the α-subunit of RNAP are not required for ExsA-dependent activation. Although occupation of binding sites 1 and 2 on the promoter is required for ExsA-dependent transcription, it is unclear whether one or both monomers interact with RNAP-σ^{70} to facilitate recruitment. The proximity of binding site 1 to the expected location for a -35 region, and the fact that ExsA compensates for the lack of a functional -35 region, is consistent with a model in which ExsA bound to site 1 interacts with region 4.2 of σ^{70} and ExsA bound to site 2 stabilizes ExsA bound to site 1 and/or the ExsA-RNAP-σ^{70} complex.

REGULATION OF ExsA ACTIVITY BY THE ExsADCE REGULATORY CASCADE

Type III secretion systems have been described as contact-dependent secretion systems because translocation of the effector proteins requires intimate contact between the pathogen and the host, and because host cell contact induces T3SS gene expression (Hayes et al., 2010). Growth of *P. aeruginosa* under Ca^{2+}-limiting conditions, or in the presence of serum, mimics the host-contact signal and is commonly used in the laboratory as a surrogate for the host-contact signal (Frank, 1997; Vallis et al., 1999; Kim et al., 2005; Urbanowski et al., 2007). Low Ca^{2+}, serum, and host cell contact are each thought to convert the injectisome to a secretion-competent state through a poorly defined mechanism (Vallis et al., 1999; McCaw et al., 2002). ExsA activity is intimately coupled to secretion by a partner-switching mechanism involving three additional proteins; ExsC, ExsD, and ExsE (Brutinel and Yahr, 2008). Both ExsC and ExsD have two potential binding partners. ExsD is an anti-activator that inhibits T3SS gene expression by disrupting both the self-association and DNA-binding activities of ExsA (Brutinel et al., 2010). ExsC functions as an anti-anti-activator and forms a 2:2 stoichiometric complex with ExsD (Dasgupta et al., 2004; Lykken et al., 2006; Zheng et al., 2007). ExsC is also a T3SS chaperone and forms a 2:1 complex with ExsE (Zheng et al., 2007). Finally, ExsE is a secreted regulator that prevents ExsC from associating with ExsD (Rietsch et al., 2005; Urbanowski et al., 2005). The current working model is that ExsA-dependent transcription is inactive under non-permissive conditions (i.e., high Ca^{2+}) because the binding equilibria favor formation of the inhibitory ExsD•ExsA and ExsC•ExsE complexes. In contrast, inducing conditions trigger secretion and/or translocation of ExsE (Urbanowski et al., 2007). The resulting decrease in the intracellular concentration of ExsE favors formation of the ExsD•ExsC complex (i.e., partner switching), thereby releasing ExsA to activate transcription. An important feature of the regulatory cascade is that minor changes in the levels of ExsC, ExsE, ExsA, or ExsD (as little as threefold) can have profound effects on T3SS gene expression (Dasgupta et al., 2004).

The partner-switching mechanism predicts that the binding affinities of ExsE, ExsC, ExsD, and ExsA are, in order of strongest affinity, ExsC•ExsE > ExsC•ExsD > ExsD•ExsA. While the binding affinity of the ExsD–ExsA complex has not been determined, the binding constants (Kd) for the ExsC•ExsE and ExsC•ExsD complexes are 1 and 18 nM, respectively (Zheng et al., 2007). Recent analyses of the ExsE•ExsC and ExsD crystal structures provide a basis for the differential binding of ExsC to ExsE and ExsD (Bernhards et al., 2009; Vogelaar et al., 2010). The ExsE monomer contains two ExsC-binding domains located in the amino- and carboxy-terminal portions of the protein and each domain contains an Arg-X-Val-X-Arg motif and a partially overlapping β-motif. In the co-complex the unstructured ExsE monomer wraps around the ExsC homodimer and each Arg-X-Val-X-Arg motif interacts with one of the ExsC monomers, thereby accounting for the 2:1 binding stoichiometry of the ExsC•ExsE complex. Interestingly, the amino-terminal region of ExsD also has an Arg-X-Val-X-Arg motif, a β-motif, and shares 22.5% sequence identity with the amino-terminus of ExsE. Whereas the entire ExsE protein (i.e., both Arg-X-Val-X-Arg motifs) is necessary for formation of the ExsC•ExsE complex, the ExsD amino-terminal region alone is sufficient to bind ExsC. This observation suggests that each ExsD monomer binds to one-half of the ExsC dimer and this likely accounts for the 2:2 binding stoichiometry of the ExsC•ExsD complex. Furthermore, the presence of a common ExsC-binding motif in ExsE and ExsD is consistent with the observation that binding of ExsE and ExsD to ExsC are mutually exclusive (Vogelaar et al., 2010).

ExsD is a dynamic protein capable of forming a homo–trimeric complex in solution, a 1:1 stoichiometric complex with ExsA, and a 2:2 stoichiometric complex with ExsC (Zheng et al., 2007; Bernhards et al., 2009; Thibault et al., 2009). Curiously, the ExsD crystal structure revealed that the amino-terminus of ExsD has structural similarity to the DNA-binding domain of the KorB transcriptional repressor (Bernhards et al., 2009). The predicted DNA-binding interface, however, is buried within the trimeric structure suggesting that dissociation of the trimer might be required for ExsD to exhibit DNA-binding activity. This property might account for the observation that trimeric ExsD lacks

DNA-binding activity (Bernhards et al., 2009). Future studies will be required to determine whether monomeric and/or dimeric ExsD has DNA-binding activity and, if so, whether such activity is of biological significance.

Studies of the ExsD•ExsA interaction have relied upon co-expression of the proteins *in vivo* and subsequent purification as an ExsD•ExsA complex. Consistent with the functional role of ExsD as an anti-activator, the purified ExsA•ExsD complex lacks DNA-binding activity (Thibault et al., 2009; Brutinel et al., 2010). ExsA dissociated from the ExsD•ExsA complex through ExsC addition, however, is able to bind to DNA, indicating that ExsD does not irreversibly inactivate ExsA (Brutinel et al., 2010). The initial and rapid transcriptional response to inducing signals, therefore, likely reflects dissociation of existing ExsD•ExsA complexes by ExsC.

The response to non-inducing signals (i.e., high Ca^{2+}) appears more complicated because neither purified trimeric ExsD, nor ExsD dissociated from the ExsC•ExsD complex, is capable of binding to ExsA *in vitro* (Thibault et al., 2009; Brutinel et al., 2010). These data, when combined with the observation that ExsD•ExsA complex formation is dependent upon concurrent expression of the proteins *in vivo*, suggest that complex formation may involve a folding intermediate of either ExsD or ExsA. ExsD dissociated from the ExsC•ExsD complex, therefore, does not contribute to the inhibition of ExsA-dependent transcription. Furthermore, the fact that trimeric ExsD is unable to bind to ExsA suggests two potential pathways for irreversible inactivation of ExsD: homo–trimerization and ExsC•ExsD complex formation. The response to inhibiting signals (i.e., high Ca^{2+}) exhibits a lag period (Diaz and Yahr, unpublished) that may reflect a requisite decay of the existing free ExsA and a requirement for *de novo* synthesis of ExsD to sequester the newly synthesized ExsA.

ExsD inhibits both the self-association and DNA-binding properties of ExsA (Thibault et al., 2009; Brutinel et al., 2010). Since the amino-terminal domain of ExsA mediates self-association and is sufficient for complex formation with ExsD, ExsD likely inhibits ExsA self-association through steric hindrance. Less clear is how ExsD inhibits the carboxy-terminal DNA-binding domain when bound to the amino-terminus of ExsA. Resolution of this question will require structural studies to determine whether bound ExsD elicits a conformational change to the DNA-binding domain of ExsA or physically occludes one or both of the helix-turn-helix DNA-binding domains.

PHYSIOLOGICAL IMPLICATIONS OF THE ExsADCE REGULATORY CASCADE

The T3SS functions as a primary defense mechanism against phagocytosis and must be rapidly activated following contact with a phagocyte. Prior to host cell contact, *P. aeruginosa* cells already express low levels of preexisting injectisomes (Rietsch and Mekalanos, 2006) and effectors that initiate the defense response. The ExsADCE regulatory cascade functions to increase the magnitude of the response by rapidly inducing expression of the injectisome components and effectors. While upregulation of T3SS gene expression would be important at the level of individual cells, in the context of an infection this requirement may not extend to the population as a whole. Single cell expression studies have demonstrated that T3SS gene expression exhibits bistability whereby only a fraction of the cells demonstrate increased expression in response to inducing signals (both low Ca^{2+} and host cell contact; Hornef et al., 2000; Rietsch et al., 2004; Urbanowski et al., 2007). Bistability is a stochastic process generally reflecting noise in the expression of a master regulator (Dubnau and Losick, 2006). Since minor changes in the expression levels of ExsA, ExsD, ExsC, or ExsE have profound effects on T3SS gene expression, bistable expression of the T3SS is likely mediated through the ExsADCE regulatory cascade. The biological significance of bistability is not entirely clear. Bistability might reflect energetic considerations whereby only a subpopulation of T3SS-expressing *P. aeruginosa* are necessary to neutralize phagocytic clearance or elicit T3SS-dependent cytotoxicity during later stages of colonization. In this context, cells lacking T3SS gene expression could be likened to the social cheaters described in quorum sensing responses (Sandoz et al., 2007). Alternatively, bistability may reflect a state of heightened activation that allows a fraction of the cells to respond rapidly to predators. Finally, there may be a selective advantage for cells that lack T3SS gene expression such as avoidance of antibody responses directed against the injectisome.

Whereas the ExsADCE system allows for rapid induction of T3SS gene expression, there is a notable lag period in down-regulation following exposure to high Ca^{2+} conditions (as described above) and this lag period likely occurs following the loss of host cell contact as well. This regulatory feature may provide for a slow taper in T3SS gene expression which, during the interim period between full induction and the return to basal expression levels, serves as a safeguard against subsequent encounters with phagocytes.

EXTRINSIC REGULATION OF T3SS GENE EXPRESSION

The ExsADCE regulatory pathway is central to T3SS gene regulation but does not operate in isolation. As described in a previous review (Yahr and Wolfgang, 2006), many extrinsic regulatory pathways involving no less than 30 gene products influence T3SS gene expression. In nearly every case, the mechanisms by which they exert control over T3SS gene expression is poorly understood. One of the future challenges facing the field is to account for regulation by each of these pathways and identify the signals to which they respond. Despite significant knowledge gaps, molecular details are beginning to emerge for some of these systems.

GLOBAL REGULATION OF T3SS GENE EXPRESSION BY Vfr

Virulence factor regulator (Vfr) is a cAMP-dependent regulator of transcription that was first identified as an activator of extracellular protease and exotoxin A expression (West et al., 1994). Now appreciated as a global regulator of virulence gene expression, the Vfr regulon consists of ~200 genes including type IV pili, a type II secretion system, quorum sensing, and the T3SS (Wolfgang et al., 2003). In addition to Vfr, the system consists of two adenylate cyclases (CyaA and CyaB) and a cAMP phosphodiesterase (**Figure 2**; CpdA; Wolfgang et al., 2003; Fuchs et al., 2010). Both high osmolarity (NaCl) and low Ca^{2+} stimulate cAMP production (Wolfgang et al., 2003), and cAMP homeostasis is tightly controlled by CpdA (Fuchs et al., 2010). Although a Vfr consensus binding sequence has been determined (Kanack et al., 2006), the mechanism by which Vfr controls T3SS gene expression remains unknown and represents a significant milestone for future studies.

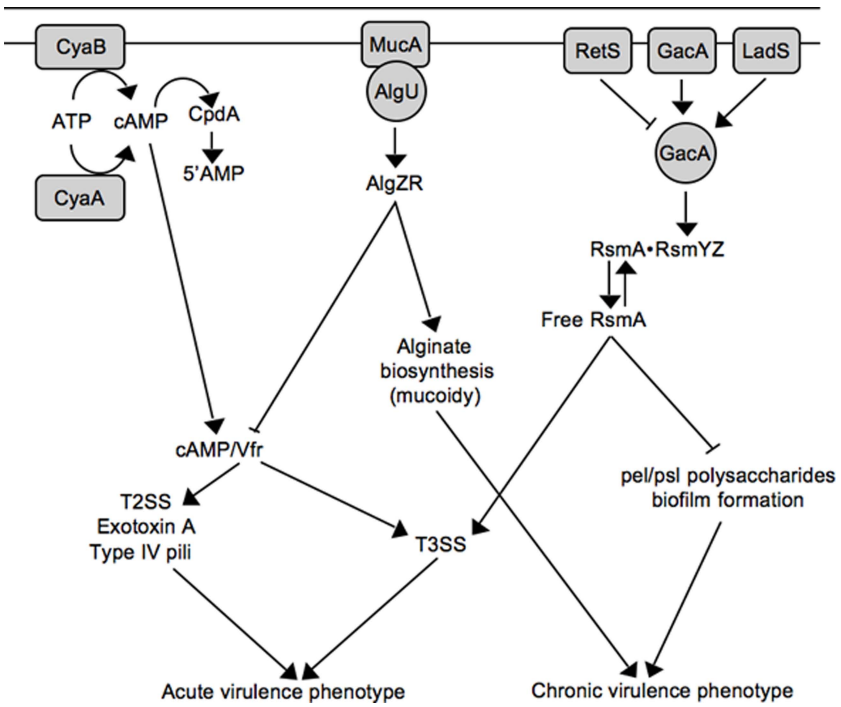

FIGURE 2 | Working model for inverse regulation of T3SS with mucoidy and biofilm formation. See text for details.

INVERSE REGULATION OF MUCOIDY AND T3SS GENE EXPRESSION

The role of the *P. aeruginosa* T3SS is well established within the context of acute infections where it contributes to tissue damage, sepsis, and mortality (Engel and Balachandran, 2009). *P. aeruginosa* is also the most common cause of chronic airway infection in individuals with cystic fibrosis (CF). CF infections are characterized by biofilm growth, slow progression, minimal tissue damage, and restriction to the airways (Gomez and Prince, 2007). The mechanistic basis for the striking difference in the progression of acute (non-CF) and chronic CF infections is not well understood. One contributing factor is that many *P. aeruginosa* virulence determinants are differentially expressed during non-CF and CF infections. Although CF and non-CF patients are initially colonized with nearly identical strains of *P. aeruginosa*, the majority of chronic CF isolates are mucoid and lack T3SS gene expression (Alonso et al., 1999; Feltman et al., 2001; Allmond et al., 2004). Mucoid conversion results from overproduction of the alginate polysaccharide and is associated with a decline in lung function and clinical prognosis (Pedersen et al., 1992; Li et al., 2005). Mucoid conversion and the loss of T3SS gene expression are now known to be linked events involving *mucA* mutation (Wu et al., 2004; Jones et al., 2010).

Alginate biosynthesis is regulated by the AlgU alternative sigma factor and the membrane-bound MucA anti-sigma factor (Wozniak and Ohman, 1994; Govan and Deretic, 1996). MucA normally sequesters AlgU to prevent expression of the AlgU regulon (**Figure 2**; Schurr et al., 1996; Mathee et al., 1997). MucA is (indirectly) responsive to a variety of environmental signals (primarily stress signals) resulting in the release of AlgU (Wood and Ohman, 2009). Liberated AlgU activates many genes including the AlgZR two-component regulatory system and the alginate biosynthetic genes (Wozniak and Ohman, 1994). While environmental signals represent one mechanism for activation of the AlgU regulon, mucoid conversion in CF isolates usually results from nonsense mutations in *mucA* (Boucher et al., 1997; Mathee et al., 1997). Jin and colleagues were the first to observe that *mucA* mutants are defective for T3SS gene expression and found the inhibition to be mediated through the AlgZR two-component system (Wu et al., 2004). Wolfgang and colleagues later noted that *vfr* and *mucA* mutants share several common phenotypes including reduced expression of the T3SS, T2SS, exotoxin A, and type IV pili, and subsequently confirmed that *mucA* mutants are defective for *vfr* transcription (**Figure 2**; Jones et al., 2010). The lack of T3SS gene expression in *mucA* mutants, therefore, reflects a defect in cAMP/Vfr signaling. Coincident mucoid conversion and loss of cAMP/Vfr signaling suggests that the selective advantage of *mucA* mutants in the CF airways may extend beyond mucoidy itself (Rau et al., 2010). Finally, the observation that *algZR* mutants have increased T3SS gene expression suggests that AlgZR modulates expression of the T3SS even in the presence of functional MucA (Jones et al., 2010). Since the MucA/AlgU system controls a generalized stress response, an interesting question for the future is whether stress signals encountered within the host modulate T3SS gene expression through MucA/AlgU.

T3SS GENE EXPRESSION AND BIOFILM FORMATION

Like alginate production, *P. aeruginosa* biofilms are thought to contribute to the chronic nature of CF infections (Singh et al., 2000). Biofilm formation is a complex adaptation subject to multiple layers of regulation (Harmsen et al., 2010). One regulatory mechanism involves the RetS and LadS sensor kinases. Whereas a *retS* (regulator

of *e*xopolysaccharide and *t*ype III *s*ecretion) mutant is defective for T3SS gene expression and displays enhanced biofilm formation, a *ladS* mutant has the reciprocal phenotypes (Goodman et al., 2004; Ventre et al., 2006). Mechanistically, RetS and LadS mediate opposite effects on activation of the GacAS system (**Figure 2**). The GacAS system regulates biofilm formation through control of the Pel and Psl polysaccharides, key components of the biofilm matrix (Ryder et al., 2007). Psl biosynthesis is controlled at the post-transcriptional level by RsmA, an RNA-binding protein that binds directly to the 5′ UTR region of the *pslA* mRNA to inhibit translational initiation (Irie et al., 2010). In response to unknown signals, GacA activates transcription of two small non-coding regulatory RNAs (RsmY and RsmZ). RsmY and RsmZ each have ~4–6 binding sites for RsmA and function by sequestering free RsmA from target mRNAs (Lapouge et al., 2008). Decreased levels of free RsmA, therefore, are required for Psl expression. In contrast, T3SS gene expression is dependent upon increased levels of free RsmA (Goodman et al., 2004; Mulcahy et al., 2006). The mechanism by which RsmA promotes T3SS gene expression is poorly understood. RsmA is a member of the CsrA family of proteins first described in *E. coli* (Romeo et al., 1993). Although CsrA family members usually prevent translation by binding to or near the Shine–Dalgarno (SD) sequence of target mRNAs (Lapouge et al., 2008), direct positive regulation by CsrA has also been reported and involves mRNA stabilization (Wei et al., 2001). This leaves several scenarios to account for positive regulation of T3SS gene expression by RsmA. RsmA may function directly at the level of intrinsic regulation by promoting expression of a positive (ExsA or ExsC) acting factor or inhibiting expression of negative (ExsD or ExsE) regulator. Alternatively, RsmA may function indirectly by modulating an extrinsic regulatory pathway that controls the T3SS. With respect to the latter possibility it is noteworthy that the RsmA regulon is extensive and includes rhamnolipid production, lipase production, swarming motility, quorum sensing, lectin, pyocyanin, hydrogen cyanide, and the type VI secretion system (Pessi et al., 2001; Heurlier et al., 2004; Brencic and Lory, 2009). The possibility exists, therefore, that the regulatory activity of RsmA toward T3SS gene expression results from pleiotropic effects that may be difficult to define.

The observation of inverse regulation by RetS and LadS implied that T3SS gene expression and biofilm formation were mutually exclusive events. It is now becoming apparent, however, that such a model is an over-simplification and that the T3SS is possible during biofilm growth (Mikkelsen et al., 2009).

T3SS GENE EXPRESSION AND MOTILITY

All three forms of *P. aeruginosa* motility (type IV pili-based twitching, swarming, and flagellar-based swimming,) are coordinated (either positively or negatively) with T3SS gene expression. As described above, type IV pili and T3SS gene expression are positively regulated by Vfr (Jones et al., 2010). The biological significance of this relationship reflects the adherence properties provided by type IV pili, which function as the primary adhesin for host-contact-dependent expression of the T3SS (Sundin et al., 2002). T3SS gene expression is also positively correlated with swarming motility, which might be advantageous during colonization of mucosal surfaces (Overhage et al., 2008). In contrast, flagellar gene expression is inversely regulated with T3SS gene expression. Whereas a *fliC* mutant is deregulated for T3SS gene expression (three- to ninefold) and displays increased cytotoxicity toward a macrophage cell line, ExsA over-expression reduces motility and expression of a *fliA* transcriptional reporter (Soscia et al., 2007). The reciprocal nature of T3SS and flagellar gene expression is interesting to contemplate in the context of inflammasome activation. Several studies suggest that flagellin (FliC) translocated into macrophages by the *P. aeruginosa* T3SS activates the inflammasome leading to a proinflammatory response (Franchi et al., 2007; Galle et al., 2007; Sutterwala et al., 2007; Miao et al., 2008). Despite experimental evidence of inflammasome activation *ex vivo*, it is unclear whether this occurs *in vivo* because the ExoS and ExoU effectors both block FliC-dependent inflammasome activation. The additional finding that FliC expression is inversely regulated with T3SS gene expression suggests that inflammasome activation is either relatively unimportant, does not normally occur during infections, or occurs under conditions that remain to be defined.

ENVIRONMENTAL SIGNALS THAT MODULATE T3SS GENE EXPRESSION

Ca^{2+}-limitation and host cell contact are well-defined signals that activate transcription through the control of T3SS secretory activity. Many additional signals, arising from both endogenous and exogenous sources, also influence T3SS gene expression and are summarized in **Table 1**. Evident is the fact that these signals are diverse with stress and metabolic signals representing the largest class. Less obvious is that the mechanisms by which these signals influence T3SS gene expression are poorly understood. In fact, besides ExsA only one additional transcriptional factor (PsrA) is known to directly regulate the T3SS. PsrA binds the P_{exsC} promoter to positively control transcription of the *exsCEBA* operon (Shen et al., 2006). Interestingly, the DNA-binding activity of PsrA is directly inhibited by long chain fatty acids. Indeed T3SS gene expression is reduced in the presence of long chain fatty acids, although the biological significance of this observation is unclear (Kang et al., 2009). The involvement of PsrA is discussed here to illustrate the point that cross-talk between different regulatory systems coordinates T3SS gene expression with diverse cellular functions. In addition to the T3SS, PsrA regulates a diverse set of genes including type IV pili (a potential link to Vfr), the *psl* biosynthesis operon (a potential link to RsmA), the stationary phase sigma factor *rpoS* (a potential link to quorum sensing) and a variety of genes involved in metabolic and energetic metabolism. Furthermore, *psrA* mutants are defective for swarming motility, biofilm formation, and resistance to some antibiotics. In just this one example, therefore, one begins to appreciate the level of complexity involved in controlling T3SS gene expression. Despite the involvement of numerous signals and gene products, it seems likely that reductionist approaches will ultimately reveal that only a minority (i.e., PsrA, Vfr, RsmA) of the genes implicated in regulation of the T3SS directly participate in the control of T3SS gene expression. Future explorations aimed as examining whether signals and gene products exert their regulatory effects at the level of ExsADCE or through upstream regulatory pathways are required to fully integrate the regulatory pathways leading to T3SS gene expression.

Table 1 | Environmental signals that influence T3SS gene expression.

Signal	Effect on T3SS	Implicated genes	Reference
ACTIVATION OF SECRETION			
Host cell contact	+	*exsADCE*	Vallis et al. (1999), Urbanowski et al. (2007)
Low calcium	+	*exsADCE*	Frank (1997)
Serum/albumin	+	*exsADCE*	Vallis et al. (1999), Kim et al. (2005)
ENVIRONMENTAL STRESS			
DNA damage	–	*recA, ptrB, prtN, prtR*	Wu and Jin (2005)
Hyperosmotic stress	+	cAMP/Vfr	Aspedon et al. (2006), Rietsch and Mekalanos (2006)
Copper stress	–	*ptrA*	Ha et al. (2004)
METABOLIC SIGNALS/STRESSES			
Pyruvate imbalance	–	*aceA, aceB*	Dacheux et al. (2002)
Unknown	–	*gltR, gltA cbrA*	Rietsch et al. (2004), Rietsch and Mekalanos (2006)
Spermidine/spermine	+	*spuE*	Zhou et al. (2007)
Tryptophan metabolites	–	*trpA, leuC*	Shen et al. (2008)
Long chain fatty acids	–	*psrA, rpoS*	Kang et al. (2009)
Mucin	±	unknown	Tralau et al. (2007)
PQS	–	*pqsR, mvfR*	Singh et al. (2010)
ANAEROBIOSIS			
NO	+	*nirS, narGH*	Van Alst et al. (2009)
ANTIBIOTIC EXPOSURE			
Azithromycin	+	*mexCD-oprJ, ptrA*	Gillis et al. (2005)
Chloramphenicol	–	*mexEF*	Linares et al. (2005)
Tetracycline	+	unknown	Linares et al. (2006)
Antimicrobial peptides	–	*psrA*	Gooderham et al. (2008)
Quorum sensing	–	*rpoS*	Hogardt et al. (2004), Bleves et al. (2005)

CHALLENGES FOR THE FUTURE

The literature contains a wealth of information regarding genes and conditions that contribute to T3SS gene expression in *P. aeruginosa*. The challenge ahead lies in assimilating this information into a unified regulatory network supportive of all prior and future observations. In absolute terms this will undoubtedly prove impossible owing to differences in mutant phenotypes, strain backgrounds, growth conditions, and experimental findings. In analyzing the primary literature consideration of the following points is important to keep in mind: (i) Nearly 20 microarray studies have noted alterations in T3SS gene expression. Most of these studies were conducted under non-permissive conditions for T3SS gene expression (i.e., high Ca^{2+}) making it difficult to ascertain whether the observed changes represent minor alterations to the basal level of T3SS transcription or more significant regulatory effects. (ii) To the best of our knowledge, plasmid-expressed ExsA is able to restore transcription to all mutants identified as defective for T3SS transcription. Reports of regulatory effects occurring upstream of ExsA, therefore, must be interpreted with caution since ExsA over-expression can easily obscure the activity of and/or requirement for other regulatory systems. (iii) Although most studies of T3SS gene expression have relied upon Ca^{2+} chelation as the inducing signal, it is unlikely that Ca^{2+} chelation and host cell contact are entirely analogous signals. Future studies are required to fully define the physiologically relevant signals encountered *in vivo* and determine how they modulate or fine-tune T3SS gene expression. (iv) The induction of T3SS gene expression is minimally dependent upon a functional injectisome, the ExsADCE cascade, and the Vfr and RsmA global regulatory systems and is an important consideration in that regulatory phenotypes that alter T3SS gene expression might result from pleiotropic effects without actually contributing to the regulatory process *per se*.

Therapeutic targeting of the T3SS is an achievable goal with potential intervention points including the inhibition of assembly, secretion, translocation, and T3SS gene expression. Since ExsA-dependent transcription is dependent upon an intact secretion and translocation machinery, each class of inhibitor offers the added benefit of reduced T3SS gene expression. Most efforts to date have logically targeted the highly conserved secretion/translocation machinery with the hope of identifying inhibitors that possess activity against multiple pathogens. A limitation of this approach lies in identification of the inhibitor target since each of the ~25 constituents of the secretion/translocation machinery as well as their interaction interfaces represent potential target sites. Direct targeting of transcription offers the advantage that many promising targets already exist including the ExsADCE regulatory cascade, and the cAMP/Vfr, MucA/AlgU, and GacA/RsmA global regulatory pathways, and in fact inhibitors of ExsA-dependent transcription have already been identified (Grier et al., 2010). Time will tell whether such inhibitors can deliver on the promise of controlling *P. aeruginosa* infections.

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REFERENCES

Allmond, L. R., Ajayi, T., Moriyama, K., Wiener-Kronish, J. P., and Sawa, T. (2004). V-antigen genotype and phenotype analyses of clinical isolates of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 42, 3857–3860.

Alonso, A., Rojo, F., and Martinez, J. L. (1999). Environmental and clinical isolates of *Pseudomonas aeruginosa* show pathogenic and biodegradative properties irrespective of their origin. *Environ. Microbiol.* 1, 421–430.

Aspedon, A., Palmer, K., and Whiteley, M. (2006). Microarray analysis of the osmotic stress response in *Pseudomonas aeruginosa*. *J. Bacteriol.* 188, 2721–2725.

Bernhards, R. C., Jing, X., Vogelaar, N. J., Robinson, H., and Schubot, F. D. (2009). Structural evidence suggests that antiactivator ExsD from *Pseudomonas aeruginosa* is a DNA binding protein. *Protein Sci.* 18, 503–513.

Bleves, S., Soscia, C., Nogueira-Orlandi, P., Lazdunski, A., and Filloux, A. (2005). Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 187, 3898–3902.

Boucher, J. C., Yu, H., Mudd, M. H., and Deretic, V. (1997). Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect. Immun.* 65, 3838–3846.

Brannon, M. K., Davis, J. M., Mathias, J. R., Hall, C. J., Emerson, J. C., Crosier, P. S., Huttenlocher, A., Ramakrishnan, L., and Moskowitz, S. M. (2009). *Pseudomonas aeruginosa* type III secretion system interacts with phagocytes to modulate systemic infection of zebrafish embryos. *Cell. Microbiol.* 11, 755–768.

Brencic, A., and Lory, S. (2009). Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol. Microbiol.* 72, 612–632.

Brutinel, E. D., Vakulskas, C. A., Brady, K. M., and Yahr, T. L. (2008). Characterization of ExsA and of ExsA-dependent promoters required for expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol. Microbiol.* 68, 657–671.

Brutinel, E. D., Vakulskas, C. A., and Yahr, T. L. (2009). Functional domains of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* type III secretion system. *J. Bacteriol.* 191, 3811–3821.

Brutinel, E. D., Vakulskas, C. A., and Yahr, T. L. (2010). ExsD inhibits expression of the *Pseudomonas aeruginosa* type III secretion system by disrupting ExsA self-association and DNA binding activity. *J. Bacteriol.* 192, 1479–1486.

Brutinel, E. D., and Yahr, T. L. (2008). Control of gene expression by type III secretory activity. *Curr. Opin. Microbiol.* 11, 128–133.

Coburn, B., Sekirov, I., and Finlay, B. B. (2007). Type III secretion systems and disease. *Clin. Microbiol. Rev.* 20, 535–549.

Dacheux, D., Epaulard, O., De Groot, A., Guery, B., Leberre, R., Attree, I., Polack, B., and Toussaint, B. (2002). Activation of the *Pseudomonas aeruginosa* type III secretion system requires an intact pyruvate dehydrogenase aceAB operon. *Infect. Immun.* 70, 3973–3977.

Dasgupta, N., Lykken, G. L., Wolfgang, M. C., and Yahr, T. L. (2004). A novel anti-anti-activator mechanism regulates expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol. Microbiol.* 53, 297–308.

Dubnau, D., and Losick, R. (2006). Bistability in bacteria. *Mol. Microbiol.* 61, 564–572.

Engel, J., and Balachandran, P. (2009). Role of *Pseudomonas aeruginosa* type III effectors in disease. *Curr. Opin. Microbiol.* 12, 61–66.

Feltman, H., Schulert, G., Khan, S., Jain, M., Peterson, L., and Hauser, A. R. (2001). Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Microbiology* 147, 2659–2669.

Franchi, L., Stoolman, J., Kanneganti, T. D., Verma, A., Ramphal, R., and Nunez, G. (2007). Critical role for Ipaf in *Pseudomonas aeruginosa*-induced caspase-1 activation. *Eur. J. Immunol.* 37, 3030–3039.

Frank, D. W. (1997). The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 26, 621–629.

Fuchs, E. L., Brutinel, E. D., Klem, E. R., Fehr, A. R., Yahr, T. L., and Wolfgang, M. C. (2010). In vitro and in vivo characterization of the *Pseudomonas aeruginosa* cyclic AMP (cAMP) phosphodiesterase CpdA, required for cAMP homeostasis and virulence factor regulation. *J. Bacteriol.* 192, 2779–2790.

Galle, M., Schotte, P., Haegman, M., Wullaert, A., Yang, H. J., Jin, S., and Beyaert, R. (2007). The *Pseudomonas aeruginosa* type III secretion system plays a dual role in the regulation of caspase-1 mediated IL-1beta maturation. *J. Cell. Mol. Med.* 12, 1767–1776.

Gillis, R. J., White, K. G., Choi, K. H., Wagner, V. E., Schweizer, H. P., and Iglewski, B. H. (2005). Molecular basis of azithromycin-resistant *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 49, 3858–3867.

Gomez, M. I., and Prince, A. (2007). Opportunistic infections in lung disease: *Pseudomonas* infections in cystic fibrosis. *Curr. Opin. Pharmacol.* 7, 244–251.

Gooderham, W. J., Bains, M., Mcphee, J. B., Wiegand, I., and Hancock, R. E. (2008). Induction by cationic antimicrobial peptides and involvement in intrinsic polymyxin and antimicrobial peptide resistance, biofilm formation, and swarming motility of PsrA in *Pseudomonas aeruginosa*. *J. Bacteriol.* 190, 5624–5634.

Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S., and Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev. Cell* 7, 745–754.

Govan, J. R., and Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol. Rev.* 60, 539–574.

Grier, M. C., Garrity-Ryan, L. K., Bartlett, V. J., Klausner, K. A., Donovan, P. J., Dudley, C., Alekshun, M. N., Tanaka, S. K., Draper, M. P., Levy, S. B., and Kim, O. K. (2010). *N*-hydroxybenzimidazole inhibitors of ExsA MAR transcription factor in *Pseudomonas aeruginosa*: in vitro anti-virulence activity and metabolic stability. *Bioorg. Med. Chem. Lett.* 20, 3380–3383.

Ha, U. H., Kim, J., Badrane, H., Jia, J., Baker, H. V., Wu, D., and Jin, S. (2004). An in vivo inducible gene of *Pseudomonas aeruginosa* encodes an anti-ExsA to suppress the type III secretion system. *Mol. Microbiol.* 54, 307–320.

Harmsen, M., Yang, L., Pamp, S. J., and Tolker-Nielsen, T. (2010). An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. *FEMS Immunol. Med. Microbiol.* 59, 253–268.

Hauser, A. R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat. Rev. Microbiol.* 7, 654–665.

Hayes, C. S., Aoki, S. K., and Low, D. A. (2010). Bacterial contact-dependent delivery systems. *Annu. Rev. Genet.* 44, 71–90.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P., and Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 186, 2936–2945.

Hogardt, M., Roeder, M., Schreff, A. M., Eberl, L., and Heesemann, J. (2004). Expression of *Pseudomonas aeruginosa* exoS is controlled by quorum sensing and RpoS. *Microbiology* 150, 843–851.

Hornef, M. W., Roggenkamp, A., Geiger, A. M., Hogardt, M., Jacobi, C. A., and Heesemann, J. (2000). Triggering the ExoS regulon of *Pseudomonas aeruginosa*: a GFP-reporter analysis of exoenzyme (Exo) S, ExoT and ExoU synthesis. *Microb. Pathog.* 29, 329–343.

Hovey, A. K., and Frank, D. W. (1995). Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* exoenzyme S regulon. *J. Bacteriol.* 177, 4427–4436.

Irie, Y., Starkey, M., Edwards, A. N., Wozniak, D. J., Romeo, T., and Parsek, M. R. (2010). *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol. Microbiol.* 78, 158–172.

Jones, A. K., Fulcher, N. B., Balzer, G. J., Urbanowski, M. L., Pritchett, C. L., Schurr, M. J., Yahr, T. L., and Wolfgang, M. C. (2010). Activation of the *Pseudomonas aeruginosa* AlgU regulon through mucA mutation inhibits cyclic AMP/Vfr signaling. *J. Bacteriol.* 192, 5709–5717.

Kanack, K. J., Runyen-Janecky, L. J., Ferrell, E. P., Suh, S. J., and West, S. E. (2006). Characterization of DNA-binding specificity and analysis of binding sites of the *Pseudomonas aeruginosa* global regulator, Vfr, a homologue of the *Escherichia coli* cAMP receptor protein. *Microbiology* 152, 3485–3496.

Kang, Y., Lunin, V. V., Skarina, T., Savchenko, A., Schurr, M. J., and Hoang, T. T. (2009). The long-chain fatty acid sensor, PsrA, modulates the expression of rpoS and the type III secretion exsCEBA operon in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 73, 120–136.

Kim, J., Ahn, K., Min, S., Jia, J., Ha, U., Wu, D., and Jin, S. (2005). Factors triggering type III secretion in *Pseudomonas aeruginosa*. *Microbiology* 151, 3575–3587.

Lapouge, K., Schubert, M., Allain, F. H., and Haas, D. (2008). Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol. Microbiol.* 67, 241–253.

Li, Z., Kosorok, M. R., Farrell, P. M., Laxova, A., West, S. E., Green, C. G., Collins, J., Rock, M. J., and Splaingard, M. L. (2005). Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease

progression in children with cystic fibrosis. *JAMA* 293, 581–588.

Linares, J. F., Gustafsson, I., Baquero, F., and Martinez, J. L. (2006). Antibiotics as intermicrobial signaling agents instead of weapons. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19484–19489.

Linares, J. F., Lopez, J. A., Camafeita, E., Albar, J. P., Rojo, F., and Martinez, J. L. (2005). Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in *Pseudomonas aeruginosa*. *J. Bacteriol.* 187, 1384–1391.

Lykken, G. L., Chen, G., Brutinel, E. D., Chen, L., and Yahr, T. L. (2006). Characterization of ExsC and ExsD self-association and heterocomplex formation. *J. Bacteriol.* 188, 6832–6840.

Mathee, K., Mcpherson, C. J., and Ohman, D. E. (1997). Posttranslational control of the algT (algU)-encoded sigma22 for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). *J. Bacteriol.* 179, 3711–3720.

McCaw, M. L., Lykken, G. L., Singh, P. K., and Yahr, T. L. (2002). ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. *Mol. Microbiol.* 46, 1123–1133.

Miao, E. A., Ernst, R. K., Dors, M., Mao, D. P., and Aderem, A. (2008). *Pseudomonas aeruginosa* activates caspase 1 through Ipaf. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2562–2567.

Mikkelsen, H., Bind, N. J., Skindersoe, M. E., Givskov, M., Lilley, K. S., and Welch, M. (2009). Biofims and type III secretion are not mutually exclusive in *Pseudomonas aeruginosa*. *Microbiology* 155, 687–698.

Miyata, S., Casey, M., Frank, D. W., Ausubel, F. M., and Drenkard, E. (2003). Use of the *Galleria mellonella* caterpillar as a model host to study the role of the type III secretion system in *Pseudomonas aeruginosa* pathogenesis. *Infect. Immun.* 71, 2404–2413.

Moraes, T. F., Spreter, T., and Strynadka, N. C. (2008). Piecing together the type III injectisome of bacterial pathogens. *Curr. Opin. Struct. Biol.* 18, 258–266.

Mulcahy, H., O'callaghan, J., O'grady, E. P., Adams, C., and O'gara, F. (2006). The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. *Infect. Immun.* 74, 3012–3015.

Overhage, J., Bains, M., Brazas, M. D., and Hancock, R. E. (2008). Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J. Bacteriol.* 190, 2671–2679.

Pedersen, S. S., Hoiby, N., Espersen, F., and Koch, C. (1992). Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* 47, 6–13.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T., Camara, M., Haas, D., and Williams, P. (2001). The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J. Bacteriol.* 183, 6676–6683.

Pukatzki, S., Kessin, R. H., and Mekalanos, J. J. (2002). The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3159–3164.

Rau, M. H., Hansen, S. K., Johansen, H. K., Thomsen, L. E., Workman, C. T., Nielsen, K. F., Jelsbak, L., Hoiby, N., Yang, L., and Molin, S. (2010). Early adaptive developments of *Pseudomonas aeruginosa* after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts. *Environ. Microbiol.* 12, 1643–1658.

Rietsch, A., and Mekalanos, J. J. (2006). Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 59, 807–820.

Rietsch, A., Vallet-Gely, I., Dove, S. L., and Mekalanos, J. J. (2005). ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8006–8011.

Rietsch, A., Wolfgang, M. C., and Mekalanos, J. J. (2004). Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. *Infect. Immun.* 72, 1383–1390.

Romeo, T., Gong, M., Liu, M. Y., and Brun-Zinkernagel, A. M. (1993). Identification and molecular characterization of csrA, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. *J. Bacteriol.* 175, 4744–4755.

Ryder, C., Byrd, M., and Wozniak, D. J. (2007). Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr. Opin. Microbiol.* 10, 644–648.

Sandoz, K. M., Mitzimberg, S. M., and Schuster, M. (2007). Social cheating in *Pseudomonas aeruginosa* quorum sensing. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15876–15881.

Schurr, M. J., Yu, H., Martinez-Salazar, J. M., Boucher, J. C., and Deretic, V. (1996). Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *J. Bacteriol.* 178, 4997–5004.

Shen, D. K., Filopon, D., Chaker, H., Boullanger, S., Derouazi, M., Polack, B., and Toussaint, B. (2008). High-cell-density regulation of the *Pseudomonas aeruginosa* type III secretion system: implications for tryptophan catabolites. *Microbiology* 154, 2195–2208.

Shen, D. K., Filopon, D., Kuhn, L., Polack, B., and Toussaint, B. (2006). PsrA is a positive transcriptional regulator of the type III secretion system in *Pseudomonas aeruginosa*. *Infect. Immun.* 74, 1121–1129.

Singh, G., Wu, B., Baek, M. S., Camargo, A., Nguyen, A., Slusher, N. A., Srinivasan, R., Wiener-Kronish, J. P., and Lynch, S. V. (2010). Secretion of *Pseudomonas aeruginosa* type III cytotoxins is dependent on *Pseudomonas* quinolone signal concentration. *Microb. Pathog.* 49, 196–203.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J., and Greenberg, E. P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407, 762–764.

Soscia, C., Hachani, A., Bernadac, A., Filloux, A., and Bleves, S. (2007). Cross talk between type III secretion and flagellar assembly systems in *Pseudomonas aeruginosa*. *J. Bacteriol.* 189, 3124–3132.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., and Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 406, 959–964.

Sundin, C., Wolfgang, M. C., Lory, S., Forsberg, A., and Frithz-Lindsten, E. (2002). Type IV pili are not specifically required for contact dependent translocation of exoenzymes by *Pseudomonas aeruginosa*. *Microb. Pathog.* 33, 265–277.

Sutterwala, F. S., Mijares, L. A., Li, L., Ogura, Y., Kazmierczak, B. I., and Flavell, R. A. (2007). Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. *J. Exp. Med.* 204, 3235–3245.

Thibault, J., Faudry, E., Ebel, C., Attree, I., and Elsen, S. (2009). Anti-activator ExsD forms a 1:1 complex with ExsA to inhibit transcription of type III secretion operons. *J. Biol. Chem.* 284, 15762–15770.

Tralau, T., Vuilleumier, S., Thibault, C., Campbell, B. J., Hart, C. A., and Kertesz, M. A. (2007). Transcriptomic analysis of the sulfate starvation response of *Pseudomonas aeruginosa*. *J. Bacteriol.* 189, 6743–6750.

Urbanowski, M. L., Brutinel, E. D., and Yahr, T. L. (2007). Translocation of ExsE into Chinese hamster ovary cells is required for transcriptional induction of the *Pseudomonas aeruginosa* type III secretion system. *Infect. Immun.* 75, 4432–4439.

Urbanowski, M. L., Lykken, G. L., and Yahr, T. L. (2005). A secreted regulatory protein couples transcription to the secretory activity of the *Pseudomonas aeruginosa* type III secretion system. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9930–9935.

Vakulskas, C. A., Brady, K. M., and Yahr, T. L. (2009). Mechanism of Transcriptional Activation by *Pseudomonas aeruginosa* ExsA. *J. Bacteriol.* 191, 6654–6664.

Vakulskas, C. A., Brutinel, E. D., and Yahr, T. L. (2010). ExsA recruits RNA polymerase to an extended-10 promoter by contacting region 4.2 of Sigma-70. *J. Bacteriol.* 192, 3597–3607.

Vallis, A. J., Yahr, T. L., Barbieri, J. T., and Frank, D. W. (1999). Regulation of ExoS production and secretion by *Pseudomonas aeruginosa* in response to tissue culture conditions. *Infect. Immun.* 67, 914–920.

Van Alst, N. E., Wellington, M., Clark, V. L., Haidaris, C. G., and Iglewski, B. H. (2009). Nitrite reductase NirS is required for type III secretion system expression and virulence in the human monocyte cell line THP-1 by *Pseudomonas aeruginosa*. *Infect. Immun.* 77, 4446–4454.

Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S., and Filloux, A. (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 103, 171–176.

Vogelaar, N. J., Jing, X., Robinson, H. H., and Schubot, F. D. (2010). Analysis of the crystal structure of the ExsC. ExsE complex reveals distinctive binding interactions of the Pseudomonas aeruginosa type III secretion chaperone ExsC with ExsE and ExsD. *Biochemistry* 49, 5870–5879.

Wagner, S., Konigsmaier, L., Lara-Tejero, M., Lefebre, M., Marlovits, T. C., and

Galan, J. E. (2010). Organization and coordinated assembly of the type III secretion export apparatus. *Proc. Natl. Acad. Sci. U.S.A.* 41, 17745–17750.

Wei, B. L., Brun-Zinkernagel, A. M., Simecka, J. W., Pruss, B. M., Babitzke, P., and Romeo, T. (2001). Positive regulation of motility and flhDC expression by the RNA-binding protein CsrA of Escherichia coli. *Mol. Microbiol.* 40, 245–256.

West, S. E., Sample, A. K., and Runyen-Janecky, L. J. (1994). The vfr gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. *J. Bacteriol.* 176, 7532–7542.

Wolfgang, M. C., Lee, V. T., Gilmore, M. E., and Lory, S. (2003). Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev. Cell* 4, 253–263.

Wood, L. F., and Ohman, D. E. (2009). Use of cell wall stress to characterize sigma 22 (AlgT/U) activation by regulated proteolysis and its regulon in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 72, 183–201.

Worrall, L. J., Lameignere, E., and Strynadka, N. C. (2011). Structural overview of the bacterial injectisome. *Curr. Opin. Microbiol.* 14, 3–8.

Wozniak, D. J., and Ohman, D. E. (1994). Transcriptional analysis of the *Pseudomonas aeruginosa* genes algR, algB, and algD reveals a hierarchy of alginate gene expression which is modulated by algT. *J. Bacteriol.* 176, 6007–6014.

Wu, W., Badrane, H., Arora, S., Baker, H. V., and Jin, S. (2004). MucA-mediated coordination of type III secretion and alginate synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 186, 7575–7585.

Wu, W., and Jin, S. (2005). PtrB of *Pseudomonas aeruginosa* suppresses the type III secretion system under the stress of DNA damage. *J. Bacteriol.* 187, 6058–6068.

Yahr, T. L., and Frank, D. W. (1994). Transcriptional organization of the trans-regulatory locus which controls exoenzyme S synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 176, 3832–3838.

Yahr, T. L., Hovey, A. K., Kulich, S. M., and Frank, D. W. (1995). Transcriptional analysis of the *Pseudomonas aeruginosa* exoenzyme S structural gene. *J. Bacteriol.* 177, 1169–1178.

Yahr, T. L., and Wolfgang, M. C. (2006). Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Mol. Microbiol.* 62, 631–640.

Zheng, Z., Chen, G., Joshi, S., Brutinel, E. D., Yahr, T. L., and Chen, L. (2007). Biochemical characterization of a regulatory cascade controlling transcription of the *Pseudomonas aeruginosa* type III secretion system. *J. Biol. Chem.* 282, 6136–6142.

Zhou, L., Wang, J., and Zhang, L. H. (2007). Modulation of bacterial type III secretion system by a spermidine transporter dependent signaling pathway. *PLoS ONE* 2, e1291. doi: 10.1371/journal.pone.0001291

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Multi-functional characteristics of the *Pseudomonas aeruginosa* type III needle-tip protein, PcrV; comparison to orthologs in other Gram-negative bacteria

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Pseudomonas aeruginosa possesses a type III secretion system (T3SS) to intoxicate host cells and evade innate immunity. This virulence-related machinery consists of a molecular syringe and needle assembled on the bacterial surface, which allows delivery of T3 effector proteins into infected cells. To accomplish a one-step effector translocation, a tip protein is required at the top end of the T3 needle structure. Strains lacking expression of the functional tip protein fail to intoxicate host cells. *P. aeruginosa* encodes a T3S that is highly homologous to the proteins encoded by *Yersinia* spp. The needle-tip proteins of *Yersinia*, LcrV, and *P. aeruginosa*, PcrV, share 37% identity and 65% similarity. Other known tip proteins are AcrV (*Aeromonas*), IpaD (*Shigella*), SipD (*Salmonella*), BipD (*Burkholderia*), EspA (EPEC, EHEC), Bsp22 (*Bordetella*), with additional proteins identified from various Gram-negative species, such as *Vibrio* and *Bordetella*. The tip proteins can serve as a protective antigen or may be critical for sensing host cells and evading innate immune responses. Recognition of the host microenvironment transcriptionally activates synthesis of T3SS components. The machinery appears to be mechanically controlled by the assemblage of specific junctions within the apparatus. These junctions include the tip and base of the T3 apparatus, the needle proteins and components within the bacterial cytoplasm. The tip proteins likely have chaperone functions for translocon proteins, allowing the proper assembly of translocation channels in the host membrane and completing vectorial delivery of effector proteins into the host cytoplasm. Multi-functional features of the needle-tip proteins appear to be intricately controlled. In this review, we highlight the functional aspects and complex controls of T3 needle-tip proteins with particular emphasis on PcrV and LcrV.

Keywords: type III secretion, needle-tip proteins, PcrV, LcrV, *Pseudomonas aeruginosa*, *Yersinia*, protective antigen

INTRODUCTION

The type III secretion system (T3SS) is likened to a molecular machine located on the surface and within the envelope of many Gram-negative bacteria. These systems are essential for virulence and act as a syringe and needle, called an injectisome, forming a channel in the eukaryotic membrane to facilitate the passage of bacterial effectors into infected cells (Galan and Collmer, 1999; Cornelis, 2006). The T3S apparatus has been identified in a variety of pathogens that infect animals, plants, and insects (Hueck, 1998; Mota and Cornelis, 2005; Troisfontaines and Cornelis, 2005). Functional attributes of T3SS effectors vary among bacterial genera, with cytotoxicity and anti-phagocytic activity being characteristic for *Pseudomonas aeruginosa* and *Yersinia* spp., invasion, intracellular survival, and the promotion of proinflammatory responses for *Shigella* and *Salmonella* spp., and profound effects on cytoskeletal structure for many other pathogens (reviewed in Hueck, 1998; Galan and Collmer, 1999; Mota and Cornelis, 2005).

More than 20 T3S systems have been identified in 16S RNA based studies (Cornelis, 2002; Troisfontaines and Cornelis, 2005). In *P. aeruginosa*, 36 genes are involved in T3S (Frank, 1997). The structure of the assembled injectisome is complex and several components constitute the apparatus. An extracellular needle structure topped with a tip complex extrudes from the basal body that consists of multiple rings spanning the bacterial inner and outer membranes (**Figure 1**). The inner and outer rings of the basal body are connected by a neck domain. A T3S-associated ATPase, another component of the basal body located at the cytosolic face, is involved in the dissociation of secretion substrates from their chaperones as well as supplying energy for protein export (**Figure 1**; Cornelis, 2006; Galan and Wolf-Watz, 2006; Blocker et al., 2008; Moraes et al., 2008; Mueller et al., 2008). Bacteria have to complete the assembly of the basal body and polymerize the needle subunit proteins to an appropriate length prior to the formation of the tip complex on the distal end, which is controlled by a substrate-switching mechanism (Journet et al., 2003; Galan and Wolf-Watz, 2006). Other proteins necessary for a functional T3SS are intracellular regulators, specialized chaperones, translocators or translocases, and effectors (Cornelis, 2006; Galan and Wolf-Watz, 2006; Schroeder and Hilbi, 2008; Hauser, 2009; Parsot, 2009).

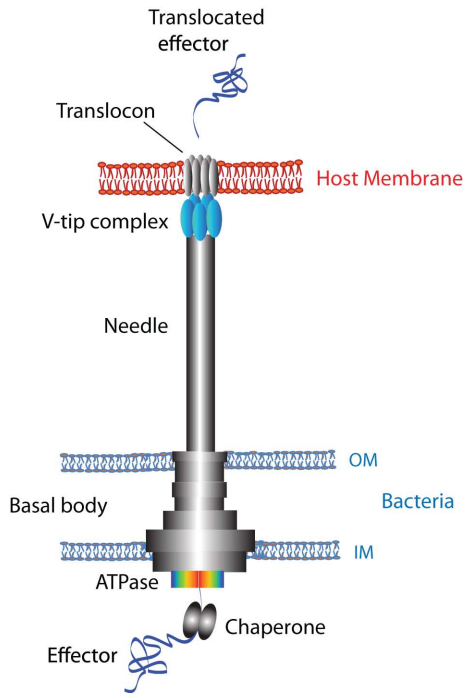

FIGURE 1 | Schematic diagram of the type III apparatus and host membrane. The T3SS injectisome comprises an extracellular needle structure from the basal body that spans the bacterial inner and outer membranes (indicated as IM and OM), the tip complex topped at the distal end of the needle (shown in blue) and a T3S-associated ATPase located at the cytosolic face of the basal body (shown in a rainbow color). The ATPase is involved in the dissociation of unfolded substrates from the specialized chaperones in addition to supplying energy for protein export (Galan and Wolf-Watz, 2006). It is postulated that the structure and assembly of tip complexes are distinct for each protein family: V-tip proteins form a homo-pentamer complex, IpaD requires IpaB to assemble the hetero-pentamer complex, and EC family proteins assemble into a filamentous structure (Knutton et al., 1998; Veenendaal et al., 2007). The tip complex is required for the assembly of a translocon (PopB and PopD for *Pseudomonas*, YopB and YopD for *Yersinia*) in the host cell membrane (Cornelis, 2006; Hauser, 2009). An unfolded effector protein is transferred through the channel of the needle and translocon and eventually delivered into the host cytosolic compartment (blue ribbon).

Activation of the T3SS is postulated to occur upon sensing the presence of host cells. Bacteria respond by turning on the transcription of T3SS genes, inserting a translocation complex or translocon assembled with translocator proteins into eukaryotic membranes, and eventually delivering effector proteins into the host to result in manipulation of cell physiology (**Figure 1**; Deane et al., 2006; Veenendaal et al., 2007; Mueller et al., 2008; Hauser, 2009; Parsot, 2009). Structural and functional aspects of the T3SS and related proteins have been summarized in numerous reviews, thus this review focuses on the needle-tip proteins pertaining to the Ysc family, PcrV from *P. aeruginosa* and LcrV from *Yersinia* spp. The tip complex likely possesses multiple roles in T3SS-mediated modulation of host cellular physiology: (1) sensing the microenvironment of host cell membranes and propagating a signal back to the bacterial cell, (2) regulation of secretion and translocation at the level of the tip complex coordinated with transcriptional activation or repression of bacterial genes, (3) assembly and insertion of a translocon in eukaryotic membranes, (4) physically bridging the needle and translocon embedded in the host membrane during effector delivery, and (5) serving as a clinically important protective antigen. In this review, we will discuss these multi-functional properties of T3 needle-tip proteins.

NEEDLE-TIP COMPLEXES OF THE T3SS

FAMILIES OF TIP PROTEINS AND STRUCTURAL CONSIDERATIONS

In animal pathogens, there are three major classes of T3SS: (1) the Ysc family that include the so-called V-tip proteins, PcrV (*P. aeruginosa*), LcrV (*Yersinia* spp.), and AcrV from a fish pathogen, *Aeromonas salmonicida*; (2) the Inv-Mxi-Spa family members identified in *Shigella flexneri* and *Salmonella typhimurium* (belonging to the SPI-1 system), and (3) the Esc family of enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *S. typhimurium* (SPI-2 system; Cornelis, 2002, 2006). In the Ysc family, PcrV and LcrV share the highest overall homology (**Table 1**). Recently, another protein Vp1659 from *Vibrio parahaemolyticus* was identified as a member of this family (Zhou et al., 2010). The amino acid sequence of the C-terminus of Vp1659

Table 1 | Needle-tip proteins of type III secretion system.

		No. of residues	Global alignment[a] with PcrV identity (similarity)	GenBank accession no.
V-tip protein (Ysc family)				
PcrV	*Pseudomonas aeruginosa*	294	Strain PA103, ours	
LcrV	*Yersinia* spp.	326	36.4% (64.4%)	P23994
AcrV	*Aeromonas* spp.	361	34.2% (59.8%)	YP_001144289
Vp1659	*Vibrio parahaemolyticus*	607	35.3%[b] (55.7%[b])	NP_798038
Tip protein containing a chaperone domain (Inv-Mxi-Spa family)				
IpaD	*Shigella flexneri*	332	18.2%	AAL72350
SipD	*Salmonella* spp.	343	20.4%	ACY89896
BipD	*Burkholderia pseudomallei*	310	21.6%	EET03029
CT584	*Chlamydia trachomatis*	183	16.4%	NP_220099
Filamentous-tip protein (EC family)				
EspA	EPEC/EHEC[c]	192	17.0%	ACG59618
Bsp22	*Bordetella* spp.	205	17.3%	CAE32114

[a] *Analyzed by using ALIGN © 1997 by William R. Pearson and the University of Virginia).*

[b] *Residues 420-607 of Vp1659 were used for alignment.*

[c] *EPEC: enteropathogenic E. coli; EHEC: enterohemorrhagic E. coli.*

(residues 420–607) is homologous to PcrV (residues 95–294) with 35% identity and 55% similarity (**Table 1**). The Inv-Mxi-Spa family, is comprised of IpaD (*S. flexneri*), SipD (*Salmonella* spp.), BipD (*Burkholderia pseudomallei*), and CT584 (*Chlamydia trachomatis*; Erskine et al., 2006; Espina et al., 2006; Johnson et al., 2007; Lara-Tejero and Galan, 2009). Although the proteins in this family contain less sequence identity to PcrV (**Table 1**), the structural similarity of IpaD, BipD, and SipD to the V-proteins has been well studied (Blocker et al., 2008; Mueller et al., 2008). CT584 is a late cycle gene product of *C. trachomatis* (Betts-Hampikian and Fields, 2010) and the tertiary structure of this protein could be modeled based on the structure of IpaD (Markham et al., 2009). Esc family members include EspA (EPEC and EHEC) and Bsp22 (*Bordetella* spp.; **Table 1**). These proteins polymerize and assemble into a distinct filamentous structure at the tip of the T3 needle (Knutton et al., 1998; Daniell et al., 2001; Crepin et al., 2005; Medhekar et al., 2009). Despite the little structural similarity to the other tip protein families, the polymerized-tip complex appears to function similarly in T3SS (Knutton et al., 1998; Crepin et al., 2005).

PcrV and LcrV tip proteins are located at the distal end of the needle structure and required for the pathogenic phenotype of host infection (Fields et al., 1999; Pettersson et al., 1999; Sato et al., 2011). Bacterial strains with a deletion of *pcrV* or *lcrV* are avirulent in mice (Carter et al., 1980; Price et al., 1991; Skrzypek and Straley, 1995; Sawa et al., 1999) and during infection of cultured epithelial or phagocytic cells (Pettersson et al., 1999; Sato et al., 2011). In cell culture systems, *pcrV*-deletion strains release effector proteins into the culture medium and are incapable of vectorial translocation of effectors into the host cytosol (Sawa et al., 1999; Goure et al., 2004; Sato et al., 2011). Complementation with wild-type *pcrV* restores translocation and cytotoxicity, suggesting the important role of the tip protein in the intoxication of eukaryotic cells.

The crystal structure of LcrV revealed an overall dumbbell shape with a "grip" formed by the coiled-coil interaction of two internal α-helices flanked by globular domains at each end (Derewenda et al., 2004). Other V-proteins have sufficient sequence similarity to construct structural models using LcrV as a template (1r6f chain A, deposited in PBD; Derewenda et al., 2004). The members of the Ysc family modeled by using the Swiss Model server[1] (Guex and Peitsch, 1997) are shown in **Figure 2**. The coiled-coil structure of two long α-helices is conserved in all three families of the tip proteins (Derewenda et al., 2004; Yip et al., 2005; Johnson et al., 2007; Blocker et al., 2008; Mueller et al., 2008).

Current structural studies indicate that tip proteins form an oligomer, possibly a pentamer, at the top end of the needle structure (Mueller et al., 2005, 2008; Deane et al., 2006; Broz et al., 2007; Caroline et al., 2008). Scanning transmission electron microscopy analyses visualized the oligomer structures of parental or chimeric V-tip proteins on the assembled needle expressed in the *lcrV* deletion strain of *Y. enterocolitica* (Mueller et al., 2005; Broz et al., 2007). The N-terminal globular domain of LcrV, PcrV, and AcrV form the base structure of the tip complex that interacts with the YscF needle (Broz et al., 2007). PcrV forms a complex with a narrower base than that of LcrV or AcrV (Mueller et al., 2005; Broz et al., 2007). Compared to LcrV translocons (YopB and YopD), PcrV expressed in *Y. pseudotuberculosis* assembles smaller translocation channels with YopB and YopD in infected erythrocyte membranes and slowly delivers effector proteins into host cells (Holmstrom et al., 2001; Broms et al., 2003). The AcrV oligomerized structure is larger in size and varied in shape (Mueller et al., 2005).

Structural studies of Inv-Mxi-Spa family members demonstrate that the N-terminal domain of IpaD, SipD, and BipD possesses structural similarity to common chaperones, suggesting a self-chaperoning function of the domain (Erskine et al., 2006; Johnson et al., 2007; Blocker et al., 2008; Wang et al., 2010b; Chatterjee et al., 2011; Rathinavelan et al., 2011). In contrast, the N-terminal structure of V-proteins is predicted to be globular and appears to have no intramolecular chaperoning domain (Blocker et al., 2008). PcrV and LcrV utilize cognate chaperones PcrG and LcrG in the bacterial cytoplasm. The chaperones and V-proteins are expressed from genes located within the same operon (Blocker et al., 2008; Lee et al., 2010). EspA is chaperoned by CesA (Yip et al., 2005).

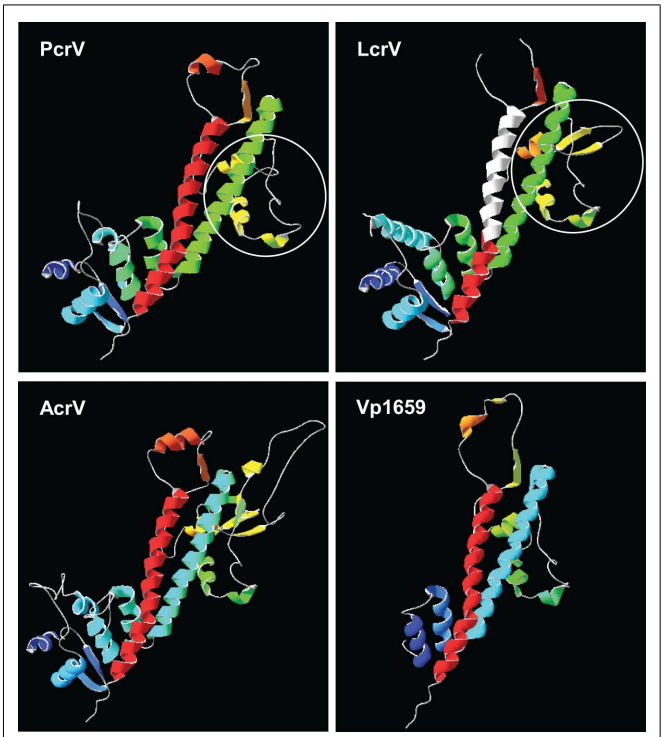

FIGURE 2 | Structural models of the V-tip protein family. V-tip proteins are modeled using LcrV (PBD: 1r6f chain A) as a template (Swiss Model server, http://www.expasy.org). The models indicate the conserved coiled-coil structure of two α-helices flanked by globular domains at each end. One of the globular domains contains protective-epitope regions identified in PcrV and LcrV (a while circle; Frank et al., 2002; Quenee et al., 2010). The immunomodulatory region of LcrV (residues 271 to 300) is located in one of the coiled-coil helices with a connected coil structure (indicated in white; Overheim et al., 2005). This region is deleted in the rV10 subunit vaccine, by which the immunomodulatory responses are eliminated (Overheim et al., 2005).

[1]http://www.expasy.org

The distinct coiled-coil structure in the center of the V-tip proteins is similar to the coiled-coil formation of monomeric needle proteins, such as MxiH from *Shigella* (Deane et al., 2006), suggesting a common mechanism of assembly and interaction of tip proteins and needle subunits (Blocker et al., 2008). One of the coiled-coil helices in LcrV is structurally homologous to a stabilizing helix of monomeric MxiH, which permits the modeling of the LcrV tip complex on the top of the MxiH needle without structural restraints (Deane et al., 2006). Also, it has been shown that the lower part of the coiled-coil structure of SipD interacts with the *Salmonella* needle protein PrgI (Rathinavelan et al., 2011).

SENSING THE MICROENVIRONMENT AND HOST CELLS

Bacteria modulate gene expression according to growth conditions to maximize advantageous strategies for survival and continued replication. It has been suggested that as bacteria sense the growth environment, the clusters of T3SS genes are transcriptionally repressed or activated (reviewed in Motin et al., 1994; Hill et al., 1997; Sawa et al., 1999; Brubaker, 2003; Yahr and Wolfgang, 2006; Brutinel and Yahr, 2008; Urbanowski and Yahr, 2008; Baer et al., 2009). For *P. aeruginosa*, a low-calcium growth environment is one signal to derepress or induce T3SS transcription by the *exsCEBA*-control system with an additional regulatory protein, ExsD (McCaw et al., 2002; Dasgupta et al., 2004; Rietsch et al., 2005; Urbanowski et al., 2005, 2007; Brutinel et al., 2009). Translocator and effector proteins are secreted into the extracellular growth medium under low-calcium conditions (Yahr et al., 1997). When calcium concentrations are sufficiently high, secretion of effectors is inhibited (Frank, 1997; Yahr et al., 1997; McCaw et al., 2002). In addition to calcium depletion, other signals that are involved in ExsA-mediated regulation of the T3SS include host–cell contact, the contribution of two component system regulators, DNA damage, and osmotic and metabolic stresses (Rietsch et al., 2004; Rietsch and Mekalanos, 2006; Yahr and Wolfgang, 2006; Kang et al., 2009). In *Yersinia*, LcrQ was identified as a transcriptional regulator protein unique to *Yersinia* with no homolog found in *Pseudomonas* (Wulff-Strobel et al., 2002). T3SS transcriptional control in *Yersinia* also involves regulators, LcrH, SycH, YopD, and YscM1/2 (Rimpilainen et al., 1992; Pettersson et al., 1996; Brutinel and Yahr, 2008). In addition, the T3SS is regulated by changes in oxygen levels or by intracellular pH as observed in cellular infections with EHEC, *Shigella*, or *Salmonella* (SPI-2; Marteyn et al., 2010; Schuller and Phillips, 2010; Yu et al., 2010). Further, the T3SS mRNA levels are post-transcriptionally modulated by small RNAs (Bordi et al., 2010).

It is postulated that the T3 needle-tip complex functions as a sensor of the growth environment and the presence of host cells for infection (Deane et al., 2006; Veenendaal et al., 2007; Mueller et al., 2008; Parsot, 2009). A physiological signal for T3SS activation is the recognition of eukaryotic membranes upon contact (Menard et al., 1994; Pettersson et al., 1996). Contact of a pathogen to the host cell induces expression of T3SS proteins, leading to the polarized transfer of effectors into the target cell cytosol (Menard et al., 1994; Rosqvist et al., 1994; Cisz et al., 2008). Contact-mediated induction of ExoS expression requires host cell integrity (Cisz et al., 2008). For *S. flexneri*, interaction with epithelial cells or incubation with serum is required to release IpaB and IpaC proteins (Menard et al., 1994).

Low-calcium conditions, induced by the addition of a calcium chelater to the growth medium, activate the T3SS in *Pseudomonas* and *Yersinia* (Carter et al., 1980; Frank et al., 1994). Mutations in *pcrV* or *lcrV* lead to a calcium-insensitive phenotype (Carter et al., 1980; Bergman et al., 1991; Sato et al., 2011). In the *Shigella* T3SS, several inducers have been shown, such as the amphipathic dye Congo Red, fetal bovine serum, cholesterol-containing lipids, and bile salts (Bahrani et al., 1997; Blocker et al., 1999; van der Goot et al., 2004; Olive et al., 2007). The *Shigella* tip protein IpaD "senses" deoxycholate or other bile salts in the environment, resulting in a conformational change in IpaD and subsequent step-wise activation of the secretion apparatus by recruitment of IpaB to the tip (Stensrud et al., 2008; Dickenson et al., 2011). After completion of IpaB surface presentation, followed by interaction with cholesterol and sphingomyelin containing lipids, IpaC moves toward the surface of the needle tip (Epler et al., 2009). At this stage, the tip complex is poised for translocation of effectors (Epler et al., 2009). *Salmonella* SipD also interacts with bile salts (Wang et al., 2010b; Chatterjee et al., 2011). In *V. parahaemolyticus*, a bile acid was identified as a host-derived inducer for T3SS genes in a transcriptional regulator-dependent manner, yet the involvement of tip proteins is unknown (Gotoh et al., 2010).

REGULATION OF TYPE III SECRETION AND TRANSLOCATION

In this section, we describe the post-translational control of the T3SS, which requires multiple levels of regulatory proteins and several types of signals (Pettersson et al., 1996; Wulff-Strobel et al., 2002; Agrain et al., 2005; Akeda and Galan, 2005; Galan and Wolf-Watz, 2006; Sorg et al., 2007; Botteaux et al., 2009). To complete the formation of a translocon and delivery of effectors into host cells, a functional injection apparatus, including the basal body, needle, and tip complex, must be assembled. This multistep process is postulated to be accomplished by a substrate-switching mechanism, a hierarchical and timely regulation of protein export, which will be discussed later. Post-translational regulation is organized into six classes based on the location of the regulatory or accessory proteins.

(1) The needle-tip complex of the injectisome: The tip complex is located at the distal end of the needle structure (Mueller et al., 2005; Broz et al., 2007; Blocker et al., 2008). V-tip proteins have been shown to be involved in the mechanical regulation of translocation and secretion (Sarker et al., 1998; Sato et al., 2011). Bacterial strains containing a deletion of a gene encoding a tip protein, *pcrV* or *ipaD*, are non-cytotoxic and possess a deregulated secretion phenotype as measured by the constitutive secretion of effector proteins (Menard et al., 1993; Frank, 1997; Sawa et al., 1999; McCaw et al., 2002; Goure et al., 2004; Picking et al., 2005; Rietsch et al., 2005; Sato et al., 2011). For *pcrV* and *lcrV* deletion strains, this is referred to as a calcium-blind or calcium independent phenotype (Carter et al., 1980; Bergman et al., 1991; Sato et al., 2011). Similarly, an *ipaD*-deletion mutant is insensitive to the inducer, Congo Red (Veenendaal et al., 2007). These data suggest that tip proteins

are involved in the control of translocation and secretion of effectors by the type III apparatus.

(2) The needle protein: It has been postulated that the type III needle propagates signals between the needle-tip sensor and regulators in the bacterial cytoplasm (Kenjale et al., 2005; Torruellas et al., 2005; Deane et al., 2006). The needle subunit proteins are related to secretory regulation and multiple YscF mutants demonstrate a constitutive-secretion phenotype (Torruellas et al., 2005; Davis and Mecsas, 2007). A dominant-negative YscF mutant, L54V, blocks secretion of parental YscF and Yop effectors and fails to assemble the injectisome (Davis et al., 2010). Mutations in the *Shigella* needle protein MixH, lead to constitutive secretion of effectors, some of which are also not inducible (Kenjale et al., 2005). The conserved tertiary structure of both needle subunits and tip proteins is coiled-coil formed by two α-helices, suggesting a similar mechanism of oligomeric assembly (Kenjale et al., 2005; Torruellas et al., 2005; Blocker et al., 2008). Biophysical analyses of common α-helical coiled-coil structures demonstrated that an allosteric potential in this type of structure induces conformational amplification involved in mechanotransduction. In other words, these proteins appear to function as a nanoswitch (Yogurtcu et al., 2010). Thus, the environmental signals sensed by the tip complex may be amplified and transmitted to the cytoplasmic side through the needle proteins by conformational changes in coiled-coil structures.

(3) Inner membrane proteins: An inner membrane component in *Yersinia*, YscU, recognizes translocators for secretion and is involved in substrate switching. Amino acid substitutions at specific residues of YscU fail to autocleave the cytoplasmic C-terminal domain and abolish export of translocator proteins LcrV, YopB, and YopD but not Yop effectors (Sorg et al., 2007; Riordan and Schneewind, 2008).

(4) The ATPase complex in the basal body and cytoplasmic chaperones: The basal body consists of the outer and inner rings connected by a neck domain and the ATPase complex (Figure 1; reviewed in Galan and Wolf-Watz, 2006). *Salmonella enterica* ATPase, InvC, has a critical role in substrate recognition and release of cognate chaperones (Akeda and Galan, 2005). T3SS-specific chaperones are classified based on their cognate interacting partners, effectors, translocators, and needle proteins (Stebbins and Galan, 2003; Cornelis, 2006). Prior to export, chaperones interact with their secreted protein substrates to target this sub-assemblage to the ATPase-sorting complex associated with the basal body. At this specific locale, the effector protein is unfolded, dissociated from the effector-chaperone complex, and then presented to the sorting complex (Boyd et al., 2000; Luo et al., 2001; Birtalan et al., 2002; Galan and Wolf-Watz, 2006; Lara-Tejero et al., 2011). An alternative interface of the effector–chaperone complex induced by conformational changes may act as a recognition signal for the controlled export of effectors (Stebbins and Galan, 2003). In addition, the ATPase provides proton motive force for export of substrates (Eichelberg et al., 1994; Wilharm et al., 2004). Tip protein-specific chaperones PcrG and LcrG for PcrV and LcrV, respectively, possess a regulatory function in T3 protein secretion in the presence of calcium (Nilles et al., 1997; Matson and Nilles, 2001; McCaw et al., 2002; Sundin et al., 2004; Rietsch et al., 2005). In *Yersinia*, a deletion of *lcrG* leads to premature release of Yop proteins (DeBord et al., 2001). In the *Pseudomonas* system, the interaction between PcrV and its cognate chaperone PcrG facilitates the export of PcrV despite the absence of an influence on the secretory regulation of effectors (Lee et al., 2010).

(5) Cytoplasmic regulators: an intrabacterial regulator YopN in *Yersinia* complexes with the co-regulatory protein TyeA to control the entry of secreted proteins to the basal body (Forsberg et al., 1991; Iriarte et al., 1998; Ferracci et al., 2005; Schubot et al., 2005). MxiC, an ortholog of YopN in *Shigella*, interacts with the Spa47 ATPase (Botteaux et al., 2009). A knockout mutant of *mxiC* constitutively secretes effectors but the secretion of translocator IpaC is weak and delayed upon type III induction (Botteaux et al., 2009; Martinez-Argudo and Blocker, 2010). Another cytoplasmic regulator is a molecular ruler or timer, YscP in *Yersinia* and Spa32 in *Shigella*. These molecules determine the needle length and are also involved in the control of substrate specificity switching (Journet et al., 2003; Agrain et al., 2005).

(6) In both *Pseudomonas* and *Yersinia* systems, translocated effector proteins ExoS and YopE play some role in a negative regulatory loop. After delivery of ExoS and YopE into the host cytosol, additional cycles of effector translocation into the same host cell are blocked in a feedback fashion (Aili et al., 2008; Cisz et al., 2008; Urbanowski and Yahr, 2008). The mechanisms of regulation or feedback signal transduction have not been defined.

TRANSLOCON ASSEMBLY AND BRIDGING TO THE NEEDLE STRUCTURE

Activation of the T3 secretory activity initiates the assembly of a translocation complex, called a translocon, in eukaryotic membranes (**Figure 1**). *P. aeruginosa* requires the products of the *pcrGVHpopBD* operon to form the translocon (Frank, 1997; Yahr et al., 1997; Frithz-Lindsten et al., 1998). PcrV, PopB, and PopD are classified as translocators or translocases necessary to form the translocon structure in eukaryotic membranes (Lee et al., 2000; Schoehn et al., 2003; Goure et al., 2004; Caroline et al., 2008). PopB and PopD are hydrophobic translocators that insert into membrane lipids while a hydrophilic translocator, PcrV, is required for assembly and insertion of the functional PopB/PopD translocon into host membranes (Frithz-Lindsten et al., 1998; Goure et al., 2004, 2005). The translocon assembly is necessary for contact-dependent lysis of erythrocytes and the one-step delivery of effector proteins into the host cytosolic compartment (Hakansson et al., 1996; Lee et al., 2000; Schoehn et al., 2003; Goure et al., 2004; Cornelis, 2006; Galan and Wolf-Watz, 2006; Blocker et al., 2008; Caroline et al., 2008). Also, contact-dependent activation of T3SS requires translocon proteins PopB and PopD as well as host cell integrity (Cisz et al., 2008). Using a *Yersinia* co-infection system, YopB, YopD, and LcrV are sufficient for channel formation when these proteins are expressed and secreted by the same bacterium (Marenne et al., 2003).

The formation of translocon channels is assessed by the release of hemoglobins from erythrocytes (Blocker et al., 1999;

Neyt and Cornelis, 1999; Goure et al., 2005). Pore formation in eukaryotic cells can be identified by infection with effectorless strains of T3SS-competent bacteria (Frithz-Lindsten et al., 1998; Viboud and Bliska, 2001; Marenne et al., 2003; Roy et al., 2004; Sato et al., 2011). Even in the absence of known effector proteins, expression of PcrV and other type III components leads to cell damage (Roy et al., 2004; El Solh et al., 2008). V-protein deletion strains are non-cytotoxic and non-hemolytic due to the inability to assemble and insert the translocon in the host membranes (Sarker et al., 1998; Sawa et al., 1999; Goure et al., 2004; Sato et al., 2011). Complementation with a wild-type copy of *pcrV* restores cytotoxicity and hemolytic activity (Sawa et al., 1999; Goure et al., 2004; Sato et al., 2011). These results suggest that PcrV is involved in the mechanical control of translocon formation and insertion in lipid bilayers. It is believed that V-proteins act as a platform for assembly and insertion of translocons (Sarker et al., 1998; Goure et al., 2004; Broz et al., 2007). V-proteins may have a chaperone function for translocon assembly and possibly for self-oligomerization (Goure et al., 2005). The Inv-Mxi-Spa family tip proteins possess a chaperone domain in the N-terminus (reviewed in Blocker et al., 2008). It has also been shown that the C-terminal α-helix of BipD is involved in contacts with translocon proteins (Erskine et al., 2006).

In cell culture systems, effector proteins are translocated into the host cytoplasm in a polarized or vectorial manner (Rosqvist et al., 1994; Sory and Cornelis, 1994; Persson et al., 1995; Vallis et al., 1999a,b). It has been suggested that the tip complex forms a physical bridge between the needle and translocon assembled within the host membrane (Knutton et al., 1998; Nilles et al., 1998; Daniell et al., 2001; Sato et al., 2011).The leakage of effector proteins in the cell culture medium is minimum and rarely detectable during infection *in vitro* (Vallis et al., 1999a,b; Sundin et al., 2004; Sato et al., 2011).

IMMUNIZATION AND THERAPEUTICS TARGETING T3SS

LcrV: A PROTECTIVE EPITOPE OF *YERSINIA* AND DETERMINANT FOR EVASION OF HOST INNATE IMMUNITY

LcrV was first identified as an antigenic factor of *Y. pestis* and named "V-antigen" by Burrows and Bacon (1956). Active immunization with V-antigen protects against pneumonic and bubonic plague in mice (Lawton et al., 1963; Leary et al., 1995; Anderson et al., 1996). Polyclonal antibodies for LcrV provide passive protection against infection with *Y. pestis* and *Y. pseudotuberculosis* (Une and Brubaker, 1984; Motin et al., 1994). LcrV is located at the tip of needles and surface exposed on bacteria so that anti-LcrV antibodies can inhibit the translocon assembly, causing a failure of effector delivery into target cells (Fields et al., 1999; Pettersson et al., 1999; Mueller et al., 2005). LcrV possesses conformational epitopes reported by several groups (Hill et al., 1997; Vernazza et al., 2009; Quenee et al., 2010). In addition to conformational epitopes, a linear epitope was identified in LcrV (amino acid residues 195–225; **Figure 2**; Quenee et al., 2010). Several laboratories developed LcrV subunit vaccines (rV) alone or in combination with other *Y. pestis* proteins, such as the fraction 1 pilus capsular antigen or rF1, which are currently in clinical trials (Powell et al., 2005; Williamson et al., 2005; Quenee and Schneewind, 2009).

Another biological function of LcrV is blocking host inflammatory responses and triggering immunosuppression by the release of IL-10 (Nakajima et al., 1995; Nedialkov et al., 1997; Sing et al., 2002a; Brubaker, 2003; Overheim et al., 2005). IL-10 release was amplified in a TLR2/TLR6 and CD14-dependent manner, suggesting that *Yersinia* evades the host innate immune response by exploiting innate pattern recognition molecules (Sing et al., 2002b, 2005; Depaolo et al., 2008). Additionally, LcrV inhibits the release of proinflammatory cytokines, IFN-γ and TNF-α (Nakajima and Brubaker, 1993; Nakajima et al., 1995). Therapeutically, the ability of LcrV to suppress IFN-γ and TNF-α release was utilized to postpone the inflammatory response in mouse skin allograft models (Motin et al., 1997). A deletion of residues 271–300 in LcrV (the position is indicated in the structural model in **Figure 2**) eliminated the immunomodulatory responses and this variant protein has been developed to the rV10 subunit vaccine (Overheim et al., 2005). Active immunization with rV10 alone or a combination with rF1 protects cynomolgus macaques from pneumonic plague, and the antibodies from macaques provide protection against bubonic plaque in mice (Cornelius et al., 2008). Active immunization with rV10 was also protective in rats and mice (DeBord et al., 2006; Anderson et al., 2009). The rV10 vaccine is undergoing the preclinical efficacy study (Cornelius et al., 2008) along with the FDA pre-investigational new drug authorization review (Quenee and Schneewind, 2009).

PcrV: A PROTECTIVE EPITOPE OF *PSEUDOMONAS*

Among three translocator proteins, only PcrV is a potent protective antigen against T3SS-mediated *Pseudomonas* infection (Sawa et al., 1999; Holder et al., 2001; Frank et al., 2002). Active immunization with recombinant PcrV protects mice from lethal infection even under induced-leukocytopenia or immunosuppression induced by a burn injury (Sawa et al., 1999; Holder et al., 2001; Moriyama et al., 2009). Passive immunization with polyclonal antisera, affinity-purified antibodies, or $F(ab')_2$ were effective against cellular intoxication, lung injury, bacteremia, and sepsis in animal models (Sawa et al., 1999; Shime et al., 2001; Frank et al., 2002; Neely et al., 2005; Imamura et al., 2007; Baer et al., 2009). Polyclonal anti-PcrV antibodies block T3SS-mediated hemolysis of erythrocytes and cytotoxicity of macrophages *in vitro* (Goure et al., 2005; Sato et al., 2011) and reduce inflammatory response and lung injury in infected BALB/c mice (Sawa et al., 1999). In addition to the inhibition of the delivery of effector proteins, anti-PcrV antibodies decrease anti-phagocytic effects mediated by host cells (Sawa et al., 1999).

More than 80 monoclonal stable cell lines were screened by *in vitro* and *in vivo* assays to identify an antibody or combination of antibodies that neutralize the cytotoxic effect of *P. aeruginosa* infection (Frank et al., 2002). Mab166 possesses this property and was subsequently developed to a humanized single-chain antibody for immunotherapy. The $F(ab')_2$ single-chain antibody conjugated with polyethylene glycol was initially produced by InterMune (Frank et al., 2002; Baer et al., 2009; Moriyama et al., 2009) and subjected to a proprietary affinity maturation procedure by Kalobios to produce KB001. Phase 1 and 1/2 clinical studies of KB001 have been completed in mechanically ventilated ICU patients and cystic fibrosis patients. The results of clinical trials indicated the

reduction in the number of inflammatory cells and markers and decreased pneumonia events (KaloBios website[2]).

The protective epitope in PcrV is conformational and located within a region between amino acids 144 and 257 (deletion mapping) or between 158 and 217 as determined by phage display (Frank et al., 2002). Only a conformational epitope has been identified in PcrV at this time (Frank et al., 2002). Mab166 is reactive to the epitope located in the globular region between the central and C-terminal helices (**Figure 2**). The location of the epitope in the PcrV-structure models overlaps the regions identified as the linear and conformational epitopes of LcrV (Vernazza et al., 2009; Quenee et al., 2010). There is no indication of PcrV-mediated immunomodulatory effects while LcrV increases IL-10 production and decreases the release of proinflammatory cytokines, IFN-γ and TNF-α (Nakajima and Brubaker, 1993; Nakajima et al., 1995; Sing et al., 2002a). PcrV has little homology to TLR2-binding motifs located in LcrV, which are responsible for IL-10 induction (Sing et al., 2005; Abramov et al., 2007). In contrast to LcrV, PcrV does not colocalize with TLR2 (Sing et al., 2002b; Overheim et al., 2005). T3SS-competent *P. aeruginosa* strains, but not the deletion mutants of *pcrV* or other translocator genes, recruit neutrophils to the lung, triggering inflammatory responses (Wangdi et al., 2010).

Pseudomonas aeruginosa is intrinsically resistant to a broad range of antibiotics. A high rate of ventilator-associated pneumonia and mortality in patients is caused by *P. aeruginosa* (Rello et al., 1993). PcrV is expressed in most of the type III positive clinical isolates from patients with acute lower respiratory tract infection and systemic infection (Roy-Burman et al., 2001). Infection with PcrV-positive isolates results in high mortality rate even in the absence of cytotoxin ExoU or ExoS (Roy-Burman et al., 2001; El Solh et al., 2008). These data indicate that there might be advantages to the use of PcrV as a therapeutic target. The neutralization of PcrV likely confers little selective pressure to bacteria for their development to a resistant phenotype as compared to selection of resistance through the use of current antibiotic therapies. Thus, passive or active immunization may be useful in combination therapies against intrinsically resistant *P. aeruginosa* isolates (El Solh and Alhajhusain, 2009).

POSSIBLE THERAPEUTICS FOR OTHER TYPE III TARGETS

Immunotherapies targeting a needle-tip protein have been also studied for other type III-positive bacteria. Antibodies recognizing the N-terminus of IpaD neutralize the hemolytic activity of *S. flexneri* (Espina et al., 2006). Antibodies against SipD (SPI-1) protect epithelial cells from invasion by *S. enterica* serovar Enteritidis (Desin et al., 2010). In contrast, active immunization of mice with recombinant BipD is not protective against experimental melioidosis (Druar et al., 2008). CT584 does not induce antibody responses in humans infected with *C. trachomatis* (Wang et al., 2010a). It will be interesting to see the efficacy of a multivalent vaccine based on tip proteins IpaD, BipD, SipD, LcrV, and PcrV, which is in the initial stages of development (Markham et al., 2010). For filamentous-tip protein family, anti-EspA antibodies inhibit cytoskeletal changes in host cells *in vitro* (La Ragione et al., 2006). One monoclonal antibody specific to a linear epitope of EspA was protective against EHEC infection (Yu et al., 2011). Polyclonal sera reactive to Bsp22 protects mice against *Bordetella* infection (Medhekar et al., 2009). Interestingly, the needle protein YscF of *Y. pestis*, another surface-exposed type III protein, induces significant antibody responses and protective effects in mice upon active immunization (Matson et al., 2005).

Another aspect of immunotherapy studies concerns improvement in delivery methods and adjuvants. For example, various and effective delivery methods of vaccines against *Yersinia* infection include liposomal delivery of epitopes or virus-based delivery of vaccines (Chattopadhyay et al., 2008; Heurtault et al., 2009; Bhattacharya et al., 2010; Van Blarcom et al., 2010). Also, whole-cell vaccines, either killed or live and attenuated have been well studied (Bumann et al., 2010; Kamei et al., 2011 and reviewed in Quenee and Schneewind, 2009).

Small molecule inhibitors targeting T3SS have been pursued for the development of novel therapeutics. Inhibitors targeting the enzymatic activity of type III effector proteins, ExoU and ExoS, are effective *in vitro* (Lee et al., 2007; Arnoldo et al., 2008). Inhibitors specific to a *Yersinia* transcription factor, LcrF (VirF), were effective against *Y. pseudotuberculosis*-mediated cytotoxicity *in vitro* and bacterial burden in the lungs in mice of pneumonia, increasing their survival (Garrity-Ryan et al., 2010). Moreover, numerous small molecules have been screened for antivirulence inhibitors targeting the T3SS in several genera of bacteria (Veenendaal et al., 2009; Baron, 2010).

CONCLUDING REMARKS

The T3SS is a potent part of the virulence machinery expressed by *P. aeruginosa* and multiple Gram-negative pathogens. Assembly of type III components and subsequent execution of this intoxication system are intricately regulated and coordinated at multiple levels. Having a comprehensive understanding of these systems as well as working out the mechanistic steps of each stage in the process will give investigators a clue toward the development of novel therapeutics. These therapeutics could target the assembly of the injectisome, the sensing of the microenvironment followed by signal transduction, transcriptional activation, translocon formation in host membranes, and effector delivery into the host cytoplasmic compartment. The PcrV tip complex is located at the distal end of the needle and responsible for several important functions. *pcrV*-deletion strains are unable to intoxicate host target cells due to a failure in the formation of the translocon channel in host plasma membranes. Thus, the structural and functional mechanism of the tip proteins and translocon proteins is particularly relevant to the design of vaccines and therapeutics targeted to neutralize T3SS-mediated intoxication (Motin et al., 1994; Hill et al., 1997; Sawa et al., 1999; Brubaker, 2003; Baer et al., 2009).

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[2]http://www.kalobios.com/kb_pipeline_001.php

REFERENCES

Abramov, V. M., Khlebnikov, V. S., Vasiliev, A. M., Kosarev, I. V., Vasilenko, R. N., Kulikova, N. L., Khodyakova, A. V., Evstigneev, V. I., Uversky, V. N., Motin, V. L., Smirnov, G. B., and Brubaker, R. R. (2007). Attachment of LcrV from *Yersinia pestis* at dual binding sites to human TLR-2 and human IFN-gamma receptor. *J. Proteome Res.* 6, 2222–2231.

Agrain, C., Callebaut, I., Journet, L., Sorg, I., Paroz, C., Mota, L. J., and Cornelis, G. R. (2005). Characterization of a type III secretion substrate specificity switch (T3S4) domain in YscP from *Yersinia enterocolitica*. *Mol. Microbiol.* 56, 54–67.

Aili, M., Isaksson, E. L., Carlsson, S. E., Wolf-Watz, H., Rosqvist, R., and Francis, M. S. (2008). Regulation of *Yersinia* Yop-effector delivery by translocated YopE. *Int. J. Med. Microbiol.* 298, 183–192.

Akeda, Y., and Galan, J. E. (2005). Chaperone release and unfolding of substrates in type III secretion. *Nature* 437, 911–915.

Anderson, D. M., Ciletti, N. A., Lee-Lewis, H., Elli, D., Segal, J., DeBord, K. L., Overheim, K. A., Tretiakova, M., Brubaker, R. R., and Schneewind, O. (2009). Pneumonic plague pathogenesis and immunity in Brown Norway rats. *Am. J. Pathol.* 174, 910–921.

Anderson, G. W. Jr., Leary, S. E., Williamson, E. D., Titball, R. W., Welkos, S. L., Worsham, P. L., and Friedlander, A. M. (1996). Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and -negative strains of *Yersinia pestis*. *Infect. Immun.* 64, 4580–4585.

Arnoldo, A., Curak, J., Kittanakom, S., Chevelev, I., Lee, V. T., Sahebol-Amri, M., Koscik, B., Ljuma, L., Roy, P. J., Bedalov, A., Giaever, G., Nislow, C., Merrill, A. R., Lory, S., and Stagljar, I. (2008). Identification of small molecule inhibitors of *Pseudomonas aeruginosa* exoenzyme S using a yeast phenotypic screen. *PLoS. Genet.* 4, e1000005. doi: 10.1371/journal.pgen.1000005

Baer, M., Sawa, T., Flynn, P., Luehrsen, K., Martinez, D., Wiener-Kronish, J. P., Yarranton, G., and Bebbington, C. (2009). An engineered human antibody fab fragment specific for *Pseudomonas aeruginosa* PcrV antigen has potent antibacterial activity. *Infect. Immun.* 77, 1083–1090.

Bahrani, F. K., Sansonetti, P. J., and Parsot, C. (1997). Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules and kinetics of activation. *Infect. Immun.* 65, 4005–4010.

Baron, C. (2010). Antivirulence drugs to target bacterial secretion systems. *Curr. Opin. Microbiol.* 13, 100–105.

Bergman, T., Hakansson, S., Forsberg, A., Norlander, L., Macellaro, A., Backman, A., Bolin, I., and Wolf-Watz, H. (1991). Analysis of the V antigen lcrGVH-yopBD operon of *Yersinia pseudotuberculosis*: evidence for a regulatory role of LcrH and LcrV. *J. Bacteriol.* 173, 1607–1616.

Betts-Hampikian, H. J., and Fields, K. A. (2010). The chlamydial type III secretion mechanism: revealing cracks in a tough nut. *Front. Microbiol.* 2:114. doi: 10.3389/fmicb.2010.00114

Bhattacharya, D., Mecsas, J., and Hu, L. T. (2010). Development of a vaccinia virus based reservoir-targeted vaccine against *Yersinia pestis*. *Vaccine* 28, 7683–7689.

Birtalan, S. C., Phillips, R. M., and Ghosh, P. (2002). Three-dimensional secretion signals in chaperone-effector complexes of bacterial pathogens. *Mol. Cell* 9, 971–980.

Blocker, A., Gounon, P., Larquet, E., Niebuhr, K., Cabiaux, V., Parsot, C., and Sansonetti, P. (1999). The tripartite type III secreton of *Shigella flexneri* inserts IpaB and IpaC into host membranes. *J. Cell Biol.* 147, 683–693.

Blocker, A. J., Deane, J. E., Veenendaal, A. K., Roversi, P., Hodgkinson, J. L., Johnson, S., and Lea, S. M. (2008). What's the point of the type III secretion system needle? *Proc. Natl. Acad. Sci. U.S.A.* 105, 6507–6513.

Bordi, C., Lamy, M. C., Ventre, I., Termine, E., Hachani, A., Fillet, S., Roche, B., Bleves, S., Mejean, V., Lazdunski, A., and Filloux, A. (2010). Regulatory RNAs and the HptB/RetS signalling pathways fine-tune *Pseudomonas aeruginosa* pathogenesis. *Mol. Microbiol.* 76, 1427–1443.

Botteaux, A., Sory, M. P., Biskri, L., Parsot, C., and Allaoui, A. (2009). MxiC is secreted by and controls the substrate specificity of the *Shigella flexneri* type III secretion apparatus. *Mol. Microbiol.* 71, 449–460.

Boyd, A. P., Lambermont, I., and Cornelis, G. R. (2000). Competition between the Yops of *Yersinia enterocolitica* for delivery into eukaryotic cells: role of the SycE chaperone binding domain of YopE. *J. Bacteriol.* 182, 4811–4821.

Broms, J. E., Sundin, C., Francis, M. S., and Forsberg, A. (2003). Comparative analysis of type III effector translocation by *Yersinia pseudotuberculosis* expressing native LcrV or PcrV from *Pseudomonas aeruginosa*. *J. Infect. Dis.* 188, 239–249.

Broz, P., Mueller, C. A., Muller, S. A., Philippsen, A., Sorg, I., Engel, A., and Cornelis, G. R. (2007). Function and molecular architecture of the *Yersinia* injectisome tip complex. *Mol. Microbiol.* 65, 1311–1320.

Brubaker, R. R. (2003). Interleukin-10 and inhibition of innate immunity to *Yersiniae*: roles of Yops and LcrV (V antigen). *Infect. Immun.* 71, 3673–3681.

Brutinel, E. D., Vakulskas, C. A., and Yahr, T. L. (2009). Functional domains of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* type III secretion system. *J. Bacteriol.* 191, 3811–3821.

Brutinel, E. D., and Yahr, T. L. (2008). Control of gene expression by type III secretory activity. *Curr. Opin. Microbiol.* 11, 128–133.

Bumann, D., Behre, C., Behre, K., Herz, S., Gewecke, B., Gessner, J. E., von Specht, B. U., and Baumann, U. (2010). Systemic, nasal and oral live vaccines against *Pseudomonas aeruginosa*: a clinical trial of immunogenicity in lower airways of human volunteers. *Vaccine* 28, 707–713.

Burrows, T. W., and Bacon, G. A. (1956). The basis of virulence in *Pasteurella pestis*: an antigen determining virulence. *Br. J. Exp. Pathol.* 37, 481–493.

Caroline, G., Eric, F., Bohn, Y. S., Sylvie, E., and Attree, I. (2008). Oligomerization of PcrV and LcrV, protective antigens of *Pseudomonas aeruginosa* and *Yersinia pestis*. *J. Biol. Chem.* 283, 23940–23949.

Carter, P. B., Zahorchak, R. J., and Brubaker, R. R. (1980). Plague virulence antigens from *Yersinia enterocolitica*. *Infect. Immun.* 28, 638–640.

Chatterjee, S., Zhong, D., Nordhues, B. A., Battaile, K. P., Lovell, S., and De Guzman, R. N. (2011). The crystal structures of the *Salmonella* type III secretion system tip protein SipD in complex with deoxycholate and chenodeoxycholate. *Protein Sci.* 20, 75–86.

Chattopadhyay, A., Park, S., Delmas, G., Suresh, R., Senina, S., Perlin, D. S., and Rose, J. K. (2008). Single-dose, virus-vectored vaccine protection against *Yersinia pestis* challenge: CD4(cells are required at the time of challenge for optimal protection. *Vaccine* 26, 6329–6337.

Cisz, M., Lee, P. C., and Rietsch, A. (2008). ExoS controls the cell contact-mediated switch to effector secretion in *Pseudomonas aeruginosa*. *J. Bacteriol.* 190, 2726–2738.

Cornelis, G. R. (2002). The *Yersinia* Ysc-Yop 'type III' weaponry. *Nat. Rev. Mol. Cell Biol.* 3, 742–752.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nat. Rev. Microbiol.* 4, 811–825.

Cornelius, C. A., Quenee, L. E., Overheim, K. A., Koster, F., Brasel, T. L., Elli, D., Ciletti, N. A., and Schneewind, O. (2008). Immunization with recombinant V10 protects cynomolgus macaques from lethal pneumonic plague. *Infect. Immun.* 76, 5588–5597.

Crepin, V. F., Shaw, R., Abe, C. M., Knutton, S., and Frankel, G. (2005). Polarity of enteropathogenic *Escherichia coli* EspA filament assembly and protein secretion. *J. Bacteriol.* 187, 2881–2889.

Daniell, S. J., Takahashi, N., Wilson, R., Friedberg, D., Rosenshine, I., Booy, F. P., Shaw, R. K., Knutton, S., Frankel, G., and Aizawa, S. (2001). The filamentous type III secretion translocon of enteropathogenic *Escherichia coli*. *Cell. Microbiol.* 3, 865–871.

Dasgupta, N., Lykken, G. L., Wolfgang, M. C., and Yahr, T. L. (2004). A novel anti-anti-activator mechanism regulates expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol. Microbiol.* 53, 297–308.

Davis, A. J., Diaz, D. A., and Mecsas, J. (2010). A dominant-negative needle mutant blocks type III secretion of early but not late substrates in *Yersinia*. *Mol. Microbiol.* 76, 236–259.

Davis, A. J., and Mecsas, J. (2007). Mutations in the *Yersinia pseudotuberculosis* type III secretion system needle protein, YscF, that specifically abrogate effector translocation into host cells. *J. Bacteriol.* 189, 83–97.

Deane, J. E., Roversi, P., Cordes, F. S., Johnson, S., Kenjale, R., Daniell, S., Booy, F., Picking, W. D., Picking, W. L., Blocker, A. J., and Lea, S. M. (2006). Molecular model of a type III secretion system needle: Implications for host-cell sensing. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12529–12533.

DeBord, K. L., Anderson, D. M., Marketon, M. M., Overheim, K. A., Depaolo, R. W., Ciletti, N. A., Jabri, B., and Schneewind, O. (2006). Immunogenicity and protective immunity against bubonic plague and pneumonic plague by immunization of mice with the recombinant V10 antigen, a variant of LcrV. *Infect. Immun.* 74, 4910–4914.

DeBord, K. L., Lee, V. T., and Schneewind, O. (2001). Roles of LcrG and LcrV during type III targeting of effector Yops by *Yersinia enterocolitica*. *J. Bacteriol.* 183, 4588–4598.

Depaolo, R. W., Tang, F., Kim, I., Han, M., Levin, N., Ciletti, N., Lin, A., Anderson, D., Schneewind, O., and Jabri, B. (2008). Toll-like receptor 6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis. *Cell Host. Microbe* 4, 350–361.

Derewenda, U., Mateja, A., Devedjiev, Y., Routzahn, K. M., Evdokimov, A. G., Derewenda, Z. S., and Waugh, D. S. (2004). The structure of *Yersinia pestis* V-antigen, an essential virulence factor and mediator of immunity against plague. *Structure* 12, 301–306.

Desin, T. S., Mickael, C. S., Lam, P. K., Potter, A. A., and Koster, W. (2010). Protection of epithelial cells from *Salmonella enterica* serovar Enteritidis invasion by antibodies against the SPI-1 type III secretion system. *Can. J. Microbiol.* 56, 522–526.

Dickenson, N. E., Zhang, L., Epler, C. R., Adam, P. R., Picking, W. L., and Picking, W. D. (2011). Conformational changes in IpaD from *Shigella flexneri* upon binding bile salts provide insight into the second step of type III secretion. *Biochemistry* 50, 172–180.

Druar, C., Yu, F., Barnes, J. L., Okinaka, R. T., Chantratita, N., Beg, S., Stratilo, C. W., Olive, A. J., Soltes, G., Russell, M. L., Limmathurotsakul, D., Norton, R. E., Ni, S. X., Picking, W. D., Jackson, P. J., Stewart, D. I., Tsvetnitsky, V., Picking, W. L., Cherwonogrodzky, J. W., Ketheesan, N., Peacock, S. J., and Wiersma, E. J. (2008). Evaluating *Burkholderia pseudomallei* Bip proteins as vaccines and Bip antibodies as detection agents. *FEMS Immunol. Med. Microbiol.* 52, 78–87.

Eichelberg, K., Ginocchio, C. C., and Galan, J. E. (1994). Molecular and functional characterization of the *Salmonella typhimurium* invasion genes invB and invC: homology of InvC to the F0F1 ATPase family of proteins. *J. Bacteriol.* 176, 4501–4510.

El Solh, A. A., Akinnusi, M. E., Wiener-Kronish, J. P., Lynch, S. V., Pineda, L. A., and Szarpa, K. (2008). Persistent infection with *Pseudomonas aeruginosa* in ventilator-associated pneumonia. *Am. J. Respir. Crit. Care Med.* 178, 513–519.

El Solh, A. A., and Alhajhusain, A. (2009). Update on the treatment of *Pseudomonas aeruginosa* pneumonia. *J. Antimicrob. Chemother.* 64, 229–238.

Epler, C. R., Dickenson, N. E., Olive, A. J., Picking, W. L., and Picking, W. D. (2009). Liposomes recruit IpaC to the *Shigella* type III secretion apparatus needle as a final step in secretion induction. *Infect. Immun.* 77, 2754–2761.

Erskine, P. T., Knight, M. J., Ruaux, A., Mikolajek, H., Sang, N. W. F., Withers, J., Gill, R., Wood, S. P., Wood, M., Fox, G. C., and Cooper, J. B. (2006). High resolution structure of BipD: an invasion protein associated with the type III secretion system of *Burkholderia pseudomallei*. *J. Mol. Biol.* 363, 125–136.

Espina, M., Olive, A. J., Kenjale, R., Moore, D. S., Ausar, S. F., Kaminski, R. W., Oaks, E. V., Middaugh, C. R., Picking, W. D., and Picking, W. L. (2006). IpaD localizes to the tip of the type III secretion system needle of *Shigella flexneri*. *Infect. Immun.* 74, 4391–4400.

Ferracci, F., Schubot, F. D., Waugh, D. S., and Plano, G. V. (2005). Selection and characterization of *Yersinia pestis* YopN mutants that constitutively block Yop secretion. *Mol. Microbiol.* 57, 970–987.

Fields, K. A., Nilles, M. L., Cowan, C., and Straley, S. C. (1999). Virulence role of V antigen of *Yersinia pestis* at the bacterial surface. *Infect. Immun.* 67, 5395–5408.

Forsberg, A., Viitanen, A. M., Skurnik, M., and Wolf-Watz, H. (1991). The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. *Mol. Microbiol.* 5, 977–986.

Frank, D. W. (1997). The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 26, 621–629.

Frank, D. W., Nair, G., and Schweizer, H. P. (1994). Construction and characterization of chromosomal insertional mutations of the *Pseudomonas aeruginosa* exoenzyme S transregulatory locus. *Infect. Immun.* 62, 554–563.

Frank, D. W., Vallis, A., Wiener-Kronish, J. P., Roy-Burman, A., Spack, E. G., Mullaney, B. P., Megdoud, M., Marks, J. D., Fritz, R., and Sawa, T. (2002). Generation and characterization of a protective monoclonal antibody to *Pseudomonas aeruginosa* PcrV. *J. Infect. Dis.* 186, 64–73.

Frithz-Lindsten, E., Holmstrom, A., Jacobsson, L., Soltani, M., Olsson, J., Rosqvist, R., and Forsberg, A. (1998). Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*. *Mol. Microbiol.* 29, 1155–1165.

Galan, J. E., and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* 284, 1322–1328.

Galan, J. E., and Wolf-Watz, H. (2006). Protein delivery into eukaryotic cells by type III secretion machines. *Nature* 444, 567–573.

Garrity-Ryan, L. K., Kim, O. K., Balada-Llasat, J. M., Bartlett, V. J., Verma, A. K., Fisher, M. L., Castillo, C., Songsungthong, W., Tanaka, S. K., Levy, S. B., Mecsas, J., and Alekshun, M. N. (2010). Small molecule inhibitors of LcrF, a *Yersinia pseudotuberculosis* transcription factor, attenuate virulence and limit infection in a murine pneumonia model. *Infect. Immun.* 78, 4683–4690.

Gotoh, K., Kodama, T., Hiyoshi, H., Izutsu, K., Park, K. S., Dryselius, R., Akeda, Y., Honda, T., and Iida, T. (2010). Bile acid-induced virulence gene expression of *Vibrio parahaemolyticus* reveals a novel therapeutic potential for bile acid sequestrants. *PLoS ONE* 5, e13365. doi: 10.1371/journal.pone.0013365

Goure, J., Broz, P., Attree, O., Cornelis, G. R., and Attree, I. (2005). Protective anti-V antibodies inhibit *Pseudomonas* and *Yersinia* translocon assembly within host membranes. *J. Infect. Dis.* 192, 218–225.

Goure, J., Pastor, A., Faudry, E., Chabert, J., Dessen, A., and Attree, I. (2004). The V antigen of *Pseudomonas aeruginosa* is required for assembly of the functional PopB/PopD translocation pore in host cell membranes. *Infect. Immun.* 72, 4741–4750.

Guex, N., and Peitsch, M. C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 18, 2714–2723.

Hakansson, S., Schesser, K., Persson, C., Galyov, E. E., Rosqvist, R., Homble, F., and Wolf-Watz, H. (1996). The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *EMBO J.* 15, 5812–5823.

Hauser, A. R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat. Rev. Microbiol.* 7, 654–665.

Heurtault, B., Gentine, P., Thomann, J. S., Baehr, C., Frisch, B., and Pons, F. (2009). Design of a liposomal candidate vaccine against *Pseudomonas aeruginosa* and its evaluation in triggering systemic and lung mucosal immunity. *Pharm. Res.* 26, 276–285.

Hill, J., Leary, S. E., Griffin, K. F., Williamson, E. D., and Titball, R. W. (1997). Regions of *Yersinia pestis* V antigen that contribute to protection against plague identified by passive and active immunization. *Infect. Immun.* 65, 4476–4482.

Holder, I. A., Neely, A. N., and Frank, D. W. (2001). PcrV immunization enhances survival of burned *Pseudomonas aeruginosa*-infected mice. *Infect. Immun.* 69, 5908–5910.

Holmstrom, A., Olsson, J., Cherepanov, P., Maier, E., Nordfelth, R., Pettersson, J., Benz, R., Wolf-Watz, H., and Forsberg, A. (2001). LcrV is a channel size-determining component of the Yop effector translocon of *Yersinia*. *Mol. Microbiol.* 39, 620–632.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* 62, 379–433.

Imamura, Y., Yanagihara, K., Fukuda, Y., Kaneko, Y., Seki, M., Izumikawa, K., Miyazaki, Y., Hirakata, Y., Sawa, T., Wiener-Kronish, J. P., and Kohno, S. (2007). Effect of anti-PcrV antibody in a murine chronic airway *Pseudomonas aeruginosa* infection model. *Eur. Respir. J.* 29, 965–968.

Iriarte, M., Sory, M. P., Boland, A., Boyd, A. P., Mills, S. D., Lambermont, I., and Cornelis, G. R. (1998). TyeA, a protein involved in control of Yop release and in translocation of *Yersinia* Yop effectors. *EMBO J.* 17, 1907–1918.

Johnson, S., Roversi, P., Espina, M., Olive, A., Deane, J. E., Birket, S., Field, T., Picking, W. D., Blocker, A. J., Galyov, E. E., Picking, W. L., and Lea, S. M. (2007). Self-chaperoning of the type III secretion system needle tip proteins IpaD and BipD. *J. Biol. Chem.* 282, 4035–4044.

Journet, L., Agrain, C., Broz, P., and Cornelis, G. R. (2003). The needle length of bacterial injectisomes is determined by a molecular ruler. *Science* 302, 1757–1760.

Kamei, A., Coutinho-Sledge, Y. S., Goldberg, J. B., Priebe, G. P., and Pier, G. B. (2011). Mucosal vaccination with a multivalent, live-attenuated vaccine induces multifactorial immunity against *Pseudomonas aeruginosa* acute lung infection. *Infect. Immun.* 79, 1289–1299.

Kang, Y., Lunin, V. V., Skarina, T., Savchenko, A., Schurr, M. J., and Hoang, T. T. (2009). The long-chain fatty acid sensor, PsrA, modulates the expression of rpoS and the type III secretion exsCEBA operon in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 73, 120–136.

Kenjale, R., Wilson, J., Zenk, S. F., Saurya, S., Picking, W. L., Picking, W. D., and Blocker, A. (2005). The needle component of the type III secreton of *Shigella* regulates the activity of the secretion apparatus. *J. Biol. Chem.* 280, 42929–42937.

Knutton, S., Rosenshine, I., Pallen, M. J., Nisan, I., Neves, B. C., Bain, C., Wolff, C., Dougan, G., and Frankel, G. (1998). A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J.* 17, 2166–2176.

La Ragione, R. M., Patel, S., Maddison, B., Woodward, M. J., Best, A., Whitelam, G. C., and Gough, K. C. (2006). Recombinant anti-EspA antibodies block *Escherichia coli* O157:H7-induced attaching and effacing lesions in vitro. *Microbes Infect.* 8, 426–433.

Lara-Tejero, M., and Galan, J. E. (2009). *Salmonella enterica* serovar typhimurium pathogenicity island 1-encoded type III secretion system translocases mediate intimate attachment to nonphagocytic cells. *Infect. Immun.* 77, 2635–2642.

Lara-Tejero, M., Kato, J., Wagner, S., Liu, X., and Galan, J. E. (2011). A Sorting Platform Determines the Order of Protein Secretion in Bacterial Type III Systems. *Science* 1188, 1188–1191.

Lawton, W. D., Erdman, R. I., and Surgalla, M. J. (1963). Biosynthesis and purification of V and W antigen in *Pasteurella pestis*. *J. Immunol.* 91, 179–184.

Leary, S. E., Williamson, E. D., Griffin, K. F., Russell, P., Eley, S. M., and Titball, R. W. (1995). Active immunization with recombinant V antigen from *Yersinia pestis* protects mice against plague. *Infect. Immun.* 63, 2854–2858.

Lee, P. C., Stopford, C. M., Svenson, A. G., and Rietsch, A. (2010). Control of effector export by the *P. aeruginosa type III secretion proteins PcrG and PcrV. Mol. Microbiol.* 75, 924–941.

Lee, V. T., Pukatzki, S., Sato, H., Kikawada, E., Kazimirova, A. A., Huang, J., Li, X., Arm, J. P., Frank, D. W., and Lory, S. (2007). Pseudolipasin A is a specific inhibitor for phospholipase A2 activity of *Pseudomonas aeruginosa* cytotoxin ExoU. *Infect. Immun.* 75, 1089–1098.

Lee, V. T., Tam, C., and Schneewind, O. (2000). LcrV, a substrate for *Yersinia enterocolitica* type III secretion, is required for toxin targeting into the cytosol of HeLa cells. *J. Biol. Chem.* 275, 36869–36875.

Luo, Y., Bertero, M. G., Frey, E. A., Pfuetzner, R. A., Wenk, M. R., Creagh, L., Marcus, S. L., Lim, D., Sicheri, F., Kay, C., Haynes, C., Finlay, B. B., and Strynadka, N. C. (2001). Structural and biochemical characterization of the type III secretion chaperones CesT and SigE. *Nat. Struct. Biol.* 8, 1031–1036.

Marenne, M. N., Journet, L., Mota, L. J., and Cornelis, G. R. (2003). Genetic analysis of the formation of the Ysc-Yop translocation pore in macrophages by *Yersinia enterocolitica*: role of LcrV, YscF and YopN. *Microb. Pathog.* 35, 243–258.

Markham, A. P., Barrett, B. S., Esfandiary, R., Picking, W. L., Picking, W. D., Joshi, S. B., and Middaugh, C. R. (2010). Formulation and immunogenicity of a potential multivalent type III secretion system-based protein vaccine. *J. Pharm. Sci.* 99, 4497–4509.

Markham, A. P., Jaafar, Z. A., Kemege, K. E., Middaugh, C. R., and Hefty, P. S. (2009). Biophysical characterization of *Chlamydia trachomatis* CT584 supports its potential role as a type III secretion needle tip protein. *Biochemistry* 48, 10353–10361.

Marteyn, B., West, N. P., Browning, D. F., Cole, J. A., Shaw, J. G., Palm, F., Mounier, J., Prevost, M. C., Sansonetti, P., and Tang, C. M. (2010). Modulation of *Shigella* virulence in response to available oxygen in vivo. *Nature* 465, 355–358.

Martinez-Argudo, I., and Blocker, A. J. (2010). The *Shigella* T3SS needle transmits a signal for MxiC release, which controls secretion of effectors. *Mol. Microbiol.* 78, 1365–1378.

Matson, J. S., Durick, K. A., Bradley, D. S., and Nilles, M. L. (2005). Immunization of mice with YscF provides protection from *Yersinia pestis* infections. *BMC Microbiol.* 5, 38. doi: 10.1186/1471-2180-5-38

Matson, J. S., and Nilles, M. L. (2001). LcrG-LcrV interaction is required for control of Yops secretion in *Yersinia pestis*. *J. Bacteriol.* 183, 5082–5091.

McCaw, M. L., Lykken, G. L., Singh, P. K., and Yahr, T. L. (2002). ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. *Mol. Microbiol.* 46, 1123–1133.

Medhekar, B., Shrivastava, R., Mattoo, S., Gingery, M., and Miller, J. F. (2009). Bordetella Bsp22 forms a filamentous type III secretion system tip complex and is immunoprotective in vitro and in vivo. *Mol. Microbiol.* 71, 492–504.

Menard, R., Sansonetti, P., and Parsot, C. (1994). The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *EMBO J.* 13, 5293–5302.

Menard, R., Sansonetti, P. J., and Parsot, C. (1993). Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J. Bacteriol.* 175, 5899–5906.

Moraes, T. F., Spreter, T., and Strynadka, N. C. (2008). Piecing together the type III injectisome of bacterial pathogens. *Curr. Opin. Struct. Biol.* 18, 258–266.

Moriyama, K., Wiener-Kronish, J. P., and Sawa, T. (2009). Protective effects of affinity-purified antibody and truncated vaccines against *Pseudomonas aeruginosa* V-antigen in neutropenic mice. *Microbiol. Immunol.* 53, 587–594.

Mota, L. J., and Cornelis, G. R. (2005). The bacterial injection kit: type III secretion systems. *Ann. Med.* 37, 234–249.

Motin, V. L., Kutas, S. M., and Brubaker, R. R. (1997). Suppression of mouse skin allograft rejection by protein A-*Yersiniae* V antigen fusion peptide. *Transplantation* 63, 1040–1042.

Motin, V. L., Nakajima, R., Smirnov, G. B., and Brubaker, R. R. (1994). Passive immunity to *Yersiniae* mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide. *Infect. Immun.* 62, 4192–4201.

Mueller, C. A., Broz, P., and Cornelis, G. R. (2008). The type III secretion system tip complex and translocon. *Mol. Microbiol.* 68, 1085–1095.

Mueller, C. A., Broz, P., Muller, S. A., Ringler, P., Erne-Brand, F., Sorg, I., Kuhn, M., Engel, A., and Cornelis, G. R. (2005). The V-antigen of *Yersinia* forms a distinct structure at the tip of injectisome needles. *Science* 310, 674–676.

Nakajima, R., and Brubaker, R. R. (1993). Association between virulence of *Yersinia pestis* and suppression of gamma interferon and tumor necrosis factor alpha. *Infect. Immun.* 61, 23–31.

Nakajima, R., Motin, V. L., and Brubaker, R. R. (1995). Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization. *Infect. Immun.* 63, 3021–3029.

Nedialkov, Y. A., Motin, V. L., and Brubaker, R. R. (1997). Resistance to lipopolysaccharide mediated by the *Yersinia pestis* V antigen-polyhistidine fusion peptide: amplification of interleukin-10. *Infect. Immun.* 65, 1196–1203.

Neely, A. N., Holder, I. A., Wiener-Kronish, J. P., and Sawa, T. (2005). Passive anti-PcrV treatment protects burned mice against *Pseudomonas aeruginosa* challenge. *Burns* 31, 153–158.

Neyt, C., and Cornelis, G. R. (1999). Insertion of a Yop translocation pore into the macrophage plasma membrane by *Yersinia enterocolitica*: requirement for translocators YopB and YopD, but not LcrG. *Mol. Microbiol.* 33, 971–981.

Nilles, M. L., Fields, K. A., and Straley, S. C. (1998). The V antigen of *Yersinia pestis* regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG. *J. Bacteriol.* 180, 3410–3420.

Nilles, M. L., Williams, A. W., Skrzypek, E., and Straley, S. C. (1997). *Yersinia pestis* LcrV forms a stable complex with LcrG and may have a secretion-related regulatory role in the low-Ca2+ response. *J. Bacteriol.* 179, 1307–1316.

Olive, A. J., Kenjale, R., Espina, M., Moore, D. S., Picking, W. L., and Picking, W. D. (2007). Bile salts stimulate recruitment of IpaB to the *Shigella flexneri* surface, where it colocalizes with IpaD at the tip of the type III secretion needle. *Infect. Immun.* 75, 2626–2629.

Overheim, K. A., Depaolo, R. W., DeBord, K. L., Morrin, E. M., Anderson, D. M., Green, N. M., Brubaker, R. R., Jabri, B., and Schneewind, O. (2005). LcrV plague vaccine with altered immunomodulatory properties. *Infect. Immun.* 73, 5152–5159.

Parsot, C. (2009). *Shigella* type III secretion effectors: how, where, when, for what purposes? Current Opinion in Microbiology 12, 110–116.

Persson, C., Nordfelth, R., Holmstrom, A., Hakansson, S., Rosqvist, R., and Wolf-Watz, H. (1995). Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. *Mol. Microbiol.* 18, 135–150.

Pettersson, J., Holmstrom, A., Hill, J., Leary, S., Frithz-Lindsten, E., Euler-Matell, A., Carlsson, E., Titball, R., Forsberg, A., and Wolf-Watz, H. (1999). The V-antigen of *Yersinia* is surface exposed before target cell contact and involved in virulence protein translocation. *Mol. Microbiol.* 32, 961–976.

Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E., and Wolf-Watz, H. (1996). Modulation of virulence factor expression by pathogen target cell contact. *Science* 273, 1231–1233.

Picking, W. L., Nishioka, H., Hearn, P. D., Baxter, M. A., Harrington, A. T., Blocker, A., and Picking, W. D. (2005). IpaD of *Shigella flexneri* is independently required for regulation of Ipa protein secretion and efficient insertion of IpaB and IpaC into host membranes. *Infect. Immun.* 73, 1432–1440.

Powell, B. S., Andrews, G. P., Enama, J. T., Jendrek, S., Bolt, C., Worsham, P., Pullen, J. K., Ribot, W., Hines, H., Smith, L., Heath, D. G., and Adamovicz, J. J. (2005). Design and testing for a nontagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague. *Biotechnol. Prog.* 21, 1490–1510.

Price, S. B., Cowan, C., Perry, R. D., and Straley, S. C. (1991). The *Yersinia pestis* V antigen is a regulatory protein necessary for Ca2+ -dependent growth and maximal expression of low-Ca2+ response virulence genes. *J. Bacteriol.* 173, 2649–2657.

Quenee, L. E., Berube, B. J., Segal, J., Elli, D., Ciletti, N. A., Anderson, D., and Schneewind, O. (2010). Amino acid residues 196-225 of LcrV represent a plague protective epitope. *Vaccine* 28, 1870–1876.

Quenee, L. E., and Schneewind, O. (2009). Plague vaccines and the molecular basis of immunity against *Yersinia pestis*. *Hum. Vaccin.* 5, 817–823.

Rathinavelan, T., Tang, C., and De Guzman, R. N. (2011). Characterization of the interaction between the *Salmonella* type III secretion system tip protein SipD and the needle protein PrgI by paramagnetic relaxation enhancement. *J. Biol. Chem.* 286, 4922–4930.

Rello, J., Ausina, V., Ricart, M., Castella, J., and Prats, G. (1993). Impact of previous antimicrobial therapy on the etiology and outcome of ventilator-associated pneumonia. *Chest* 104, 1230–1235.

Rietsch, A., and Mekalanos, J. J. (2006). Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 59, 807–820.

Rietsch, A., Vallet-Gely, I., Dove, S. L., and Mekalanos, J. J. (2005). ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8006–8011.

Rietsch, A., Wolfgang, M. C., and Mekalanos, J. J. (2004). Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. *Infect. Immun.* 72, 1383–1390.

Rimpilainen, M., Forsberg, A., and Wolf-Watz, H. (1992). A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. *J. Bacteriol.* 174, 3355–3363.

Riordan, K. E., and Schneewind, O. (2008). YscU cleavage and the assembly of *Yersinia* type III secretion machine complexes. *Mol. Microbiol.* 68, 1485–1501.

Rosqvist, R., Magnusson, K. E., and Wolf-Watz, H. (1994). Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *EMBO J.* 13, 964–972.

Roy, D., Liston, D. R., Idone, V. J., Di, A., Nelson, D. J., Pujol, C., Bliska, J. B., Chakrabarti, S., and Andrews, N. W. (2004). A process for controlling intracellular bacterial infections induced by membrane injury. *Science* 304, 1515–1518.

Roy-Burman, A., Savel, R. H., Racine, S., Swanson, B. L., Revadigar, N. S., Fujimoto, J., Sawa, T., Frank, D. W., and Wiener-Kronish, J. P. (2001). Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J. Infect. Dis.* 183, 1767–1774.

Sarker, M. R., Neyt, C., Stainier, I., and Cornelis, G. R. (1998). The *Yersinia* Yop virulon: LcrV is required for extrusion of the translocators YopB and YopD. *J. Bacteriol.* 180, 1207–1214.

Sato, H., Hunt, M. L., Weiner, J. J., Hansen, A. T., and Frank, D. W. (2011). Modified needle-tip PcrV proteins reveal distinct phenotypes relevant to the control of type III secretion and intoxication by *Pseudomonas aeruginosa*. *PLoS ONE* 6, e18356.

Sawa, T., Yahr, T. L., Ohara, M., Kurahashi, K., Gropper, M. A., Wiener-Kronish, J. P., and Frank, D. W. (1999). Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury. *Nat. Med.* 5, 392–398.

Schoehn, G., Di Guilmi, A. M., Lemaire, D., Attree, I., Weissenhorn, W., and Dessen, A. (2003). Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in *Pseudomonas*. *EMBO J.* 22, 4957–4967.

Schroeder, G. N., and Hilbi, H. (2008). Molecular pathogenesis of *Shigella* spp.: *Controlling host cell signaling, invasion, and death by type III secretion*. *Clin. Microbiol. Rev.* 21, 134–156.

Schubot, F. D., Jackson, M. W., Penrose, K. J., Cherry, S., Tropea, J. E., Plano, G. V., and Waugh, D. S. (2005). Three-dimensional structure of a macromolecular assembly that regulates type III secretion in *Yersinia pestis*. *J. Mol. Biol.* 346, 1147–1161.

Schuller, S., and Phillips, A. D. (2010). Microaerobic conditions enhance type III secretion and adherence of enterohaemorrhagic *Escherichia coli* to polarized human intestinal epithelial cells. *Environ. Microbiol.* 12, 2426–2435.

Shime, N., Sawa, T., Fujimoto, J., Faure, K., Allmond, L. R., Karaca, T., Swanson, B. L., Spack, E. G., and Wiener-Kronish, J. P. (2001). Therapeutic administration of anti-PcrV F(ab′)(2) in sepsis associated with *Pseudomonas aeruginosa*. *J. Immunol.* 167, 5880–5886.

Sing, A., Reithmeier-Rost, D., Granfors, K., Hill, J., Roggenkamp, A., and Heesemann, J. (2005). A hypervariable N-terminal region of *Yersinia* LcrV determines Toll-like receptor 2-mediated IL-10 induction and mouse virulence. *Proc. Natl. Acad. Sci. U.S.A.* 102, 16049–16054.

Sing, A., Roggenkamp, A., Geiger, A. M., and Heesemann, J. (2002a). *Yersinia enterocolitica* evasion of the host innate immune response by V antigen-induced IL-10 production of macrophages is abrogated in IL-10-deficient mice. *J. Immunol.* 168, 1315–1321.

Sing, A., Rost, D., Tvardovskaia, N., Roggenkamp, A., Wiedemann, A., Kirschning, C. J., Aepfelbacher, M., and Heesemann, J. (2002b). *Yersinia* V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *J. Exp. Med.* 196, 1017–1024.

Skrzypek, E., and Straley, S. C. (1995). Differential effects of deletions in lcrV on secretion of V antigen, regulation of the low-Ca2+ response, and virulence of *Yersinia pestis*. *J. Bacteriol.* 177, 2530–2542.

Sorg, I., Wagner, S., Amstutz, M., Muller, S. A., Broz, P., Lussi, Y., Engel, A., and Cornelis, G. R. (2007). YscU recognizes translocators as export substrates of the *Yersinia* injectisome. *EMBO J.* 26, 3015–3024.

Sory, M. P., and Cornelis, G. R. (1994). Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. *Mol. Microbiol.* 14, 583–594.

Stebbins, C. E., and Galan, J. E. (2003). Priming virulence factors for delivery into the host. *Nat. Rev. Mol. Cell Biol.* 4, 738–743.

Stensrud, K. F., Adam, P. R., La Mar, C. D., Olive, A. J., Lushington, G. H., Sudharsan, R., Shelton, N. L., Givens, R. S., Picking, W. L., and Picking, W. D. (2008). Deoxycholate interacts with IpaD of *Shigella flexneri* in inducing the recruitment of IpaB to the type III secretion apparatus needle tip. *J. Biol. Chem.* 283, 18646–18654.

Sundin, C., Thelaus, J., Broms, J. E., and Forsberg, A. (2004). Polarisation of type III translocation by *Pseudomonas aeruginosa* requires PcrG, PcrV and PopN. *Microb. Pathog.* 37, 313–322.

Torruellas, J., Jackson, M. W., Pennock, J. W., and Plano, G. V. (2005). The *Yersinia pestis* type III secretion needle plays a role in the regulation of Yop secretion. *Mol. Microbiol.* 57, 1719–1733.

Troisfontaines, P., and Cornelis, G. R. (2005). Type III secretion: more systems than you think. *Physiology* 20, 326–339.

Une, T., and Brubaker, R. R. (1984). Roles of V antigen in promoting virulence and immunity in *Yersiniae*. *J. Immunol.* 133, 2226–2230.

Urbanowski, M. L., Brutinel, E. D., and Yahr, T. L. (2007). Translocation of ExsE into Chinese hamster ovary cells is required for transcriptional induction of the *Pseudomonas aeruginosa* type III secretion system. *Infect. Immun.* 75, 4432–4439.

Urbanowski, M. L., Lykken, G. L., and Yahr, T. L. (2005). A secreted regulatory protein couples transcription to the secretory activity of the *Pseudomonas aeruginosa* type III secretion system. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9930–9935.

Urbanowski, M. L., and Yahr, T. L. (2008). Limiting too much of a good thing: a negative feedback mechanism prevents unregulated translocation of type III effector proteins. *J. Bacteriol.* 190, 2643–2644.

Vallis, A. J., Finck-Barbancon, V., Yahr, T. L., and Frank, D. W. (1999a). Biological effects of *Pseudomonas aeruginosa* type III-secreted proteins on CHO cells. *Infect. Immun.* 67, 2040–2044.

Vallis, A. J., Yahr, T. L., Barbieri, J. T., and Frank, D. W. (1999b). Regulation of ExoS production and secretion by *Pseudomonas aeruginosa* in response to tissue culture conditions. *Infect. Immun.* 67, 914–920.

Van Blarcom, T. J., Sofer-Podesta, C., Ang, J., Boyer, J. L., Crystal, R. G., and Georgiou, G. (2010). Affinity maturation of an anti-V antigen IgG expressed in situ through adenovirus gene delivery confers enhanced protection against *Yersinia pestis* challenge. *Gene Ther.* 17, 913–921.

van der Goot, F. G., Tran, v. N., Allaoui, A., Sansonetti, P., and Lafont, F. (2004). Rafts can trigger contact-mediated secretion of bacterial effectors via a lipid-based mechanism. *J. Biol. Chem.* 279, 47792–47798.

Veenendaal, A. K., Hodgkinson, J. L., Schwarzer, L., Stabat, D., Zenk, S. F., and Blocker, A. J. (2007). The type III secretion system needle tip complex mediates host cell sensing and translocon insertion. *Mol. Microbiol.* 63, 1719–1730.

Veenendaal, A. K., Sundin, C., and Blocker, A. J. (2009). Small-molecule type III secretion system inhibitors block assembly of the *Shigella* type III secreton. *J. Bacteriol.* 191, 563–570.

Vernazza, C., Lingard, B., Flick-Smith, H. C., Baillie, L. W., Hill, J., and Atkins, H. S. (2009). Small protective fragments of the *Yersinia pestis* V antigen. *Vaccine* 27, 2775–2780.

Viboud, G. I., and Bliska, J. B. (2001). A bacterial type III secretion system inhibits actin polymerization to prevent pore formation in host cell membranes. *EMBO J.* 20, 5373–5382.

Wang, J., Zhang, Y., Lu, C., Lei, L., Yu, P., and Zhong, G. (2010a). A genome-wide profiling of the humoral immune response to *Chlamydia trachomatis* infection reveals vaccine candidate antigens expressed in humans. *J. Immunol.* 185, 1670–1680.

Wang, Y., Nordhues, B. A., Zhong, D., and De Guzman, R. N. (2010b). NMR characterization of the interaction of the *Salmonella* type III secretion system protein SipD and bile salts. *Biochemistry* 49, 4220–4226.

Wangdi, T., Mijares, L. A., and Kazmierczak, B. I. (2010). In vivo discrimination of type 3 secretion system-positive and -negative *Pseudomonas aeruginosa* via a caspase-1-dependent pathway. *Infect. Immun.* 78, 4744–4753.

Wilharm, G., Lehmann, V., Krauss, K., Lehnert, B., Richter, S., Ruckdeschel, K., Heesemann, J., and Trulzsch, K. (2004). *Yersinia enterocolitica* type III secretion depends on the proton motive force but not on the flagellar motor components MotA and MotB. *Infect. Immun.* 72, 4004–4009.

Williamson, E. D., Flick-Smith, H. C., Lebutt, C., Rowland, C. A., Jones, S. M., Waters, E. L., Gwyther, R. J., Miller, J., Packer, P. J., and Irving, M. (2005). Human immune response to a plague vaccine comprising recombinant F1 and V antigens. *Infect. Immun.* 73, 3598–3608.

Wulff-Strobel, C. R., Williams, A. W., and Straley, S. C. (2002). LcrQ and SycH function together at the Ysc type III secretion system in *Yersinia pestis* to impose a hierarchy of secretion. *Mol. Microbiol.* 43, 411–423.

Yahr, T. L., Mende-Mueller, L. M., Friese, M. B., and Frank, D. W. (1997). Identification of type III secreted products of the *Pseudomonas aeruginosa* exoenzyme S regulon. *J. Bacteriol.* 179, 7165–7168.

Yahr, T. L., and Wolfgang, M. C. (2006). Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Mol. Microbiol.* 62, 631–640.

Yip, C. K., Finlay, B. B., and Strynadka, N. C. J. (2005). Structural characterization of a type III secretion system filament protein in complex with its chaperone. *Nat. Struct. Mol. Biol.* 12, 75–81.

Yogurtcu, O. N., Wolgemuth, C. W., and Sun, S. X. (2010). Mechanical response and conformational amplification in alpha-helical coiled coils. *Biophys. J.* 99, 3895–3904.

Yu, S., Gu, J., Wang, H. G., Wang, Q. X., Luo, P., Wu, C., Zhang, W. J., Guo, G., Tong, W. D., Zou, Q. M., and Mao, X. H. (2011). Identification of a novel linear epitope on EspA from enterohemorrhagic *E. coli* using a neutralizing and protective monoclonal antibody. *Clin. Immunol.* 138, 77–84.

Yu, X. J., McGourty, K., Liu, M., Unsworth, K. E., and Holden, D. W. (2010). pH sensing by intracellular *Salmonella* induces effector translocation. *Science* 328, 1040–1043.

Zhou, X., Konkel, M. E., and Call, D. R. (2010). Vp1659 is a *Vibrio parahaemolyticus* type III secretion system 1 protein that contributes to translocation of effector proteins needed to induce cytolysis, autophagy, and disruption of actin structure in HeLa cells. *J. Bacteriol.* 192, 3491–3502.

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Subversion of mucosal barrier polarity by *Pseudomonas aeruginosa*

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The lumenal surfaces of human body are lined by a monolayer of epithelia that together with mucus secreting cells and specialized immune cells form the mucosal barrier. This barrier is one of the most fundamental components of the innate immune system, protecting organisms from the vast environmental microbiota. The mucosal epithelium is comprised of polarized epithelial cells with distinct apical and basolateral surfaces that are defined by unique set of protein and lipid composition and are separated by tight junctions. The apical surface serves as a barrier to the outside world and is specialized for the exchange of materials with the lumen. The basolateral surface is adapted for interaction with other cells and for exchange with the bloodstream. A wide network of proteins and lipids regulates the formation and maintenance of the epithelium polarity. Many human pathogens have evolved virulence mechanisms that target this network and interfere with epithelial polarity to enhance binding to the apical surface, enter into cells, and/or cross the mucosal barrier. This review highlights recent advances in our understanding of how *Pseudomonas aeruginosa*, an important opportunistic human pathogen that preferentially infects damaged epithelial tissues, exploits the epithelial cell polarization machinery to enhance infection.

Keywords: *Pseudomonas aeruginosa*, microbial pathogenesis, host–pathogen interactions, cell polarity, epithelial barrier, tight junctions, adherens junctions

THE MUCOSAL BARRIER

The mucosal surfaces of our body are a primary component of our innate immune system and serve as a barrier against endogenous microflora as well as against external pathogens. This barrier is made of polarized epithelial cells, specialized immune cells, and secreted mucus. Many pathogens have evolved strategies to circumvent this barrier, including entering into cells or traveling through them by transcytosis, crossing through intercellular junctions, or directly disrupting the barrier by killing cells in the epithelium (Kazmierczak et al., 2001a).

The mucosal barrier epithelium is comprised of one or more layers of epithelial cells that have specialized and distinct apical and basolateral surfaces, separated by tight junctions (TJs), that form selective permeability barriers between biological compartments (Wang and Margolis, 2007; Martin-Belmonte and Mostov, 2008). The apical surface faces the lumen of the cavity, while the basolateral surface faces adjoining cells and the underlying basement membrane. The apical and basolateral membrane domains are distinguished by unique assemblies of proteins and lipids, creating specific membrane domains with distinct roles in formation and maintenance of barrier function, as well as the myriad of physiological barrier functions, such as nutrient exchange.

The apical surface contains transporters and enzymes that are specialized to interact with the external environment. The outer leaflet of the apical surface is highly enriched in glycosphingolipids and cholesterol. The basolateral plasma membrane of the epithelial cell contains many transporters and receptors that are involved in uptake of nutrients and hormones from the circulation. The basolateral surface can be divided into lateral domains, which contact other cells, and basal domains, which contact the basement membrane and blood vessels. The lateral surface contains specialized cell–cell contact domains, including TJs and adherens junctions (AJs).

The TJ is located at the apical-most region of the lateral surface and defines the boundary between the apical and basolateral surfaces (Ebnet, 2008). The TJ serves two functions. First, it acts as a "gate" or "barrier" to prevent paracellular diffusion between the cells. This function enables the epithelial monolayer to restrict permeability to solutes or larger particles, including pathogens. Second, the TJ acts as a fence to prevent diffusion or intermixing of plasma membrane components between the apical and basolateral domains. The TJ contains three classes of integral membrane proteins: occludins, claudins, and JAMs, each of which forms homophilic interactions that are responsible for gate function of the TJ. The TJ is attached to the cytoskeleton by a set of adaptor proteins including zonula occludens protein 1 (ZO-1).

The AJ lies underneath the TJ. The AJ consists mainly of classical cadherin family members and nectins, which are integral membrane proteins whose large extracellular domains interact in a homophilic or heterophilic manner to connect adjacent cells. Cadherins are linked to the cytoskeleton through β-catenin, α-catenin, and p120-catenin. In addition to providing the structural "linking" of neighboring cells, cadherins function as organizing nodes for multiprotein complexes that regulate cell–cell contacts, an essential function for morphogenesis and remodeling of tissues and organs (Meng and Takeichi, 2009).

A wide network of proteins and lipids regulates the formation and maintenance of epithelial cell polarity. The first step in the formation of apical–basolateral polarity is the formation of

cell–cell junctions. E-cadherins from adjacent cells interact to create homophilic intercellular adhesions. Activation of small Rho GTPase family members, leads to cytoskeleton rearrangement and recruitment of structural and regulatory proteins, resulting in the formation of mature TJs and AJs (Iden and Collard, 2008). Junction maturation is coupled to the development of apical–basolateral asymmetry in the cell, where the newly formed AJ serves as a site for basolateral protein sorting (Yeaman et al., 2004). Maintenance of cell polarity and junction integrity involves continuous sensing of external cues such as extracellular matrix content and cell–cell contacts. These cues are translated into cellular signals that are received by a regulatory core of three protein complexes: Par3/Par6/aPKC, the Crumbs complex (Crumbs-3, PALS1, and PATJ), and the Scribble complex (Scribble, LGL1/2, and DLG1). The mutually exclusive localization of these three complexes helps to stabilize apical–basolateral polarity (Bryant and Mostov, 2008; Iden and Collard, 2008).

In addition to the asymmetric distribution of key polarity proteins described above, phosphoinositides have emerged as important determinants of membrane identity. These lipids bind to specific protein domains, particularly to those involved in the regulation of the cytoskeleton. In mammalian cells, phosphatidylinositol 4,5-bisphosphate (PIP_2) is found primarily on the apical surface whereas phosphatidylinositol 3,4,5-trisphosphate (PIP_3) localizes to the basolateral surface (Martin-Belmonte and Mostov, 2008).

The apical–basolateral polarity regulation system is increasingly recognized as an important target for pathogens. Our understanding of the interactions between pathogens with the mucosal barrier has been greatly aided by the use of epithelial cell lines, such as dog kidney (MDCK) cells, Calu-3 (a cell line derived from a human adenocarcinoma), and 16HBE (derived from human bronchial epithelial cells) cells that grow as a single confluent monolayer and recapitulate the development of polarized epithelium when grown on porous filter supports (transwells; Mostov et al., 2005). When grown at high densities under these conditions, the cells can obtain nutrients from the basolateral medium and will form polarized epithelium with distinct apical and basolateral surfaces and functional TJs and AJs within 24 h. With continued culture, cell polarity develops further. One advantage of this system is that pathogen–epithelial interactions can be studied without confounding effects contributed by immune cells. Furthermore, by using confluent monolayers, it is possible to compare microbe interactions between the apical and basolateral surfaces without having to take into account the effect of increased access to the basolateral surface that occurs in subconfluent cells or in the setting of epithelial injury (Kazmierczak et al., 2001a). Finally, some of the epithelial cells, including MDCK cells and primary mouse alveolar type II cells, can be grown as three-dimensional (3D) cysts when cultured on extracellular matrix in which the basolateral surface faces outward (Bryant and Mostov, 2008). These models may more closely mimic organs; in addition, they facilitate the examination of interactions of pathogens with the basolateral surface in the absence of the porous filter support (Barrila et al., 2010; Bucior et al., 2010). These reductionist systems provide a platform to analyze host–pathogen interactions, which can then be further validated in animal studies.

PATHOGENS SUBVERT CELL POLARITY: AN EMERGING THEME

Important human mucosal pathogens, including viruses and bacteria, have been shown to directly target components of the polarity regulation network. Here we discuss a few recently described examples (**Figure 1**). *Neisseria meningitidis*, a devastating and common cause of bacterial meningitis, recruits Par3/Par6/aPKC to subvert junctional proteins at the endothelial cell surface. Ectopic intercellular junctional domains are formed at the site of binding of microcolonies by subverting an endothelial specific G-protein coupled receptor, the β2-adrenergic receptor, and an associated scaffolding protein, β-arrestin (Coureuil et al., 2010). This event leads to depletion of junctional proteins at the cell–cell interface and opening of intercellular junctions at the brain–endothelial interface, potentially explaining the tropism of this pathogen for the human central nervous system (Coureuil et al., 2009). Notably, these events are not observed in epithelial cells, as they appear to require the endothelial specific proteins VE-cadherin and β2-adrenergic receptor. Enteropathogenic *Escherichia coli*, a leading cause of diarrhea in children in the third world, induces PIP_2 and PIP_3 clustering at the bacterial-induced actin-rich pedestal in MDCK cells (Sason et al., 2009; Smith et al., 2010). CagA, the only known *Helicobacter pylori* effector translocated through its type IV secretion system, recruits the TJ proteins ZO-1 and JAM-A to the site of bacterial attachment in polarized MDCK cells (Amieva et al., 2003). CagA also directly interacts with and inhibits the serine/threonine kinase Par-1, causing it to dissociate from the membrane and leading to junction and polarity defects (Saadat et al., 2007). These events may contribute to the *H. pylori*-induced disorganization of gastric epithelial architecture and subsequent mucosal damage, inflammation, and cancer (Saadat et al., 2007). The disruption of epithelial barrier polarity by CagA has also been reported to create a nutrient-rich niche for *H. pylori* replication at the apical surface, allowing growth of microcolonies directly over the intercellular junctions (Tan et al., 2009). *Listeria monocytogenes*, a Gram-positive, food-borne pathogen uses E-cadherin as a binding receptor and as an internalization platform. Binding of the bacterial surface protein internalin A to E-cadherin initiates a process of actin rearrangement and AJ protein recruitment near the bacterial binding site that stimulates endocytosis and internalization of the bacteria (Bonazzi et al., 2009). Several viruses, including adenovirus, α-herpes viruses, reoviruses, and Hepatitis C target junctional complexes and polarity regulators as well (Bergelson, 2009).

PSEUDOMONAS AERUGINOSA, AN OPPORTUNISTIC PATHOGEN, EXPLOITS EPITHELIAL DAMAGE AND LOSS OF POLARITY

For opportunistic pathogens, of which *Pseudomonas aeruginosa* is an important example, the mucosal barrier represents a formidable challenge to bacterial-mediated damage or entry. However, in the setting of injured or incompletely polarized epithelium, *P. aeruginosa* can initiate colonization and unleash its arsenal of potent virulence factors, which include the type III secretion system (T3SS) and its secreted effectors (Engel, 2003; Engel and Balachandran, 2009). Indeed, this Gram-negative pathogen is a leading cause of nosocomial infections in hospitalized patients (Mandell et al., 2010). Its predilection for injured tissue explains

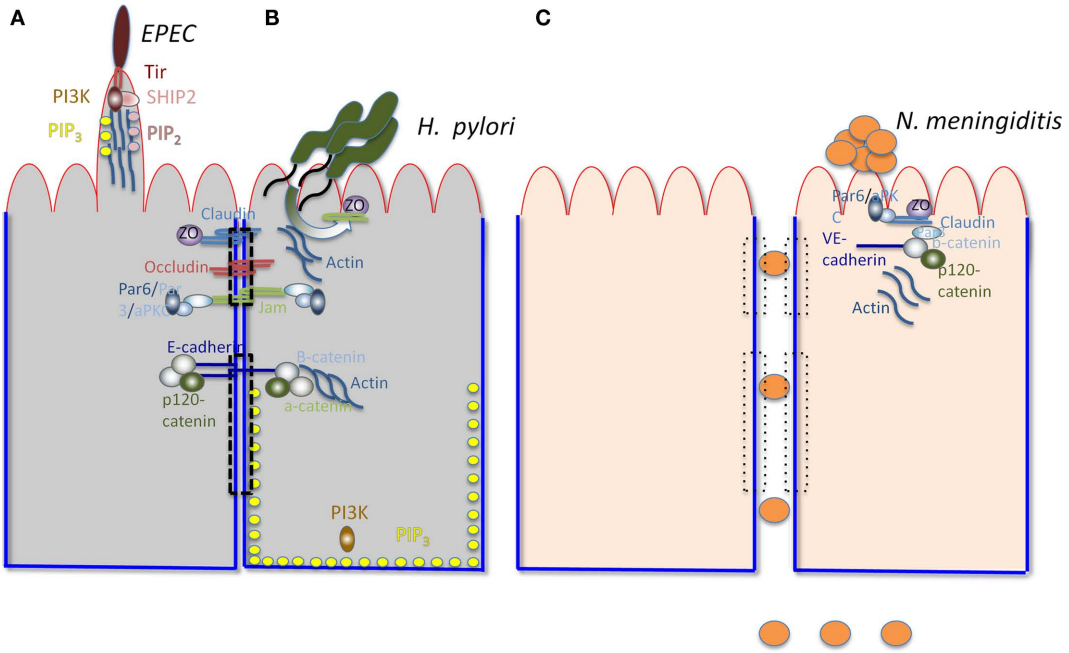

FIGURE 1 | Interactions of various pathogens with polarized cells. The apical surface is outlined in red and the basolateral surface in blue. The TJ (upper) and AJ (lower) are indicated by a dashed rectangle. The major components of TJs (claudin, occludin, ZO, and JAMs) and AJs (E-cadherin, b-catenin, and a-catenin) are shown. The Par3/Par6/aPKC complex is shown to associate with JAMs. Actin is associated with AJs. PI3K and PIP_3 are associated with the BL surface. **(A)** Illustrates relevant characteristics of EPEC-induced pedestals. PI3K, PIP_3, SHIP2, and PIP2 are recruited by Tir (the translocated intimin receptor) to the actin-containing pedestal, along with actin, Arp2/3, Nck, and N-wasp (not shown). **(B)** Illustrates *H. pylori* recruiting junctional components, including JAMs, ZO-1, and Par (not shown) to form a replicative niche at the AP surface. **(C)** Represents endothelial cells, shows *N. meningitidis* disrupting intracellular junctions and breaching the blood–brain barrier by recruiting components of the TJ and the AJ (including Par-6, aPKC, Par-3, Claudin, ZO-5, VE-cadherin, b-catenin, and p-120 catenin) to the site of binding of the bacterial microcolony at the AP surface. The loss of the TJ and AJ is illustrated by the rectangles with dotted lines.

its propensity to cause ventilator-associated pneumonia, skin infections in burn patients or at the site of surgical incisions, and catheter-related infections, amongst others. *P. aeruginosa* is also a cause of chronic lung infections and ultimately death in patients with cystic fibrosis (CF; Mandell et al., 2010). Studies of the interaction of *P. aeruginosa* with polarized epithelium in culture and *in vivo* show that the degree of polarity significantly affects the final outcome of infection. Bacterial-induced host cell cytotoxicity or internalization is enhanced when bacteria are added to cells whose polarity has been altered by various manipulations (Fleiszig et al., 1997, 1998; Kazmierczak et al., 2001b, 2004). Finally, *P. aeruginosa* preferentially adheres to, enters, and injures wounded epithelium (Yamaguchi and Yamada, 1991; Zahm et al., 1991; Tsang et al., 1994; de Bentzmann et al., 1996a,b,c; Geiser et al., 2001). In this review, we describe selected recent advances in our understanding of *P. aeruginosa* interactions with polarized epithelium at each step of the infection process.

FIRST MINUTES OF INFECTION: BINDING TO THE EPITHELIUM

The primary adhesins for *P. aeruginosa* binding to epithelial cells include type IV pili (TFP), polarly localized pili that can extend and retract, and flagella, that power swimming motility. For both adhesins, the molecular details of attachment to polarized epithelium remain to be clearly defined (Engel, 2007). In recent exciting studies, the role of retractile TFP in adherence of *P. aeruginosa* (strain PAK) to human lung epithelial primary cells grown as pseudostratified epithelium on a porous filter support was further examined (Heiniger et al., 2010). The authors compared infection of the apical surface of the intact pseudostratified epithelium to infection of mechanically injured cells, in which the cells comprising the basolateral layer of the pseudostratified epithelium are exposed. Binding to the intact pseudostratified epithelium required TFP but was not dependent upon pilin retraction. In contrast, pilin retraction (powered by the ATPase PilT) as well as the surface pilin associated protein PilY1, was required to penetrate into the basolateral portions of the monolayer. In the future, it will be of interest to determine whether PilY1 can directly bind to eukaryotic cells, whether antibodies directed against PilY1 can block this binding, and whether PilY1 binds specifically to one or more host cell proteins.

Bucior et al. (2010) examined the binding and subsequent downstream events during exposure of *P. aeruginosa* to the apical or basolateral surfaces of epithelial cells grown at various states of polarization. Confluent monolayers of MDCK and Calu-3 cells were plated on transwells and grown for different lengths of time to recapitulate various stages of polarization. These cells were infected with two different *P. aeruginosa* strains (PA01 and PAK) and the role of glycans or heparan sulfate proteoglycans (HSPGs) was examined. N-glycans were of special interest because previous work had revealed that MDCK cells with defects in cell surface glycosylation were resistant to *P. aeruginosa*

infection, indicating that bacteria might require N-glycans for initial adhesion and subsequent host cell injury (Apodaca et al., 1995). HSPGs were examined because they are enriched at the basolateral surface. Indeed, the authors found that N-glycan chains at the apical surface were necessary and sufficient for *P. aeruginosa* binding, invasion, and cytotoxicity to MDCK and Calu-3 cells grown at various states of polarization. Enhanced expression and/or expression of more complex mannose-containing glycans increased binding, invasion, and cytotoxicity toward well-polarized epithelial cell monolayers, whereas pharmacologic inhibition of N-glycosylation or infection of concanavalin A-resistant MDCK cells resulted in decreased binding, invasion, and cytotoxicity. At the basolateral surface, the sulfation of heparan sulfate (HS) chains of HSPGs was found to be critical for *P. aeruginosa* binding, cytotoxicity, and invasion. In incompletely polarized epithelium, HSPG abundance was increased at the apical surface, explaining at least in part the increased susceptibility of injured epithelium to *P. aeruginosa* colonization and damage. Using MDCK cells grown as 3D cysts as a model for epithelial organs, *P. aeruginosa* was shown to specifically co-localized with HS-rich areas at the basolateral membrane. *P. aeruginosa* was also shown to bind HS chains and N-glycans coated onto plastic surfaces, with the highest binding affinity toward HS chains, suggesting that N-glycans and HS may be major regulators of *P. aeruginosa* binding to apical and basolateral membranes, respectively. Together, these findings demonstrate that *P. aeruginosa* recognizes distinct receptors on the apical and basolateral surface of polarized epithelium.

In the future, it will be of interest to examine whether TFP and flagella play different roles in adhesion at the apical versus basolateral surface, whether they display different binding specificities toward N-glycans and HSPGs, and whether these specificities are observed *in vivo* as well. It would also be informative to test whether N-glycosylation or HSPGs are upregulated in lungs of CF patients. Identifying the specific N-glycosylated apical receptor for *P. aeruginosa* will be an important step for our understanding of *P. aeruginosa* pathogenesis. Even in the absence of a defined apical receptor, this work suggests that therapies that target both N-glycosylation and HSPG synthesis, that compete with binding to N-glycans or HSPGs (such as simple sugars or heparin), or that restore polarized segregation of these molecules may be useful adjuncts to more standard antibiotic therapy.

THE EARLY HOURS: SUBVERSION OF EPITHELIAL POLARITY TO ENHANCE INFECTION

Pseudomonas aeruginosa binding activates a central host signaling molecule, phosphatidylinositol 3-kinase (PI3K), which is required for the synthesis of PIP_3 and for activation of a downstream effector, the serine/threonine kinase Akt. Activation of the PI3K/PIP_3/Akt pathway was shown to be necessary and sufficient for *P. aeruginosa* entry from the apical surface of polarized epithelial cells (Kierbel et al., 2005a). Interestingly, PIP_3 has recently emerged as a key polarity regulator that serves as a scaffold at the basolateral cytoplasm (Di Paolo and De Camilli, 2006; Gassama-Diagne et al., 2006). In follow-up studies, Kierbel and colleagues (Gassama-Diagne et al., 2006; Kierbel et al., 2007) have shown that *P. aeruginosa* subverts the PI3K/PIP_3/Akt pathway to transform a patch of the apical surface into one with basolateral characteristics and to gain entry from the apical surface (**Figure 2**). In polarized monolayers, *P. aeruginosa* binds near cell–cell junctions, where it activates and recruits PI3K to the apical surface. This event leads to a remarkable remodeling of the apical membrane in which protrusions enriched for PIP_3 and actin form at the apical surface at the site of bacterial binding. These protrusions are deficient in apical membrane markers and contain basolateral constituents. Notably, no disruption of the TJs could be detected, suggesting that the bacterial-induced rerouting of basolateral markers involved transcytosis rather than diffusion. Consistent with this notion, a dominant negative mutant of dynamin also blocked delocalization of basolateral proteins to the bacterial-induced apical protrusion. The end result is that this bacterium transforms apical into basolateral membrane, creating a local microenvironment that facilitates its colonization and entry into to the mucosal barrier.

Since apical–basolateral polarity is highly regulated in epithelial cells, it will be interesting to identify the initial events that lead to protrusion formation. Preliminary results suggest that polarity regulators are recruited to the bacterial binding site prior to PI3K (unpublished results). Analysis of the dynamics of protrusion formation, determining whether AJs or TJs are formed at the site of aggregate binding, and elucidating the molecular mechanisms by which they are redirected to the apical surface, will be key to understanding PA-induced apical protrusions.

For both *P. aeruginosa* and *N. meningitidis*, protrusion formation involves aggregates of bacteria as opposed to individual bacteria. It is possible that a threshold number of bacteria are required to activate this response or that these structures represent a specific host response to a physically large cluster. While bacterial aggregates are observed in stationary phase cultures in the absence of host cells (Allesen-Holm et al., 2006), it will be interesting to determine whether they are preformed or whether they form in response to host cells, as suggested by earlier work that described pack-swarming motility (Dacheux et al., 2001). Finally, comparison of the interactions of the polarized epithelium with single bacteria versus aggregates, the requirement for the major adhesins TFP and flagella, and the role of the T3SS in the formation of protrusions will be of interest to examine.

What function does protrusion formation serve and who benefits from it? One attractive possibility is that the bacteria use these protrusions for entry. Indeed, treatments that inhibit entry, such as inhibitors of PI3K or of the actin cytoskeleton, also inhibit protrusion formation (Kierbel et al., 2005b). Alternatively or in addition, protrusion formation may represent a response that could be beneficial to the host. For example, recruitment of basolateral constituents to the apical surface could trigger the innate immune response in a spatially restricted manner. The relationship of the PI3K/Akt pathway to other signaling pathways activated upon *P. aeruginosa* binding and internalization, including translocation of NF-kB to the nucleus and cytokine production, remains to be explored.

LATE INFECTION: DISRUPTION OF CELL–CELL CONTACTS IN THE EPITHELIUM OR SIMPLY CELL DEATH?

An important question in the study *P. aeruginosa* pathogenesis is the fate of the adherent bacteria, whether they be aggregates or single bacterial cells. For chronic infection, such as in lungs of CF patients, *P. aeruginosa* is thought to grow as biofilms in the thick layer of mucus overlying airway epithelial cells rather than adhering and invading directly into to the airway epithelium (Moreau-Marquis et al., 2008). In contrast, during acute infection, bacterial internalization, host cell

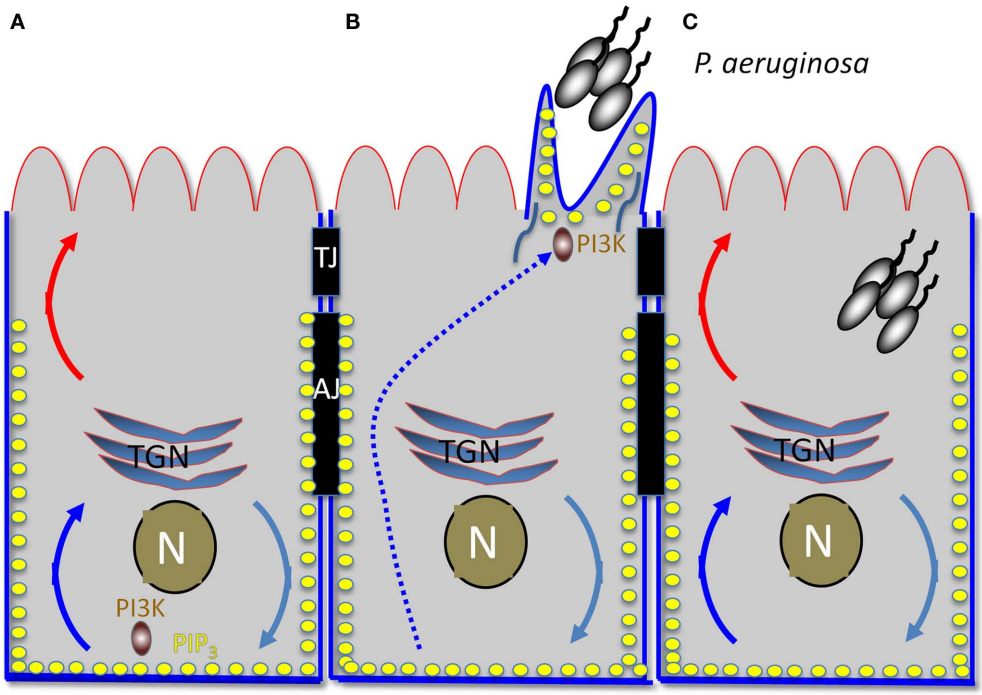

FIGURE 2 | Early hours of *P. aeruginosa* infection: subversion of host cell polarity to transform apical into a basolateral membrane. (A) Diagram of polarized epithelial cell. The apical surface is outlined in red and the basolateral surface in blue. TJ, tight junction; AJ, adherens junction; TGN, *trans*-Golgi network; N, nucleus. Turquoise, blue, and red curved arrows represent vesicular trafficking. PIP_3 is represented by the yellow dots. **(B)** Protrusion formation. An aggregate of *P. aeruginosa* recruits PI3K to the apical surface, generating local production of PIP_3. BL recycling of proteins is redirected to the AP surface, creating a basolateral environment at the apical surface. **(C)** An aggregate of *P. aeruginosa* is internalized into the host cell, possibly at least in part through the transient protrusion.

destruction, and penetration to the basolateral surface are observed (Kazmierczak et al., 2001a). The general trend emerging from these studies is that loss of cell polarity enhances adhesion and favors subsequent downstream events, including cell death, disruption of cell junctions, inhibition of wound repair, and/or bacterial internalization (Kazmierczak et al., 2001a; Garrity-Ryan et al., 2004; Bucior et al., 2010), but differences in cell types, infection conditions and in the spectrum of T3SS toxins influences the outcome. For example, ExoU-secreting strains are associated with rapid host cell death by necrosis; ExoS-secreting *P. aeruginosa* strains lead to slower host cell death with features of apoptosis; and strains engineered to produce only ExoT lead to apoptotic cell death by ADP ribosylation of Crk (Alaoui-El-Azher et al., 2006; Shafikhani et al., 2008; Hauser, 2009).

Out of the four known *P. aeruginosa* T3SS effectors proteins, ExoS and ExoT are logical candidates for interfering with cellular polarity and disrupting cell junction integrity (Engel and Balachandran, 2009; Hauser, 2009). These two highly homologous bifunctional enzymes encode an N-terminal GTPase-activating protein (GAP) domain with activity toward Rho family GTPases and a C-terminal ADP ribosyltransferase domain. Each domain interacts with central components of the polarity regulation network. The GAP domains of both ExoT and ExoS inactivate the RhoA family GTPases, Ccd42, Rac1, and RhoA. The ExoS ADPRT domain targets a variety of effectors involved in regulating the actin cytoskeleton, including Ras, Ral, Rabs, and ERM proteins, while the ADPRT domain of ExoT specifically targets CrkI and CrkII, scaffolding proteins associated with focal adhesions (Sun et al., 2004).

Recent studies from the Prince lab have examined in more detail the effect of T3SS effectors on epithelial barrier function (Soong et al., 2008). Human airway cells grown on transwell filters were infected with various informative isogenic mutants of PAK, a representative non-ExoU-producing strain. ExoS was found to be necessary and sufficient to alter the integrity of TJs in the absence of host cell death, as measured by accessibility to biotin, permeability to small and large molecular weight dextrans, bacterial transmigration, and the absence of LDH release or trypan blue staining under the conditions of the experiments. In PAK strains engineered to express only ExoS, the ADPRT domain was necessary and sufficient to alter epithelial cell permeability. PAK strains expressing ExoS disrupted ZO-1, occludin, and ezrin localization, decreased membrane-associated occludin, and blocked ezrin phosphorylation as would be predicted from ADP ribosylation of ezrin. The authors conclude that the ADPRT domain of ExoS is sufficient to disrupt epithelial barrier integrity. Of note, the exact mechanism by which ExoS disrupts TJs remains to be determined. It could represent a general cellular response to ExoS-mediated cell death or to the disruption of the actin cytoskeleton, or it could be a direct consequence of ExoS on junctional components.

SUMMARY

An emerging theme in microbial pathogenesis is the recognition that pathogens exploit or disrupt components of the mucosal barrier in order to facilitate colonization, to create a specialized niche

for replication where they remain shielded from the host immune response, and/or to disseminate to distant tissues or to new hosts. Host cell polarity determinants are logical targets for pathogens, as control of epithelial cell polarity requires constant sensing of external cues. In addition, the signaling nodes emanating from polarity determinants may interface with the host innate immune response, although this connection has just begun to be explored. This review highlights recent advances and suggests future directions in furthering our understanding of how *P. aeruginosa* exploit the host polarity machinery to establish infection. These studies may lead us to discover new bacterial virulence mechanisms and therapeutic targets against this highly antibiotic resistance pathogen. Finally, it will extend our understanding of the initiation and maintenance of epithelial cell polarity, an area important in development, cancer biology, and pathogenesis.

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REFERENCES

Alaoui-El-Azher, M., Jia, J., Lian, W., and Jin, S. (2006). ExoS of *Pseudomonas aeruginosa* induces apoptosis through a Fas receptor/caspase 8-independent pathway in HeLa cells. *Cell. Microbiol.* 8, 326–338.

Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M., and Tolker-Nielsen, T. (2006). A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol. Microbiol.* 59, 1114–1128.

Amieva, M. R., Vogelmann, R., Covacci, A., Tompkins, L. S., Nelson, W. J., and Falkow, S. (2003). Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* 300, 1430–1434.

Apodaca, G., Bomsel, M., Lindstedt, R., Engel, J., Frank, D., Mostov, K., and Wiener-Kronish, J. (1995). Characterization of *Pseudomonas aeruginosa*-induced MDCK cell injury: glycosylation defective host cells are resistant to bacterial killing. *Infect. Immun.* 63, 1541–1551.

Barrila, J., Radtke, A. L., Crabbe, A., Sarker, S. F., Herbst-Kralovetz, M. M., Ott, C. M., and Nickerson, C. A. (2010). Organotypic 3D cell culture models: using the rotating wall vessel to study host–pathogen interactions. *Nat. Rev. Microbiol.* 8, 791–801.

Bergelson, J. M. (2009). Intercellular junctional proteins as receptors and barriers to virus infection and spread. *Cell Host Microbe* 5, 517–521.

Bonazzi, M., Lecuit, M., and Cossart, P. (2009). *Listeria monocytogenes* internalin and E-cadherin: from bench to bedside. *Cold Spring Harb. Perspect. Biol.* 1, a003087.

Bryant, D. M., and Mostov, K. E. (2008). From cells to organs: building polarized tissue. *Nat. Rev. Mol. Cell Biol.* 9, 887–901.

Bucior, I., Mostov, K., and Engel, J. N. (2010). *Pseudomonas aeruginosa*-mediated damage requires distinct receptors at the apical and basolateral surfaces of the polarized epithelium. *Infect. Immun.* 78, 939–953.

Coureuil, M., Lecuyer, H., Scott, M. G., Boularan, C., Enslen, H., Soyer, M., Mikaty, G., Bourdoulous, S., Nassif, X., and Marullo, S. (2010). Meningococcus hijacks a beta2-adrenoceptor/beta-arrestin pathway to cross brain microvasculature endothelium. *Cell* 143, 1149–1160.

Coureuil, M., Mikaty, G., Miller, F., Lecuyer, H., Bernard, C., Bourdoulous, S., Dumenil, G., Mege, R. M., Weksler, B. B., Romero, I. A., Couraud, P. O., and Nassif, X. (2009). Meningococcal type IV pili recruit the polarity complex to cross the brain endothelium. *Science* 325, 83–87.

Dacheux, D., Goure, J., Chabert, J., Usson, Y., and Attree, I. (2001). Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas aeruginosa*-infected macrophages. *Mol. Microbiol.* 40, 76–85.

de Bentzmann, S., Plotkowski, C., and Puchelle, E. (1996a). Receptors in the *Pseudomonas aeruginosa* adherence to injured and repairing airway epithelium. *Am. J. Respir. Crit. Care Med.* 154, S155–S162.

de Bentzmann, S., Roger, P., Dupuit, F., Bajolet-Laudinat, O., Fuchey, C., Plotkowski, M. C., and Puchelle, E. (1996b). Asialo GM1 is a receptor for *Pseudomonas aeruginosa* adherence to regenerating respiratory epithelial cells. *Infect. Immun.* 64, 1582–1588.

de Bentzmann, S., Roger, P., and Puchelle, E. (1996c). *Pseudomonas aeruginosa* adherence to remodelling respiratory epithelium. *Eur. Respir. J.* 10, 2145–2150.

Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443, 651–657.

Ebnet, K. (2008). Organization of multiprotein complexes at cell–cell junctions. *Histochem. Cell Biol.* 130, 1–20.

Engel, J. (2007). "*Pseudomonas aeruginosa* internalization by non-phagocytic cells," in *Pseudomonas: A Model System in Biology*, eds. J.-L. Ramos and A. Filloux (New York, NY: Springer) 343–368.

Engel, J., and Balachandran, P. (2009). Role of *Pseudomonas aeruginosa* type III effectors in disease. *Curr. Opin. Microbiol.* 12, 61–66.

Engel, J. N. (2003). "Molecular pathogenesis of acute *Pseudomonas aeruginosa* infections," in *Severe Infections Caused by Pseudomonas aeruginosa*, eds A. Hauser and J. Rello (New York: Kluwer Academic/Plenum Press), 201–230.

Fleiszig, S. M., Vallas, V., Jun, C. H., Mok, L., Balkovetz, D. F., Roth, M. G., and Mostov, K. E. (1998). Susceptibility of epithelial cells to *Pseudomonas aeruginosa* invasion and cytotoxicity is upregulated by hepatocyte growth factor. *Infect. Immun.* 66, 3443–3446.

Fleiszig, S. M., Wiener-Kronish, J. P., Miyazaki, H., Vallas, V., Mostov, K. E., Kanada, D., Sawa, T., Yen, T. S., and Frank, D. W. (1997). *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. *Infect. Immun.* 65, 579–586.

Garrity-Ryan, L., Shafikhani, S., Balachandran, P., Nguyen, L., Oza, J., Jakobsen, T., Sargent, J., Fang, X., Cordwell, S., Matthay, M. A., and Engel, J. N. (2004). The ADP ribosyltransferase domain of *Pseudomonas aeruginosa* ExoT contributes to its biological activities. *Infect. Immun.* 72, 546–558.

Gassama-Diagne, A., Yu, W., Ter Beest, M., Martin-Belmonte, F., Kierbel, A., Engel, J., and Mostov, K. (2006). Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. *Nat. Cell Biol.* 8, 963.

Geiser, T. K., Kazmierczak, B. I., Garrity-Ryan, L. K., Matthay, M. A., and Engel, J. N. (2001). *Pseudomonas aeruginosa* ExoT inhibits in vitro lung epithelial wound repair. *Cell. Microbiol.* 3, 223–236.

Hauser, A. R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat. Rev. Microbiol.* 7, 654–665.

Heiniger, R. W., Winther-Larsen, H. C., Pickles, R. J., Koomey, M., and Wolfgang, M. C. (2010). Infection of human mucosal tissue by *Pseudomonas aeruginosa* requires sequential and mutually dependent virulence factors and a novel pilus-associated adhesin. *Cell. Microbiol.* 12, 1158–1173.

Iden, S., and Collard, J. G. (2008). Crosstalk between small GTPases and polarity proteins in cell polarization. *Nat. Rev. Mol. Cell Biol.* 9, 846–859.

Kazmierczak, B., Mostov, K., and Engel, J. (2001a). Interaction of bacterial pathogens with polarized epithelium. *Annu. Rev. Microbiol.* 55, 407–435.

Kazmierczak, B. I., Jou, T.-S., Mostov, K., and Engel, J. (2001b). Rho-GTPase activity modulates *Pseudomonas aeruginosa* internalization by epithelial cells. *Cell. Microbiol.* 3, 85–98.

Kazmierczak, B. I., Mostov, K., and Engel, J. N. (2004). Epithelial cell polarity alters Rho-GTPase responses to *Pseudomonas aeruginosa*. *Mol. Biol. Cell* 15, 411–419.

Kierbel, A., Gassam, A., Mostov, K., and Engel, J. (2005a). The phosphoinositol-3-kinase-protein kinase B/Akt pathway is critical for *Pseudomonas aeruginosa* strain PAK internalization. *Mol. Biol. Cell* 16, 2577.

Kierbel, A., Gassama-Diagne, A., Mostov, K., and Engel, J. N. (2005b). The phosphoinositol-3-kinase-protein kinase B/Akt pathway is critical for *Pseudomonas aeruginosa* strain PAK internalization. *Mol. Biol. Cell* 16, 2577–2585.

Kierbel, A., Gassama-Diagne, A., Rocha, C., Radoshevich, L., Olson, J., Mostov, K., and Engel, J. (2007). *Pseudomonas aeruginosa* exploits a PIP3-dependent pathway to transform apical into basolateral membrane. *J. Cell Biol.* 177, 21–27.

Mandell, G. L., Bennett, J. E., and Dolin, R. (2010). *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, 7th Edn. Philadelphia, PA: Churchill Livingstone/Elsevier.

Martin-Belmonte, F., and Mostov, K. (2008). Regulation of cell polarity during epithelial morphogenesis. *Curr. Opin. Cell Biol.* 20, 227–234.

Meng, W., and Takeichi, M. (2009). Adherens junction: molecular architecture and regulation. *Cold Spring Harb. Perspect. Biol.* 1, a002899.

Moreau-Marquis, S., Stanton, B. A., and O'Toole, G. A. (2008). *Pseudomonas aeruginosa* biofilm formation in

the cystic fibrosis airway. *Pulm. Pharmacol. Ther.* 21, 595–599.

Mostov, K., Brakeman, P., Datta, A., Gassama, A., Katz, L., Kim, M., Leroy, P., Levin, M., Liu, K., Martin, F., O'Brien, L. E., Verges, M., Su, T., Tang, K., Tanimizu, N., Yamaji, T., and Yu, W. (2005). Formation of multicellular epithelial structures. *Novartis Found. Symp.* 269, 193–200; discussion 200–205, 223–230.

Saadat, I., Higashi, H., Obuse, C., Umeda, M., Murata-Kamiya, N., Saito, Y., Lu, H., Ohnishi, N., Azuma, T., Suzuki, A., Ohno, S., and Hatakeyama, M. (2007). *Helicobacter pylori* CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. *Nature* 447, 330–333.

Sason, H., Milgrom, M., Weiss, A. M., Melamed-Book, N., Balla, T., Grinstein, S., Backert, S., Rosenshine, I., and Aroeti, B. (2009). Enteropathogenic *Escherichia coli* subverts phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate upon epithelial cell infection. *Mol. Biol. Cell* 20, 544–555.

Shafikhani, S. H., Morales, C., and Engel, J. (2008). The *Pseudomonas aeruginosa* type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. *Cell. Microbiol.* 10, 994–1007.

Smith, K., Humphreys, D., Hume, P. J., and Koronakis, V. (2010). Enteropathogenic *Escherichia coli* recruits the cellular inositol phosphatase SHIP2 to regulate actin-pedestal formation. *Cell Host Microbe* 7, 13–24.

Soong, G., Parker, D., Magargee, M., and Prince, A. S. (2008). The type III toxins of *Pseudomonas aeruginosa* disrupt epithelial barrier function. *J. Bacteriol.* 190, 2814–2821.

Sun, J., Maresso, A. W., Kim, J. J., and Barbieri, J. T. (2004). How bacterial ADP-ribosylating toxins recognize substrates. *Nat. Struct. Mol. Biol.* 11, 868–876.

Tan, S., Tompkins, L. S., and Amieva, M. R. (2009). *Helicobacter pylori* usurps cell polarity to turn the cell surface into a replicative niche. *PLoS Pathog.* 5, e1000407. doi: 10.1371/journal.ppat.1000407

Tsang, K. W. T., Rutman, A., Tanaka, E., Lundt, V., Dewar, A., Cole, P. J., and Wilson, R. (1994). Interaction of *Pseudomonas aeruginosa* with human respiratory mucosa in vitro. *Eur. Respir. J.* 7, 1746–1753.

Wang, Q., and Margolis, B. (2007). Apical junctional complexes and cell polarity. *Kidney Int.* 72, 1448–1458.

Yamaguchi, T., and Yamada, H. (1991). Role of mechanical injury on airway surface in the pathogenesis of *Pseudomonas aeruginosa*. *Am. Rev. Respir. Dis.* 144, 1147–1152.

Yeaman, C., Grindstaff, K. K., and Nelson, W. J. (2004). Mechanism of recruiting Sec6/8 (exocyst) complex to the apical junctional complex during polarization of epithelial cells. *J. Cell. Sci.* 117, 559–570.

Zahm, J. M., Chevillard, M., and Puchelle, E. (1991). Wound repair of human surface respiratory epithelium. *Am. J. Respir. Cell Mol. Biol.* 5, 242–248.

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