MECHANISMS OF ANTIBIOTIC RESISTANCE

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MECHANISMS OF ANTIBIOTIC RESISTANCE

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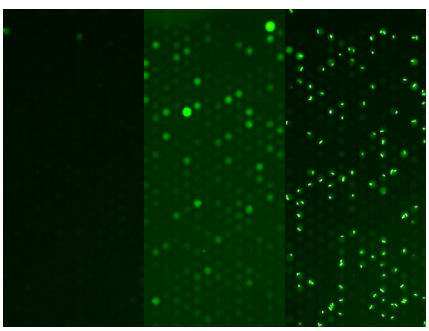

Single-cell drug efflux assay using fluorogenic substrate fluorescein-di-β-D-galactopyranoside (FDG) and femtoliter droplet array. In the wild-type resistant *E. coli* (left), FDG is pumped out of cells before hydrolysis so no fluorescence is seen. In contrast, in ΔacrB (center) and ΔtolC (right) mutants FDG is hydrolyzed to fluorescein. In ΔacrB cells, not only the cells but also the droplets emit fluorescence because the remaining minor RND efflux pumps slowly pump out the fluorescein. Although only a small amount of the dye is pumped out, it can be easily detected because it is confined to the femtoliter droplet. In ΔtolC cells, fluorescein is accumulated in the cell because TolC is a channel protein common to both the major and minor RND efflux pumps in *E. coli*.

Antibiotics represent one of the most successful forms of therapy in medicine. But the efficiency of antibiotics is compromised by the growing number of antibiotic-resistant pathogens. Antibiotic resistance, which is implicated in elevated morbidity and mortality rates as well as in the increased treatment costs, is considered to be one of the major global public health threats (www.who.int/drugresistance/en/) and the magnitude of the problem recently prompted a number of international and national bodies to take actions to protect the public (http://ec.europa.eu/dgs/health_consumer/docs/road-map-amr_en.pdf: http://www.who.int/drugresistance/amr_global_action_plan/en/; http://www.whitehouse.gov/sites/default/files/docs/carb_national_strategy.pdf). Understanding the mechanisms by which bacteria successfully defend themselves against the antibiotic assault represent the main theme of this eBook published as a Research Topic in Frontiers in Microbiology, section of Antimicrobials, Resistance, and Chemotherapy. The articles in the eBook update the reader on various aspects and mechanisms of antibiotic resistance. A better understanding of these mechanisms should facilitate the development of means to potentiate the efficacy and increase the lifespan of antibiotics while minimizing the emergence of antibiotic resistance among pathogens.

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Mechanisms of antibiotic resistance

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Antibiotics represent one of the most successful forms of therapy in medicine. But the efficiency of antibiotics is compromised by a growing number of antibiotic-resistant pathogens. Antibiotic resistance, which is implicated in elevated morbidity and mortality rates as well as in the increased treatment costs, is considered to be one of the major global public health threats (www.who.int/drugresistance/en/) and the magnitude of the problem recently prompted a number of international and national bodies to take actions to protect the public (http://ec.europa.eu/dgs/health_consumer/docs/road-map-amr_en.pdf: http://www.who.int/drugresistance/amr_global_action_plan/en/; http://www.whitehouse.gov/sites/default/files/docs/carb_national_strategy.pdf). Understanding the mechanisms by which bacteria successfully defend themselves against the antibiotic assault represent the main theme of this eBook published as a Research Topic in *Frontiers in Microbiology: Antimicrobials, Resistance, and Chemotherapy*. The articles in the eBook update the reader on various aspects and mechanisms of antibiotic resistance. A better understanding of these mechanisms should facilitate the development of means to potentiate the efficacy and increase the lifespan of antibiotics while minimizing the emergence of antibiotic resistance.

The multidrug efflux systems contribute significantly to the increased resistance to multiple antibiotics in bacteria. A major challenge in developing efficacious antibiotics against drug-resistant pathogens is to identify compounds that can counteract the efflux functions. The wealth of bacterial genomics information available suggests the presence of a variety of efflux systems in bacteria. Even a single bacterium may possess multiple efflux transporters of different families, with the overlapping substrate spectra. Accumulating evidence has indicated that the MexXY multidrug efflux system is a primary determinant of aminoglycoside resistance in *Pseudomonas aeruginosa*. Morita et al. (2012) provided a timely review on the *P. aeruginosa* MexXY pump and other aminoglycoside efflux pumps in a range of different bacteria. The expression of bacterial multidrug efflux system is usually controlled by transcriptional regulators that either repress or activate the transcription of the multidrug efflux genes. The articles by Usui et al. (2013) and Deng et al. (2013) further illustrated the complexity of regulation of multidrug efflux systems. However, the importance of multidrug efflux system may not be overstated for a specific antibiotic or organism, which is supported by the findings of Baucheron et al. (2014).

β-lactam antibiotics, which inhibit the biosynthesis of bacterial cell wall, are the most widely available antibiotics used to treat a number of bacterial infections. Resistance to β-lactam antibiotics, however, has become a worldwide health care problem. Production of β-lactamases is a major and threatening resistance mechanism toward β-lactam antibiotics. Epidemiological work by Chuma et al. (2013) demonstrated a recent emergence of β-lactamase-mediated cefotaxime resistance in *Salmonella enetrica* Serovar Infantis. To counteract β-lactam resistance in pathogenic bacteria, extensive research in the past three decades has focused on the discovery of novel compounds inhibiting the β-lactamase function. Watkins et al. (2013) reviewed the novel β-lactamase inhibitors that are close to being introduced in the clinical practice. Despite the successful development of β-lactamase inhibitors for the combination therapy, the use of β-lactamase inhibitors is still challenged by the variable affinity of inhibitors to different β-lactamases and by the vast quantity of β-lactamases produced by the resistant strains. To address this issue and optimize the existing β-lactam-based therapy, Zeng and Lin (2013) proposed to inhibit the induction of β-lactamases by targeting the key players required for β-lactamase induction, such as lytic transglycosylase.

Aminoglycosides are another class of clinically important antibiotics for treating various bacterial pathogens. The increasing resistance of clinical isolates against aminoglycosides, however, has compromised the effectiveness of this class of antibiotics. A major mechanism of aminoglycoside resistance is the production of aminoglycoside-modifying enzymes. Two enzymes with aminoglycoside-modifying activities are discussed in this research topic. Shi et al. (2013) provided a comprehensive overview of the structure of aminoglycoside kinase and reported on the recent progress in the discovery of aminoglycoside phosphotransferase inhibitors using structure-guided strategies. Aminoglycoside 6′-*N*-acetyltransferase type Ib is another clinically important

enzyme prevalent in a wide variety of Gram-negative pathogens. Ramirez et al. (2013) reviewed the unique sequence, genomics and functional features of this type of aminoglycoside-modifying enzymes. They also provided an insightful discussion on the development of innovative antisense technologies to combat this type of aminoglycoside resistance.

Several articles are focused on the discovery of innovative antimicrobials by harnessing our knowledge in bacterial physiology and pathogenesis. Quorum sensing is a unique cell-to-cell communication that modulates the expression of antibiotic resistance as well as virulence genes. Thus, the key compounds mediating quorum sensing such as acylated homoserine lactone have been attractive targets for antimicrobial chemotherapy. Hirakawa and Tomita (2013) reviewed recent progress in the discovery of acylated homoserine lactone inhibitors/modulators and discussed the feasibility of targeting other molecular components involved in signal transduction (e.g., regulatory elements) to modulate quorum sensing. Olivares et al. (2013) provided a timely review to analyze recent works on the intrinsic resistome, that is the concerted activity of elements required for the intrinsic resistance in *E. coli* and *P. aeruginosa*. The feasibility of using intrinsic resistome inhibitors for potentiating the effects of clinical drugs is also discussed in this review. Iino et al. (2013) used a large-scale femtoliter droplet array for single-cell analysis to assess the heterogeneity among the individual cells, enabling the identification of the novel or unconventional mechanisms of antibiotic resistance or resistance against novel antibiotics.

This research topic also includes a panel of articles focused on specific resistance mechanisms in different pathogens. Tuberculosis (TB) is notoriously known for its resistance to multiple drugs. Green and Garneau-Tsodikova (2013) focused on various mechanisms of resistance to the currently available anti-TB drugs and provided perspectives for novel strategies and lead scaffolds/compounds that are aimed at deterring these resistance mechanisms. In an effort to reduce emerging resistance, combinations of small molecules and a high-throughput synergy screening have been also explored (Zhang et al., 2007). Ilina et al. (2013) observed that a mutation in the ribosomal protein S5 is responsible for the resistance of *Neisseria gonorrhoeae* strains against multiple drugs including the decreased susceptibility to spectinomycin, cefixime and ceftriaxone. Zaheer et al. (2013) found that both therapeutic and subtherapeutic macrolide administration significantly increased the proportion of erythromycin resistant enterococci but had no effect on the development of macrolide resistance in *Mannheimia haemolytica,* both isolated from the nasopharynx. Sun et al. (2013) reported that sterol C-22 desaturase ERG5, which is highly conserved among various fungal species, is involved in azole resistance and may serve as a novel target for antifungal drugs, in particular against *Neurospora crassa* and *Fusarium verticillioides*. Using the population-based multivariate analysis, Abbes et al. (2013) observed that fluconazole resistance in *Candida glabrata* involves complex interactions between drug resistance gene expression and/or copy number.

Recent metagenomics and functional genomics studies have provided a compelling evidence that antibiotic resistance genes are widespread and the natural reservoirs of potential antibiotic resistance include many ecosystems such as in agriculture (e.g., animal manure, soil, water, wastewater lagoons), the gut of humans and food animals, and even ancient soils. The diverse range of novel antibiotic resistance genes could be accessible to clinically relevant bacteria and play a critical role in the emergence of antibiotic resistance among pathogens. Pehrsson et al. (2013) provided an insightful review for the novel resistance functions uncovered using the functional metagenomic examination of various resistance reservoirs. Municipal biosolids that are produced during the activated sludge treatment are also a significant reservoir of antibiotic resistance as assessed by Kaplan et al. (2013). Burch et al. (2013) explored an alternative approach, aerobic digestion, to reduce the quantity of antibiotic resistance genes in wastewater solids. The occurrence of antibiotics resistance genes in finfish aquaculture environments is further discussed by Miranda et al. (2013). The presence of antibiotic resistance genes in the aquatic environment is also demonstrated by Fahrenfeld et al. (2013) and Suzuki et al. (2013). To address the key issue concerning the role of environmental resistance gene reservoir in the emergence of clinically important resistant pathogens, Perry and Wright (2013) reviewed recent works suggesting genetic exchange between the environmental and clinical resistomes. Community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a major cause of disease in the general population. Roberts et al. (2013) examined 55 environmental MRSA isolates from 805 samples and found that 98% of them are also resistant to other classes of antibiotics in addition to methicillin, thus most likely representing the antibiotic resistance gene pool in the environment. Clearly, with the aid of high-throughput sequencing and metagenomics approaches, recent studies of natural antibiotic resistance gene reservoirs have revealed a much higher level of diversity and novelty than anticipated. However, there is still a significant knowledge gap regarding the mechanisms of horizontal gene transfer that are involved in the exchange of genes among different ecological compartments. A better understanding of these *in situ* processes is required in order to control the development, transmission, and evolution of antibiotic resistant genes.

In summary, the articles within this eBook address various timely issues related to antibiotic resistance mechanisms. Clearly, discovery of new antimicrobials as well as finding strategies to expand the useful life of existing antibiotics is important to combat the ever-increasing antimicrobial resistance. Bacteria, however, possess an enormous diversity of genes that allow them, sooner or later, to counteract the action of newly invented antibiotics. As reflected in many articles in this eBook, the natural resistomes are common and exist in diverse environmental niches. Misuse of antibiotics, in terms of application and dosage, is an additional contributing factor for the development of antibiotic resistance (Nosanchuk et al., 2014). Consequently, to mitigate antibiotic resistance, we should cautiously use antibiotics from a One Health perspective (http://www.onehealthinitiative.com/). On the other hand, as it is also reflected in this Research Topic, in parallel to the development of new of antibiotics, it is imperative to study the molecular basis of resistance development so that we can prevent and overcome antibiotic resistance by targeting resistance mechanisms, which will make the existing and novel antibiotics more effective and sustainable.

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ORIGINAL RESEARCH ARTICLE
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Lack of efflux mediated quinolone resistance in *Salmonella enterica* serovars Typhi and Paratyphi A

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Salmonella enterica serovars Typhi and Paratyphi A isolates from human patients in France displaying different levels of resistance to quinolones or fluoroquinolones were studied for resistance mechanisms to these antimicrobial agents. All resistant isolates carried either single or multiple target gene mutations (i.e., in *gyrA*, *gyrB*, or *parC*) correlating with the resistance levels observed. Active efflux, through upregulation of multipartite efflux systems, has also been previously reported as contributing mechanism for other serovars. Therefore, we investigated also the occurrence of non-target gene mutations in regulatory regions affecting efflux pump expression. However, no mutation was detected in these regions in both Typhi and Paratyphi isolates of this study. Besides, no overexpression of the major efflux systems was observed for these isolates. Nevertheless, a large deletion of 2334 bp was identified in the *acrS-acrE* region of all *S.* Typhi strains but which did not affect the resistance phenotype. As being specific to *S.* Typhi, this deletion could be used for specific molecular detection purposes. In conclusion, the different levels of quinolone or FQ resistance in both *S.* Typhi and *S.* Paratyphi A seem to rely only on target modifications.

Keywords: *Salmonella*, ciprofloxacin, transcriptional regulatory genes, *acrS*, efflux pumps

INTRODUCTION

Enteric fever caused by the human-adapted pathogens *Salmonella enterica* serovars Typhi (*S.* Typhi) and Paratyphi A (*S.* Paratyphi A), B, and C, remains a major health problem (Crump and Mintz, 2010). A global epidemiologic study estimated that during the year 2000 typhoid fever caused 21.7 million illnesses and 21,7000 deaths and paratyphoid fever caused 5.4 million illnesses (Crump et al., 2004). During the past decade *S.* Paratyphi A was responsible for a growing proportion of enteric fever in Asia (Ochiai et al., 2005; Crump and Mintz, 2010). Enteric fever being associated with poor sanitation and unsafe food and water, it particularly affects children and adolescents in developing countries of Asia, Africa and Latin America (Crump et al., 2004; Bhan et al., 2005; Crump and Mintz, 2010). In developed countries, patients are most often ill-returned travellers or migrant workers (Bhan et al., 2005; Connor and Schwartz, 2005; Hassing et al., 2013).

To treat these infections, fluoroquinolones (FQ) and third-generation cephalosporins have been considered as first-line drugs, owing to the resistance to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole that appeared during the 1980s (Hassing et al., 2011, 2013). Multidrug resistance (MDR) in *S.* Typhi is encoded mainly by resistance genes carried by large conjugative plasmids and has been reported worldwide (Le et al., 2007). As a consequence of a widespread FQ usage, *S.* Typhi and *S.* Paratyphi A isolates resistant to nalidixic acid (NAL^R, minimum inhibitory concentration [MIC] > 16 mg/L) and with decreased susceptibility to ciprofloxacin (CIP^{DS}, MIC 0.125–1.0 mg/L) have also emerged. Such NAL^R-CIP^{DS} *S.* Typhi and *S.* Paratyphi A have been isolated in endemic areas and also in developed countries (Roumagnac et al., 2006; Le et al., 2007; Gaborieau et al., 2010; Accou-Demartin et al., 2011; Hassing et al., 2011, 2013).

Resistance to quinolones in *Salmonella* spp. is mostly attributed to point mutations in the quinolone resistance-determining regions (QRDRs) of the target genes *gyrA*, *gyrB*, *parC*, and *parE* (Cloeckaert and Chaslus-Dancla, 2001; Piddock, 2002; Velge et al., 2005; Giraud et al., 2006). For the *gyrA* gene, coding the A subunit of DNA gyrase, a single mutation resulting in an amino acid substitution at the position 83 (Serine to Phenylalanine or to Tyrosine) or at the position 87 (Aspartic acid to Asparagine or Glycine) has been the most frequently described in NAL^R-CIP^{DS} *S.* Typhi and *S.* Paratyphi A isolates (Bhan et al., 2005; Renuka et al., 2005; Le et al., 2007; Gaborieau et al., 2010; Hassing et al., 2011). A second mutation leading to the amino acid change at the position 80 (Serine to Isoleucine or to Arginine) of the ParC subunit of topoisomerase IV was described to increase the CIP MIC ($\geq$ 0.5 mg/L) in *S.* Typhi and *S.* Paratyphi A human isolates from India (Gaind et al., 2006). Whereas three mutations, i.e., a double mutation in *gyrA* at both codons 83 and 87 and one mutation in *parC*, were shown to confer CIP resistance (MIC >1 mg/L) in *S.* Typhi and *S.* Paratyphi A human isolates from India or from Taiwan (Gaind et al., 2006; Lee et al., 2013).

Moreover, the varying levels of CIP resistance observed in *S.* Typhi and *S.* Paratyphi A isolates with only a single *gyrA* mutation suggest that other mechanisms could be involved in quinolone resistance in this serovar (Renuka et al., 2005).

Resistance to FQ in *S.* Typhimurium has also been attributed to active efflux mechanism, due to overproduction of the AcrAB-TolC efflux system (Giraud et al., 2000, 2006; Cloeckaert and Chaslus-Dancla, 2001; Piddock, 2006). We have previously reported the contribution of the AcrAB-TolC efflux system in resistance to FQ in several MDR epidemic clones of *S.* Typhimurium, such as *S.* Typhimurium of phage types DT204 or DT104 (Baucheron et al., 2002, 2004a,b). Among the chromosomal loci affecting AcrAB-TolC expression, the *ramRA* locus appears to be the most important in *Salmonella* spp. (Abouzeed et al., 2008; Kehrenberg et al., 2009). *ramR* encodes a repressor protein (RamR) belonging to the TetR family of repressor proteins, and has been shown to be the local repressor protein of *ramA* transcription (Abouzeed et al., 2008; Baucheron et al., 2012); while *ramA* encodes a transcriptional activator protein (RamA) belonging to the AraC/XylS family of regulatory proteins (Nikaido et al., 2008; Bailey et al., 2010). The latter is involved in upregulating expression of the AcrAB-TolC system (Nikaido et al., 2008; Bailey et al., 2010). Several mutations in *ramR* or its binding site upstream of *ramA*, affecting expression of this efflux system, have been detected in clinical isolates of serovar Typhimurium or Kentucky and of minor serovars Hadar, Infantis, Livingstone, or Schwarzengrund (Abouzeed et al., 2008; Kehrenberg et al., 2009; Hentschke et al., 2010; Akiyama and Khan, 2012; Baucheron et al., 2013).

In the present study, we have characterized mechanisms involved in resistance to quinolones or fluroquinolones in 21 *S.* Typhi and *S.* Paratyphi A strains displaying different levels of resistance to these drugs and isolated from patients in France during the period 1997–2008. For a subset of strains, with suspected increased efflux activity, we investigated the occurrence of mutations in the global *ram*, *sox* and *mar* regulatory loci of AcrAB-TolC, and in the local *acrR* and *acrS* repressor genes of the AcrAB and AcrEF pumps, respectively (Abouzeed et al., 2008; Kehrenberg et al., 2009).

MATERIALS AND METHODS

BACTERIAL STRAINS

The twenty one strains including 16 *S.* Typhi and 5 *S.* Paratyphi A selected for this study were collected by the French National Reference Center for *Salmonella*, Institut Pasteur, Paris, France. They were isolated in France from travellers or migrants between 1997 and 2008 (**Table 1**). The selection was made to obtain diversity in terms of geographic origin, year of isolation, genetic lineages (haplotype for *S.* Typhi; Roumagnac et al., 2006), and phenotype of resistance to quinolones (**Table 1**).

Table 1 | *Salmonella enterica* serovars Typhi and Paratyphi A strains analyzed in this study.

Strain	Country	Year of isolation	Haplo type	Antimicrobial resistance pattern	MIC (mg/L)		Substitution(s) in the QRDR of:				AcrA production ratio*
					NAL	CIP	GyrA	GyrB	ParC	ParE	
***SALMONELLA* TYPHI**											
06–423	India	2006	ND	Pansusceptible	4	0.015	WT	WT	WT	WT	1
06–426	India	2006	ND	CIP^{DS}	16	0.125	WT	S464Y	WT	WT	1
02–1180	India	2002	H45	$NALCIP^{DS}$	64	0.125	D87G	WT	WT	WT	1
05–3275	Morocco	2005	H6	$NALCIP^{DS}$	64	0.125	D87N	WT	WT	WT	1
4(02)MB	Vietnam	1997	H58	ASCSulTmpTeNAL	128	0.03	S83Y	WT	WT	WT	0.5
222(97)MN	Vietnam	1996	ND	$ASCSulTmpTeNALCIP^{DS}$	128	0.125	S83F	WT	WT	WT	0.5
43(97)MN	Vietnam	1996	H63	$ASCSulTmpTeNALCIP^{DS}$	128	0.125	S83F	WT	WT	WT	0.5
98–3139	Mexico	1998	H50	$NALCIP^{DS}$	128	0.125	S83F	WT	WT	WT	0.5
02–7744	India	2002	H52	$NALCIP^{DS}$	128	0.125	S83F	WT	WT	WT	0.5
226(97)MN	Vietnam	1996	H61	$ASCSulTmpTeNALCIP^{DS}$	128	0.25	S83F	WT	WT	WT	0.5
97–2307	India	1997	H63	$NALCIP^{DS}$	256	0.125	S83F	WT	WT	WT	0.5
318(98)MB	Vietnam	1998	H58	$ASCSulTmpTeNALCIP^{DS}$	512	0.25	S83Y	WT	WT	WT	1
39(98)MN	Vietnam	1998	H58	$ASCSulTmpTeNALCIP^{DS}$	512	0.25	S83F	WT	WT	WT	1
4(02)MN	Vietnam	2000	H58	$ASCSulTmpTeNALCIP^{DS}$	1024	0.25	S83F	WT	WT	D420N	1
5(04)MN	Vietnam	2004	ND	$NALCIP^{DS}$	1024	0.25	S83F	WT	WT	D420N	1
04–2176	India	2004	H58	SSpSulTmpTeNALCIP	1024	8	S83F; D87N	WT	S80I	WT	1
***SALMONELLA* PARATYPHI A**											
08–8903	Senegal	2008		Pansusceptible	8	0.030	WT	WT	WT	WT	2
07–6329	Burkina Faso	2007		CIP^{DS}	16	0.25	WT	S464F	WT	WT	2
05–208	India	2005		$NALCIP^{DS}$	256	0.50	S83F	WT	WT	WT	2
08–4271	Guinea Bissau	2008		$NACIP^{DS}$	1024	1	S83F	WT	WT	WT	2
08–2580	India	2008		$NALCIP^{DS}$	1024	1	S83F	WT	WT	WT	3

**AcrA expression was measured by dot blotting with an anti-AcrA polyclonal antibody.*

ND, not determined.

WT, wild type.

A, amoxicillin; S, streptomycin; Sp, spectinomycin; C, chloramphenicol; Sul, sulfamethoxazole; Tmp, trimethoprim; Te, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CIP^{DS}, decreased susceptibility to ciprofloxacin.

D, aspartic acid; F, phenylalanine; G, glycine; I, isoleucine; N, asparagine; S, serine; Y, tyrosine.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility was investigated by the standard disk diffusion method according to the recommendations of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (www.sfm-microbiologie.org/). The MICs of NAL and CIP were determined by the standard agar doubling dilution method as described previously (Baucheron et al., 2002). The NAL^R isolates were defined as having a MIC $>$ 16 mg/L. The CIP^R isolates were defined as having a MIC $>$ 1 mg/L and CIP^{DS} isolates as having a MIC comprised between 0.125 and 1.0 mg/L (Accou-Demartin et al., 2011; Hassing et al., 2013). MICs of these antibiotics were also determined in the presence of the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN, Sigma) at the following concentrations: 10, 20, 30, 40, 50, and 60 mg/L.

ASSESSMENT OF TARGET-AFFECTING MECHANISMS

Mutations in the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes were detected as described previously (Le et al., 2007; Song et al., 2010; Accou-Demartin et al., 2011).

The search of plasmid-mediated quinolone resistances genes, *qnrA*, *qnrB*, *qnrS*, *qnrD*, *aac*(6')-Ib-cr, and *qepA* was performed as described previously (Accou-Demartin et al., 2011).

ASSESSMENT OF EFFLUX MECHANISMS

Efflux pump production was assessed by dot blot using an anti-AcrA polyclonal antibody as described previously (Abouzeed et al., 2008). Occurrence of mutations affecting *acrAB, acrEF*, and *tolC* expression was determined by PCR and sequencing the regulatory regions *ramR-ramA*, *acrR-acrA*, *marC*-marO-*marR-marA*, *soxS-soxR*, and *acrS-acrE* using primers listed in (**Table 2**).

Table 2 | Primers used for PCRs.

Primer used and target region	Primer	Nucleotide position relative to the LT2 strain genome sequence*	Oligonucleotide sequence(s) (5′–3′)	Size (bp)	Annealing temp (°C)	Reference
DETECTION OF MUTATIONS						
ramR-ramA	ram5	638085	TCGGTAAAAGGCAGTTCCAG	958	60	Baucheron et al., 2013
	ramA6	639042	GTCGATAACCTGAGCGGAAA			
acrR-acrA	acrR1	533463	CAGTGGTTCCGTTTTTAGTG	992	58	Olliver et al., 2005
	acrR2	534454	ACAGAATAGCGACACAGAAA			
marC-marO-*marR-marA*	marR1	1597459	CAGTGTTGCGTCTGGACATC	787	60	Baucheron et al., 2013
	marR2	1598245	GCTAACGGGAGCAGTACGAC			
soxS-soxR	sox1	4503970	CTACAGGCGGTGACGGTAAT	915	60	Baucheron et al., 2013
	sox2	4504884	CGGCGCTTTAGTTTTAGGTG			
acrS-acrE	acrS3	3559106	AAAACGAACGGGAACTGATG	2874 ***	58	This study
	acrS4	3561978	ACAAACATACCGGGAAGCAG			
qRT-PCR						
gmk	gmk-f	3933294	TTGGCAGGGAGGCGTTT	62	60	Baucheron et al., 2012
	gmk-r	3933355	GCGCGAAGTGCCGTAGTAAT			
gyrB	gyrB-f	4040275	TCTCCTCACAGACCAAAGATAAGCT	81	60	Baucheron et al., 2012
	gyrB-r	4040195	CGCTCAGCAGTTCGTTCATC			
rrs	rrs-f	NA**	CCAGCAGCCGCGGTAAT	57	60	Baucheron et al., 2012
	rrs-r	NA**	TTTACGCCCAGTAATTCCGATT			
ramA	ramA-f	639180	GCGTGAACGGAAGCTAAAAC	167	60	Baucheron et al., 2012
	ramA-r	639346	GGCCATGCTTTTCTTTACGA			
ramR	ramR-f	638623	TAACGCAGGTGTTGCAGAAG	192	64	Baucheron et al., 2012
	ramR-r	638432	TGGTTCAGACCCCAACTGAT			
acrA	acrA-f	533120	GAAACCGCACGTATCAACCT	220	60	Baucheron et al., 2012
	acrA-r	532901	CCTGTTTCAGCGAACCATTT			
acrB	acrB-f	531348	TCGTGTTCCTGGTGATGTACCT	68	66	Baucheron et al., 2012
	acrB-r	531281	AACCGCAATAGTCGGAATCAA			
acrF	acrF-f	3563042	GCTCTGTCGTCCATCTCAAAGA	70	66	This study
	acrF-r	3563111	CGCGCTACAACGTTATAGTTTTCA			
tolC	tolC-f	3349107	GCCCGTGCGCAATATGAT	67	60	Baucheron et al., 2012
	tolC-r	3349173	CCGCGTTATCCAGGTTGTTG			

*GenBank NC_003197.1.

**NA: Not Applicable due to the number of copies of this gene in Salmonella.

2874 bp for S. Typhimurium or S. Paratyphi A and 539 bp for S. Typhi (see ***Figure 1).

Transcription levels of efflux related genes *acrA*, *acrB*, *acrF*, *tolC*, *ramA*, and *ramR* were done by quantitative reverse transcription-PCR (qRT-PCR) as described previously (Baucheron et al., 2012; Giraud et al., 2013). Primers used for qRT-PCR are listed in (**Table 2**).

RESULTS AND DISCUSSION

RESISTANCE PHENOTYPES AND TARGET-AFFECTING MECHANISMS

The twenty one *S.* Typhi and *S.* Paratyphi A strains of this study were isolated in France but acquired abroad, mainly in Asia and Africa (**Table 1**). Among the *S.* Typhi strains, all but two were NAL^R (MIC > 16 mg/L). One of the two strains was pan-susceptible and the second was CIP^{DS} but only categorized as intermediate for NAL (NAL^I, MIC 16 mg/L). Of the 14 NAL^R strains, one was CIP^R, 12 were CIP^{DS} and one was CIP^S (MIC 0.03 mg/L). Eight NAL^R *S.* Typhi strains were also multidrug resistant. The majority of the NAL^R *S.* Typhi strains belonged to haplotype H58 which had emerged in Southern Asia during the mid 1990s (Roumagnac et al., 2006; Le et al., 2007). Among the *S.* Paratyphi A strains, all but two were NAL^R. One of the two strains was pansusceptible and the second was NAL^I-CIP^{DS}. The three others were NAL^R- CIP^{DS}.

As shown in **Table 1**, both NAL^I-CIP^{DS} had a mutation resulting in an amino acid substitution at position 464 of GyrB: serine to tyrosine for the *S.* Typhi isolate and serine to phenylalanine for the *S.* Paratyphi A isolate. The most frequent mechanism of resistance of NAL^R-CIP^{DS} ($n = 17$) and NAL^R-CIP^S ($n = 1$) strains, whatever the serovar, was a substitution at position 83 (serine to phenylalanine, $n = 12$, 66.6%) of GyrA. Other GyrA modifications were observed at position 83 (serine to tyrosine) in two isolates or at position 87 (aspartic acid to glycine, aspartic acid to asparagine) in one isolate for each. As described previously, a single substitution in GyrA was associated with resistance to nalidixic acid and decreased susceptibility to CIP (Bhan et al., 2005; Le et al., 2007; Gaborieau et al., 2010; Hassing et al., 2011). One exception was the *S.* Typhi strain 4 (02) MB, which was NAL^R-CIP^S (and not CIP^{DS}) despite a mutation in *gyrA* resulting in substitution serine to tyrosine at position 83.

Additional substitutions were found in ParE of 2 NAL^R-CIP^{DS} *S.* Typhi strains that led to amino acid substitution aspartic acid to asparagine at position 420. In both cases, a 2-fold increase of NAL MICs was observed.

In the CIP^R *S.* Typhi isolate, three mutations leading to a double substitution in GyrA at positions 83 (serine to phenylalanine) and 87 (aspartic acid to asparagine) and one substitution at the position 80 of ParC (serine to isoleucine), as observed in previous studies (Renuka et al., 2005; Gaind et al., 2006; Lee et al., 2013).

The NAL^R and CIP^{DS} *S.* Typhi and *S.* Paratyphi A strains harboring a single substitution in GyrA showed various values for NAL (64–1024 mg/L) and CIP (0.03–0.5 mg/L) MICs which suggested the presence of other mechanisms of resistance. Since the plasmid-mediated quinolone resistance-conferring genes *qnrA*, *qnrB*, *qnrD* or *qnrS*, *qepA*, *and aac(6′)-Ib-cr* were not detected, we investigated the role of the AcrAB-TolC efflux system.

INVOLVEMENT OF EFFLUX

None of the *S.* Typhi strains showed significant AcrA overproduction by dot blot, but nevertheless all *S.* Paratyphi A isolates showed a 2 or 3-fold increased AcrA production relative to the susceptible *S.* Typhi isolate (**Table 1**). Thus, overproduction of AcrA seems not to be involved in CIP^{DS} isolates compared with the susceptible isolates of *S.* Typhi or *S.* Paratyphi A. In presence of the efflux pump inhibitor PAβN (20 or 40 mg/L), the CIP MICs similarly decreased (4 or 8-fold) in CIP^{DS} and in susceptible strains (**Table 3** and data not shown), which is in accordance with previous studies on *S.* Typhimurium and corresponds to

Table 3 | Study of efflux in a subset of *Salmonella enterica* serovars Typhi and Paratyphi A strains.

Strain	Antimicrobial resistance pattern[a]	MIC (mg/L)[b]		Substitution(s) in the QRDR of [c]:				*acrSE* sequencing	Transcription level of:					
		NAL	CIP	GyrA	GyrB	ParC	ParE		*acrA*	*acrF*	*acrB*	*tolC*	*ramA*	*ramR*
***SALMONELLA* TYPHI**														
06–423	Pansusceptible	4	0.015 [0.004]	WT	WT	WT	WT	Deletion[d]	1.0	1.0	1.0	1.0	1.0	1.0
02–1180	NALCIP^{DS}	64	0.125 [0.015]	D87G	WT	WT	WT	Deletion[d]	1.5	0.7	0.5	0.8	0.9	1.4
05–3275	NALCIP^{DS}	64	0.125 [0.030]	D87N	WT	WT	WT	Deletion[d]	0.5	1.4	0.2	0.5	0.3	0.7
97–2307	NALCIP^{DS}	256	0.125 [0.030]	S83F	WT	WT	WT	Deletion[d]	1.7	0.8	0.8	0.7	1.9	2.2
04–2176	SSpSulTmpTeNALCIP	1024	8 [2]	S83F; A87N	WT	S80I	WT	Deletion[d]	1.5	0.8	1.3	0.9	1.2	2.2
***SALMONELLA* PARATYPHI A**														
08–8903	Pansusceptible	8	0.030 [0.008]	WT	WT	WT	WT	WT	1.0	1.0	1.0	1.0	1.0	1.0
07–6329	CIP^{DS}	16	0.25 [0.060]	WT	S464F	WT	WT	WT	1.6	1.4	1.0	1.0	1.0	1.2
05–208	NALCIP^{DS}	256	0.50 [0.030]	S83F	WT	WT	WT	WT	1.3	0.9	1.4	1.0	1.1	1.0
08–4271	NALCIP^{DS}	1024	1 [0.25]	S83F	WT	WT	WT	WT	1.2	1.1	0.7	1.4	1.3	1.3
08–2580	NALCIP^{DS}	1024	1 [0.25]	S83F	WT	WT	WT	WT	2.0	1.6	1.4	1.4	2.0	1.3

[a] *S, streptomycin; Sp, spectinomycin; Sul, sulfamethoxazole; Tmp, trimethoprim; Te, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CIP^{DS}, decreased susceptibility to ciprofloxacin.*

[b] *Values in brackets are MICs in the presence of the efflux pump inhibitor PAβN at 40 mg/L.*

[c] *WT, wild type; D, aspartic acid; F, phenylalanine; G, glycine; I, isoleucine; N, asparagine; S, serine.*

[d] *2334 bp deleted.*

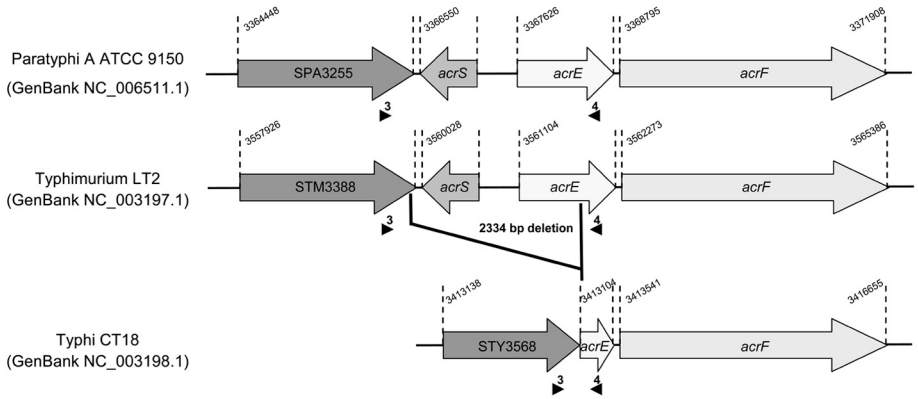

FIGURE 1 | Deletion identified in the *acrSE* region relative to the genome sequence of *S.* Typhi strain CT18 (GenBank NC_003198.1). The sequence of the *acrSE* region of *S.* Typhi strain CT18 is compared to those of *S.* Paratyphi A strain ATCC9150 (GenBank NC_006511.1) and *S.* Typhi strain LT2 (GenBank NC_003197.1). Small black arrows indicate primers positions used for PCR to amplify and sequence the *acrSE* region. The 2334 bp chromosomal deletion was found in all *S.* Typhi strains studied.

a decrease of resistance level observed for *acrB* or *tolC* deletion mutants (Baucheron et al., 2002, 2004b).

Despite a lack of evidence of increased efflux in the resistance phenotype, we measured by qRT-PCR the transcription levels of efflux related genes *acrA*, *acrF*, *acrB*, *tolC*, *ramA*, and *ramR* in CIP^{DS} non-MDR strains and in the CIP^{R} strain. No differences were detected in the transcription levels of these genes, between susceptible, CIP^{DS} and CIP^{R} strains, whatever the serovar (**Table 3**). In addition, no mutations were detected in the regulatory regions of the AcrAB-TolC efflux system. However, during the screening of the regulatory regions, we identified a single large deletion of 2334 bp in the *acrS-acrE* region of all *S.* Typhi strains, including the susceptible one (**Table 3**). This deletion encompassed the *acrS* gene, that encodes a transcriptional repressor, and a large part of the *acrE* gene that encodes the AcrE periplasmic lipoprotein, which is homologous to AcrA (Olliver et al., 2005). This 2334 bp deletion was also observed in the *acrS-acrE* region of sequenced genomes of MDR *S.* Typhi CT18 strain (Parkhill et al., 2001) and pansusceptible TY2 strain (Deng et al., 2003) (**Figure 1**). Previously, it has been shown that *acrS* deletion in *S.* Typhimurium does not affect *acrEF* expression (Olliver et al., 2005). Similarly the natural *acrSE* deletion detected in *S.* Typhi had no impact on the *acrF* transcription level as observed in this study. To our knowledge, this is the first description of such a natural *acrS-acrE* chromosomal deletion and seems specific to *S.* Typhi since it was not detected in all currently sequenced genomes of the other serovars (not shown).

CONCLUSIONS

The main mechanisms involved in quinolone or FQ resistance in both *S.* Typhi and *S.* Paratyphi A are target modifications. In contrast to what is seen in enteric pathogenic serovars, such as Typhimurium or the emerging CIP^{R} Kentucky ST198 clone (Baucheron et al., 2013), increased efflux pump production-mediated mechanisms seem to be totally absent in both *S.* Typhi and *S.* Paratyphi A. The deletion identified in the *acrSEF* region, although not involved in the resistance phenotype, may be helpful for the specific detection of *S.* Typhi.

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Aerobic digestion reduces the quantity of antibiotic resistance genes in residual municipal wastewater solids

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Numerous initiatives have been undertaken to circumvent the problem of antibiotic resistance, including the development of new antibiotics, the use of narrow spectrum antibiotics, and the reduction of inappropriate antibiotic use. We propose an alternative but complimentary approach to reduce antibiotic resistant bacteria (ARB) by implementing more stringent technologies for treating municipal wastewater, which is known to contain large quantities of ARB and antibiotic resistance genes (ARGs). In this study, we investigated the ability of conventional aerobic digestion to reduce the quantity of ARGs in untreated wastewater solids. A bench-scale aerobic digester was fed untreated wastewater solids collected from a full-scale municipal wastewater treatment facility. The reactor was operated under semi-continuous flow conditions for more than 200 days at a residence time of approximately 40 days. During this time, the quantities of *tet*(A), *tet*(W), and *erm*(B) decreased by more than 90%. In contrast, *intI1* did not decrease, and *tet*(X) increased in quantity by 5-fold. Following operation in semi-continuous flow mode, the aerobic digester was converted to batch mode to determine the first-order decay coefficients, with half-lives ranging from as short as 2.8 days for *tet*(W) to as long as 6.3 days for *intI1*. These results demonstrated that aerobic digestion can be used to reduce the quantity of ARGs in untreated wastewater solids, but that rates can vary substantially depending on the reactor design (i.e., batch vs. continuous-flow) and the specific ARG.

Keywords: antibiotic resistance genes, municipal wastewater treatment, aerobic digestion, class 1 integrons, qPCR

INTRODUCTION

The resistance of pathogenic bacteria to antibiotic chemotherapy is a growing problem with significant consequences for public health. In the United States, methicillin-resistant *Staphylococcus aureus* (MRSA) infections lead to more fatalities than the combination of HIV/AIDs, Parkinson's disease, and homicides (Spellberg et al., 2011). The estimated economic cost of antibiotic resistance ranges from $21 to 34 billion dollars per year (Spellberg et al., 2011). In response, medical practitioners have attempted to reduce the number of inappropriate and unnecessary antibiotic prescriptions. The biomedical research community is also focusing its research efforts to develop new antibiotics as well as alternatives to antibiotic chemotherapy (Kohanski et al., 2010; Jabes, 2011; Edgar et al., 2012). Finally, in Sweden and Switzerland, the use of antibiotics in agriculture for growth promotion and prophylaxis has been banned (Wierup, 2001; Arnold et al., 2004).

Despite these initiatives, a significant body of research suggests that antibiotic resistant bacteria (ARB) are becoming increasingly more prevalent (Palumbi, 2001; Levy and Marshall, 2004; Levy, 2005). An alternative, but complementary, approach to reducing the prevalence of ARB would be to identify pertinent reservoirs of resistance and then to implement appropriate technologies to ameliorate these reservoirs. Consistent with this approach, numerous studies have identified untreated municipal wastewater (raw sewage) as a significant reservoir of ARB and antibiotic resistance genes (ARGs) (Bönemann et al., 2006; da Silva et al., 2006; Auerbach et al., 2007; Schlüter et al., 2007; Szczepanowski et al., 2009; Zhang et al., 2009a; Galvin et al., 2010; Uyaguari et al., 2011; Zhang and Zhang, 2011). Municipal wastewater treatment processes, therefore, should represent an important opportunity to mitigate the quantity of this reservoir of antibiotic resistance.

Although prior research has demonstrated that the treated municipal wastewater also contains substantial concentrations of ARB and ARGs (da Silva et al., 2006; Pruden et al., 2006; Graham et al., 2011; LaPara et al., 2011), a mass balance on wastewater treatment operations suggests that >99% of the ARB and ARGs in untreated municipal wastewater accumulate in the residual wastewater solids. These are subsequently treated by numerous technologies to reduce their nutrient and pathogen content (to varying degrees) prior to their disposal on agricultural land (Tchobanoglous et al., 2003). There have been relatively few investigations on the different technologies used for treating residual wastewater solids and their associated effectiveness at mitigating ARB and ARGs. Diehl and LaPara (2010) observed relatively little removal of ARGs in aerobic digestion processes operated at 22–55°C, but observed increasingly effective removal of ARGs in anaerobic digestion processes at temperature >37°C. In contrast, Ma et al. (2011) observed little benefit of increasing the temperature of anaerobic digestion beyond 37°C.

In the present research, we undertook a detailed investigation of the effectiveness of a bench-scale conventional aerobic digestion process at mitigating the quantity of ARGs in untreated residual wastewater solids. Although our prior research had observed no effect of aerobic digestion on the quantity of ARGs in wastewater solids (Diehl and LaPara, 2010), these previous experiments were performed in relatively small bioreactors with a mean hydraulic residence time of 4 days. Assuming that ARGs decay at a relatively slow rate (i.e., half-lives > 4 days), this experimental design would have been insufficient to observe significant reductions in ARGs. This short time period used in our prior experimental design is also pertinent because the United States Environmental Protection Agency requires that aerobic digestion processes have a mean hydraulic residence time of 40 days (when operated at 20°C) to qualify as a "process to significantly reduce pathogens" (PSRP), which must be achieved before these treated wastewater solids can be applied to agricultural land for their disposal (albeit with some restrictions) (Tchobanoglous et al., 2003). This research is of considerable practical importance because numerous full-scale municipal wastewater treatment facilities currently utilize aerobic digestion processes to treat their wastewater solids, particularly those that treat less than 10 million gallons of wastewater each day (at higher flow rates, other technologies are considered more practical and economical).

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

A 10-L aerobic digester was operated at room temperature with a mean residence time of 40 days and a minimum dissolved oxygen (DO) concentration of 2 mg/L. The digester was inoculated with 10 L of untreated residual municipal wastewater solids from a full-scale municipal wastewater treatment plant. Mixing and aeration were provided by pumping atmospheric air through a stone diffuser located at the bottom of the reactor vessel at a rate sufficient to prevent settling of solids and to maintain the minimum DO concentration. Typical operating variables, including temperature, DO, pH, total solids, volatile solids, and inert solids, were monitored throughout the entire time period the digester was in operation (Clesceri et al., 1999). The total solids concentration represents the quantity of material remaining after drying at 103°C (i.e., the sum of inorganic and organic material dissolved and suspended in the sample). In contrast, the volatile solids concentration represents the fraction of the total solids concentration that is lost upon ignition at 550°C, whereas the IS concentration represents the fraction of the total solids that is not lost upon ignition at 550°C (i.e., the fraction of the total solids that represents that ash-material). Water loss from the digestor due to evaporation was monitored and replaced by adding appropriate volumes of deionized water to the digester.

The digester was operated for more than 175 days while being fed on a weekly basis untreated residual municipal wastewater solids. The digester was considered to have reached steady-state conditions once the residence time for inert solids (i.e., the average amount of time that an "inert solid" would reside in the aerobic digester) had been maintained at 41.1 ± 0.5 days (mean $\pm$ standard deviation) for a time period of 35 days. Once steady-state conditions had been established, the operating mode of the digester was shifted to better reflect continuous-flow operating conditions by feeding untreated residual municipal wastewater solids on a daily basis from Day 180 to Day 191. Following this semi-continuous flow phase, the aerobic digester was operated for an additional 27 days while being fed on a weekly basis untreated residual municipal wastewater solids. On Day 218, half of the digester contents (i.e., 5 L) were replaced with untreated residual municipal wastewater solids to allow the determination of decay coefficients in a batch-like reactor.

SAMPLE COLLECTION AND GENOMIC DNA EXTRACTION AND PURIFICATION

Triplicate samples (100 μL) were collected from larger aliquots (50–300 mL) of digester contents to ensure accurate sample collection volumes. Samples were then diluted with 500 μL of lysis buffer (120 mM sodium phosphate buffer, 5% dodecyl sulfate, pH 8.0 ± 0.1) and subjected to three consecutive freeze-thaw cycles followed by incubation at 70°C for 90 min. Genomic DNA was then extracted using a FastDNA Spin Kit (MP Biomedicals LLC, Solon, OH) according to the manufacturer's instructions.

REAL-TIME PCR

Real-time PCR was used to quantify the concentrations of three different genes that encode resistance to tetracycline [*tet*(A), *tet*(W), and *tet*(X)], one gene that encodes resistance to erythromycin [*erm*(B)], one gene that encodes resistance to sulfonamides (*sul1*), and the integrase gene of class 1 integrons (*intI1*). The three tetracycline resistance genes were selected because they represent each of the three known mechanisms of tetracycline resistance (efflux pumps, ribosomal protection proteins, and enzymatic modification) (Levy et al., 1999). The erythromycin gene, *erm*(B), was chosen because it encodes an rRNA methyltransferase that confers resistance to macrolides, lincosamides, and streptogramin B (Roberts et al., 1999). Prior work has demonstrated that all five of these ARGs are present at substantial concentrations in wastewater and/or wastewater solids (Diehl and LaPara, 2010; LaPara et al., 2011; Munir et al., 2011). Class 1 integrons were quantified because of their association with multiple antibiotic resistance. These integrons enable bacteria to collect multiple, exogenous ARGs and modulate their expression (Mazel, 2006). qPCR was also used to determine the concentrations of 16S rRNA genes (a measure of total bacterial biomass), all *Bacteroides* spp. (a measure of total fecal bacteria), and human-specific *Bacteroides* spp. (a measure of human fecal bacteria) (Muyzer et al., 1993; Bernhard and Field, 2000; Layton et al., 2006). Additional information regarding the use of qPCR to quantify these genes can be found in **Table 1**.

Real-time PCR was carried out on an Eppendorf Mastercycler EP Realplex thermal cycler (Eppendorf, Westbury, NY). PCR assays were optimized to reduce or eliminate the formation of primer-dimers and non-specific products. Typical PCR assays began with a 1 min initial denaturation at 95°C. This step was followed by 40 cycles of denaturation at 95°C for 15 s and combined annealing and extension at the primer-specific annealing temperature for 1 min. Typical reaction volumes were 25 μL and consisted of 12.5 μL of BioRad iTaq SYBR Green Supermix with ROX

Table 1 | Gene targets, resistance mechanisms, primer sequences, amplicon sizes, and annealing temperatures for real-time PCR assays.

Gene target	Resistance mechanism	Primer sequence (5′ → 3′)	Size (bp)	Annealing temperature (°C)	References
16S rRNA gene	NA	F: CCT ACG GGA GGC AGC AG R: ATT ACC GCG GCT GCT GG	202	60	Muyzer et al., 1993
Bacteroides spp.	NA	F: GAG AGG AAG GTC CCC CAC R: CGC TAC TTG GCT GGT TCA G	116	60	Layton et al., 2006
Human-specific *Bacteroides*	NA	F: ATC ATG AGT TCA CAT GTC CG R: TAC CCC GCC TAC TAT CTA ATG	82	56	Bernhard and Field, 2000; Seurinck et al., 2005
erm(B)	Ribosomal protection	F: GAT ACC GTT TAC GAA ATT GG R: GAA TCG AGA CTT GAG TGT GC	364	58	Chen et al., 2007
intI1	Class 1 integron	F: CCT CCC GCA CGA TGA TC R: TCC ACG CAT CGT CAG GC	280	60	Goldstein et al., 2001
sul1	Enzymatic modification	F: CCG TTG GCC TTC CTG TAA AG R: TTG CCG ATC GCG TGA AGT	67	60	Heuer and Smalla, 2007
tet(A)	Efflux	F: GCT ACA TCC TGC TTG CCT TC R: CAT AGA TCG CCG TGA AGA GG	210	60	Ng et al., 2001
tet(W)	Ribosomal protection	F: GAG AGC CTG CTA TAT GCC AGC R: GGG CGT ATC CAC AAT GTT AAC	168	60	Aminov et al., 2001
tet(X)	Enzymatic modification	F: AGC CTT ACC AAT GGG TGT AAA R: TTC TTA CCT TGG ACA TCC CG	278	60	Ghosh et al., 2009

(Life Science Research, Hercules, CA), 25 μg of bovine serum albumin, optimized quantities of forward and reverse primers, and approximately 1 ng of template genomic DNA. Each analysis consisted of three replicates. Standards were made from PCR products that targeted specific genes from either well-described bacterial isolates or from municipal wastewater solids. PCR products were ligated into a pGEM-T Easy cloning vector, transformed into JM109 competent cells, and extracted from cell cultures using the alkaline lysis procedure (Sambrook et al., 1989). It was confirmed that all plasmids contained the specified gene by nucleotide sequence analysis of the extracted plasmid. The DNA concentration of plasmid extracts was quantified using a TD-700 fluorometer and Hoechst 33258 dye. Each standard curve consisted of a 10-fold dilution series containing at least 5 standards ($r^2 \geq 0.99$). Amplification efficiencies were 100 ± 10%.

DATA ANALYSIS

All data obtained from groups of triplicate samples were treated as if they had been obtained from a normal distribution (i.e., means and standard deviations were used to describe the data). This assumption of normal distributions was based on results from Shapiro–Wilk normality tests performed in SigmaPlot 12.0 that indicated the complete semi-continuous flow data series for most gene targets could not be distinguished from a normal distribution ($P > 0.05$). Analysis of variance (ANOVA; Microsoft Excel 2010) was used with data from the semi-continuous flow experiment to determine the statistical significance of differences in gene target concentrations between untreated and treated residual solids samples.

Simple linear regression (Arc 1.06) was used with log-transformed data from the batch experiment to determine the goodness of fit of the data to a first-order kinetic model. The first-order kinetic model was chosen based on previous empirical observations that it tends to fit this type of data well. Values of P used to compare the relative statistical significance of different kinetic coefficients (**Table 4**) were determined using Welch's t-test for unequal n and unequal sample variance. The sample variance for each estimated first-order kinetic coefficient was obtained from the estimated standard error for that coefficient as provided by Arc 1.06.

RESULTS

SEMI-CONTINUOUS FLOW OPERATING MODE

With respect to typically monitored operating variables, the lab-scale aerobic digester performed as an appropriate experimental model simulation during the semi-continuous flow experimental period (**Table 2**). The pH was circumneutral, digester temperature was the same as the ambient air temperature, and DO concentrations were well above the target DO concentration of 2 mg/L. The total and volatile solids concentrations for the untreated and treated solids were well within ranges typically encountered in practice (2–5% total solids, 0.6–3% volatile solids) (Tchobanoglous et al., 2003). The fractions of total and volatile solids destroyed were 26 and 41%, respectively, which is also typical for a full-scale aerobic digestion process (Tchobanoglous et al., 2003).

The aerobic digester eliminated a substantial fraction of bacterial biomass and fecal indicator bacteria (FIB) as measured by

Table 2 | Operating variables during operation in semi-continuous flow mode.

Variable	Mean ± SD	*n* values
pH	7.5 ± 0.2	*n* = 12
Temperature (°C)	17.0 ± 0.3	*n* = 12
Dissolved oxygen (mg/L)	5.8 ± 1.5	*n* = 12
Hydraulic residence time (days)	13.5 ± 0.7	*n* = 12
Untreated total solids	4.6% ± 0.04%	*n* = 4
Treated total solids	3.4% ± 0.3%	*n* = 4
Total solids destruction	26.3% ± 6.0%	*n* = 4
Total solids residence time (days)	33.1 ± 0.4	*n* = 12
Untreated volatile solids	3.2% ± 0.05%	*n* = 4
Treated volatile solids	1.9% ± 0.1%	*n* = 4
Volatile solids destruction	41.1% ± 4.8%	*n* = 4
Volatile solids residence time (days)	27.7 ± 0.6	*n* = 12
Untreated inert solids	1.5% ± 0.02%	*n* = 4
Treated inert solids	1.5% ± 0.1%	*n* = 4
Inert solids destruction	−5.7% ± 8.4%	*n* = 4
Inert solids residence time (days)	41.7 ± 0.1	*n* = 12

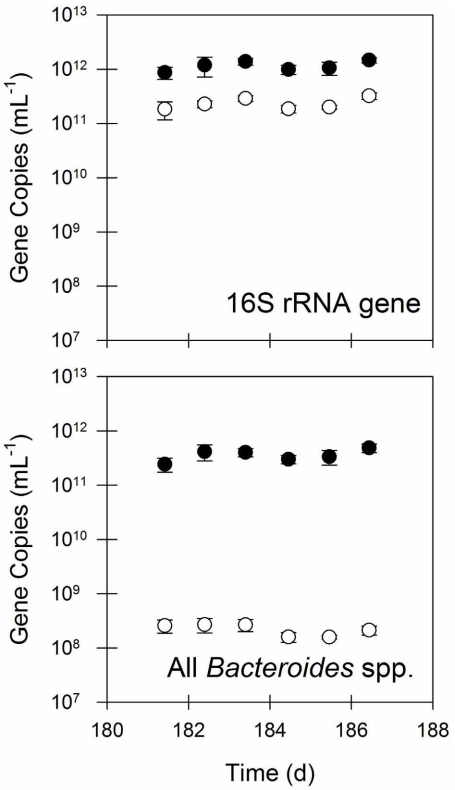

FIGURE 1 | The quantities of 16S rRNA genes and fecal indicator bacteria as measured by 16S rRNA genes of all *Bacteroides* spp. in untreated (closed circles) and treated (open circles) residual solids. Values are the arithmetic mean of triplicate samples; error bars represent one standard deviation. The concentrations of human-specific *Bacteroides* spp. were approximately 5×10^8 gene copies mL^{-1} in untreated samples, but were below the detection limit (1×10^8 gene copies mL^{-1}) in treated samples.

qPCR targeting the 16S rRNA gene and 16S rRNA genes specific for all *Bacteroides* spp. and for human-specific *Bacteroides* spp. (**Figure 1**). The concentrations of 16S rRNA genes were 77% lower in treated samples compared to the untreated samples. This indicates net destruction of total bacterial biomass in the digester, consistent with the total and volatile solids removal. Significant removal was observed for both types of FIB. The concentrations of all *Bacteroides* spp. were 99.9% lower in treated samples compared to untreated samples. Similarly, the concentrations of human-specific *Bacteroides* spp. were approximately 5×10^8 gene copies mL^{-1} in untreated samples, but were below the detection limit (1×10^8 gene copies mL^{-1}) in treated samples.

The untreated wastewater solids contained substantial quantities of each of the ARGs investigated in this study. The quantities of *intI1*, *sul1*, and *tet*(W) were similar, present at a concentration of approximately 10^{10} gene copies mL^{-1}. In contrast, the concentration of *erm*(B) was approximately 10^{11} gene copies mL^{-1}, and the concentrations of *tet*(A) and *tet*(X) were approximately 10^9 gene copies mL^{-1}. Given that the concentrations of 16S rRNA genes were approximately 10^{12} gene copies mL^{-1} in the untreated solids, the ratio of the various antibiotic resistance determinants examined in this study to bacterial cells ranged from approximately 0.1% for *tet*(A) and *tet*(X) to 1% for *intI1*, *sul1*, and *tet*(W), and to 10% for *erm*(B).

The bench-scale aerobic digester removed between 85 and 98% of *erm*(B), *sul1*, *tet*(A), and *tet*(W) during the semi-continuous flow experimental period (**Figure 2**), which was substantially greater than that for bacterial biomass (i.e., 16S rRNA genes). In contrast, the quantity of *intI1* was not statistically different ($P = 0.17$) in the untreated and treated solids, suggesting that aerobic digestion operated in semi-continuous flow mode does not eliminate *intI1* (**Figure 3**). Furthermore, the ratio of *intI1* to 16S rRNA genes increased in the treatment process from 0.8 to 3%, indicating that aerobic digestion likely selects for bacterial cells possessing a class 1 integron. Interestingly, the aerobic digestion process also appeared to select for bacterial cells containing *tet*(X), as the quantity of this gene was 5-fold greater in the treated solids than in the untreated solids.

BATCH OPERATING MODE

Following the semi-continuous flow experimental phase, the aerobic digester was shifted to batch mode to determine decay rates for each target gene. As with the previous experimental phase, typically monitored operating variables indicated that the digester operated as an appropriate simulation of a full-scale aerobic digester. The pH rose from a semi-continuous phase value between 7 and 7.5 to just above 8 following the addition of untreated residual solids, but then gradually decreased to between 7 and 7.5. A substantial decrease in DO concentration to less than 1 mg/L was initially observed, but increased to > 4 mg/L within 24 h and remained so for the duration of the batch experiment.

A significant decay rate was observed for 16S rRNA genes and FIB during operation in batch mode (**Figure 4**). The quantities of 16S rRNA genes decayed by 90% during the 20-day batch

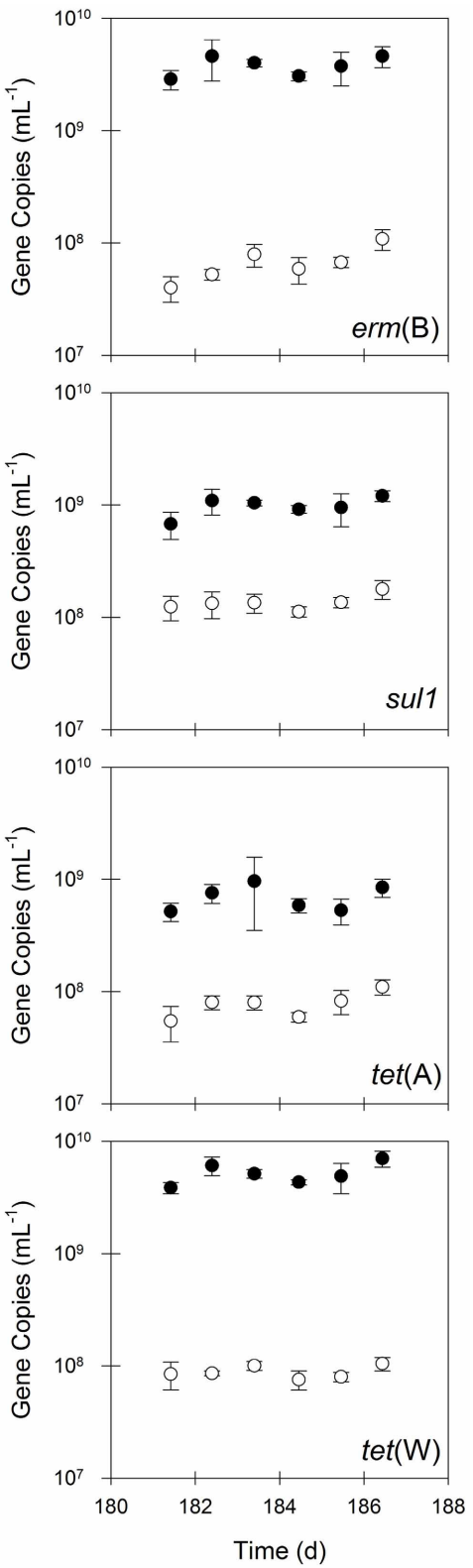

FIGURE 2 | The quantities of *erm*(B), *sul1*, *tet*(A), and *tet*(W) in untreated (closed circles) and treated (open circles) residual solids. Values are the arithmetic mean of triplicate samples; error bars represent one standard deviation.

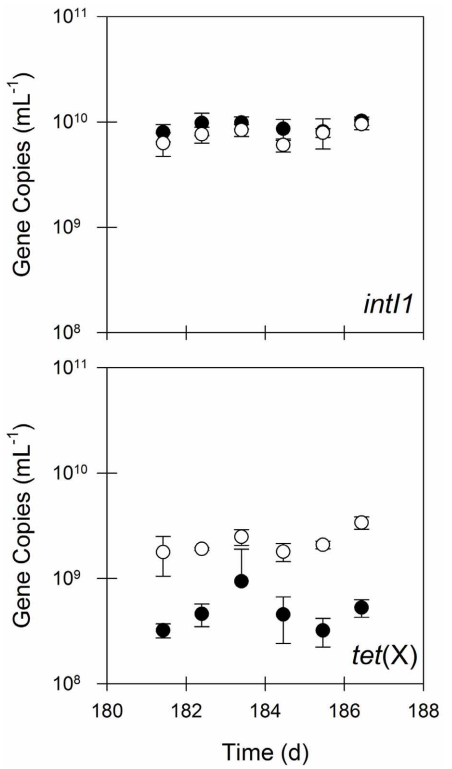

FIGURE 3 | The quantities of *intI1* and *tet*(X) in untreated (closed circles) and treated (open circles) residual solids. Values are the arithmetic mean of triplicate samples; error bars represent one standard deviation.

experiment ($t_{1/2} = 5.5$ d; **Table 3**). In contrast, all *Bacteroides* spp. decayed by nearly four orders of magnitude over 20 days ($t_{1/2} = 1.4$ d; **Table 3**), whereas human-specific *Bacteroides* spp. decayed to below the detection limit within one week of beginning the batch experimental phase ($t_{1/2} = 4.6$ d; **Table 3**).

In contrast to operation in semi-continuous flow mode, the quantities of all of the antibiotic resistance determinants, including *intI1* and *tet*(X), declined in the batch experimental phase (**Figure 5**). The quantities of *erm*(B) and *tet*(W) declined by approximately two orders of magnitude during the 20-day experiment, whereas the quantities of *intI1*, *sul1*, *tet*(A), and *tet*(X) each declined by one order of magnitude during the same time period. Correspondingly, the first-order decay rates varied considerably among individual gene targets. The *intI1* and *tet*(X) genes decayed the most slowly, each with a half-life of approximately 6 days (**Table 3**). These rates of decay were statistically similar to each other as well as to the rate of decay for the 16S rRNA gene (**Table 4**). In contrast, the first-order decay rates were significantly ($P < 0.05$) more rapid for the remaining gene targets, with half-lives ranging from 2.8 to 4.6 days (**Table 3**). These rates of decay were significantly faster than the decay rate for 16S rRNA genes (**Table 4**).

DISCUSSION

The long-term goal of our research is to determine how the numerous technologies used to treat municipal wastewater can

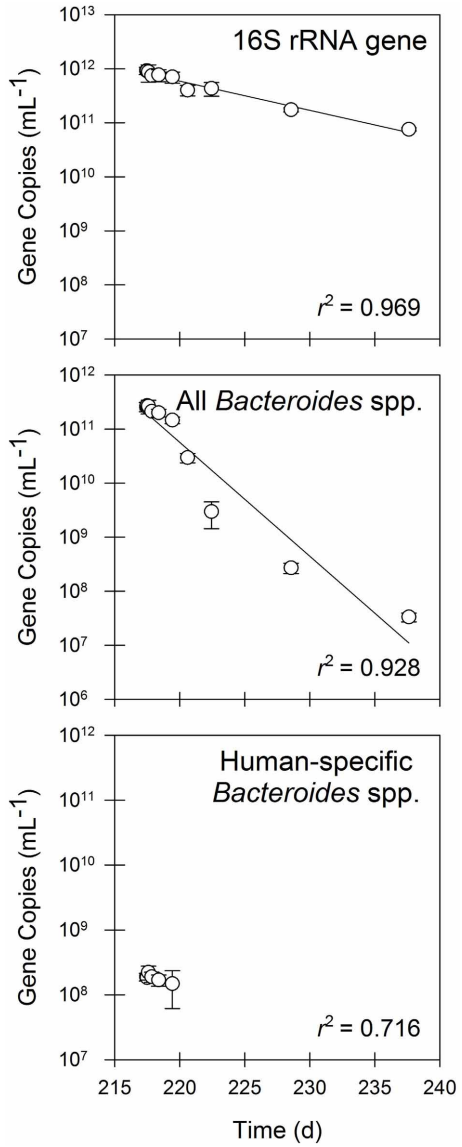

FIGURE 4 | The quantities of 16S rRNA genes, fecal indicator bacteria as measured by 16S rRNA genes of all *Bacteroides* spp., and fecal indicator bacteria as measured by 16S rRNA genes of human-specific *Bacteroides* spp. in residual solids undergoing batch treatment. Values are the arithmetic mean of triplicate samples; error bars represent one standard deviation.

Table 3 | Summary of first-order degradation kinetic model parameter estimates for the 16S rRNA gene, fecal indicator bacteria as measured by 16S rRNA genes of all *Bacteroides* spp., fecal indicator bacteria as measured by 16S rRNA genes of human-specific *Bacteroides* spp., *erm*(B), *intI1*, *sul1*, *tet*(A), *tet*(W), and *tet*(X) during batch mode operation.

Gene target	k (day^{-1}) ± standard error (day^{-1})	$t_{1/2}$ (days)
16S rRNA gene	0.13 ± 0.008	5.5
Bacteroides spp.	0.49 ± 0.048	1.4
Human-specific *Bacteroides* spp.	0.15 ± 0.047	4.6
erm(B)	0.19 ± 0.025	3.6
intI1	0.11 ± 0.011	6.3
sul1	0.15 ± 0.009	4.6
tet(A)	0.16 ± 0.011	4.4
tet(W)	0.25 ± 0.025	2.8
tet(X)	0.12 ± 0.006	5.7

All rates were regressed from 10 data points (except human-specific Bacteroides spp., n = 6) and are statistically significant (P < 0.05).

simultaneously be used to eliminate the substantial quantities of ARB and ARGs that are known to exist in untreated sewage. The present study makes an important advance in our knowledge by elucidating the extent and rate by which aerobic digestion can be used to eliminate ARGs. This research is practically important because the overwhelming majority of ARB and ARGs in raw sewage ultimately end up in the residual wastewater solids, and a recent study suggested that more than 2200 municipal wastewater treatment facilities use this technology to produce more than 85,000 dry tons of treated wastewater solids in the United States each year (Beecher et al., 2007).

In most cases, the rate of disappearance of different ARGs exceeded that of the total number of bacteria (as measured by 16S rRNA gene copies), suggesting that these ARGs were actively eliminated during the aerobic digestion process. Although there is only very limited data presently available in the published literature, these disappearance rates are generally similar to the rates that were previously observed during anaerobic digestion at 37°C (Diehl and LaPara, 2010). In contrast, the quantities of *tet*(X) and *intI1* decayed at a rate similar to that of all bacteria, suggesting that these genes were passively eliminated, paralleling the decline in the total number of bacteria. This observation is substantially different than our previous study, in which both *tet*(X) and *intI1* rapidly declined in bench-scale anaerobic digestion processes operated at temperatures of 37°C or higher (Diehl and LaPara, 2010). Therefore, the rates by which different ARGs decay in a conventional aerobic digestion process are either similar to or slower than the decay rates observed in anaerobic digestion processes. This apparent inferiority of aerobic digestion is pertinent because anaerobic digestion is also a commonly used technology to treat residual wastewater solids (Tchobanoglous et al., 2003).

This research also demonstrated that reactor design has a major effect on the fate of ARGs during the treatment of residual municipal wastewater solids. The most obvious difference occurred with *tet*(X), which declined under batch experimental conditions but increased substantially in semi-continuous flow conditions. Similarly, the quantity of *intI1* declined under batch conditions but remained static during semi-continuous flow operation. In contrast, model results suggest that the quantities of *erm*(B) and *tet*(W) declined more rapidly in semi-continuous flow operation than would be suggested by the first-order decay coefficient elucidated under batch conditions (analyses not shown). Previous researchers have suggested that reactor design affects the removal of ARGs during wastewater solids digestion (Ma et al., 2011); our results support this

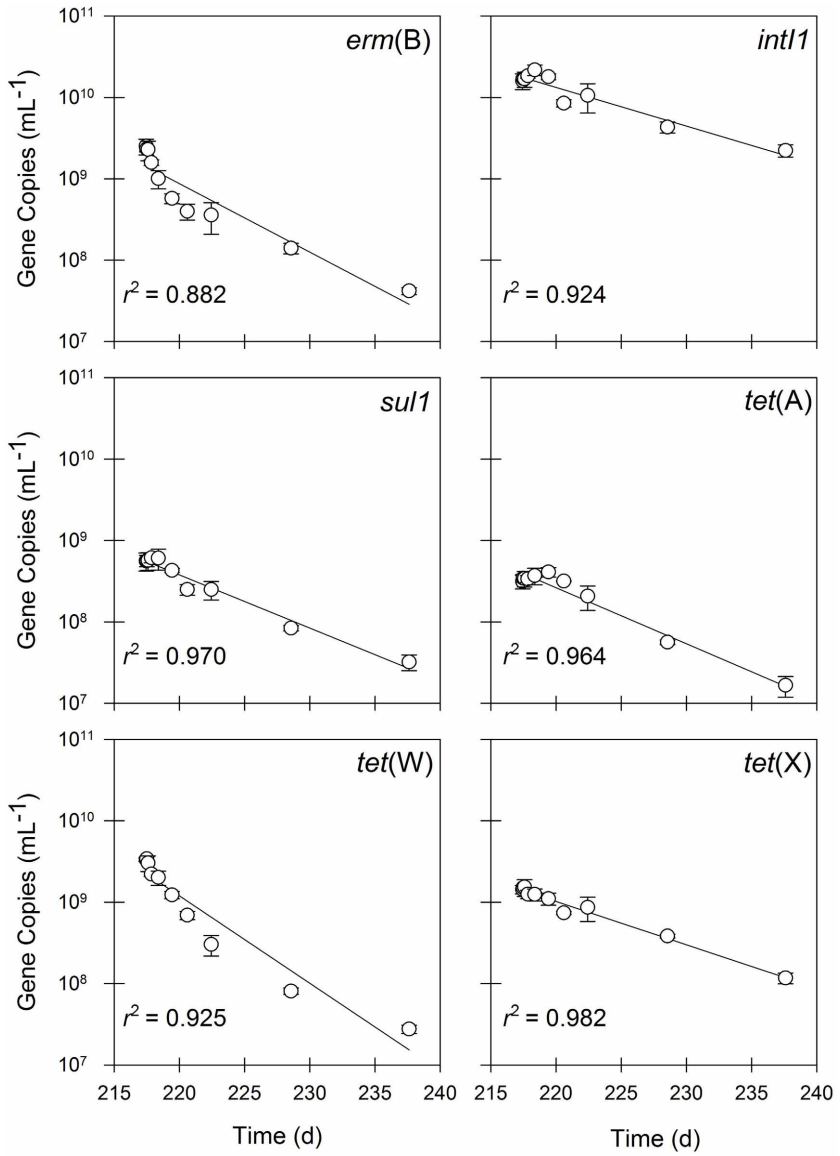

FIGURE 5 | The quantities of *erm*(B), *intI1*, *sul1*, *tet*(A), *tet*(W), and *tet*(X) in residual solids undergoing batch treatment. Values are the arithmetic mean of triplicate samples; error bars represent one standard deviation.

hypothesis. Additional research is needed to clarify the importance of reactor design and the disappearance of ARGs during the digestion of wastewater solids.

A growing body of evidence suggests that class 1 integrons, which are linked to multiple antibiotic resistance, are particularly prominent in wastewater (Ghosh et al., 2009; Zhang et al., 2009b; Ramsden et al., 2010; Ma et al., 2011; Stalder et al., 2012). Our research has shown significant variation in removal efficiencies of *intI1* depending on the specific technology (i.e., anaerobic digestion achieves better and more efficient removal than aerobic digestion) and the specific operating conditions (temperature, flow regime, etc.). Additional research is needed to better understand the fate and gene cassette content of class 1 integrons in residual solids treatment systems.

Prior research has suggested that *sul1* flanks all class 1 integrons (Mazel, 2006). When the aerobic digester was operated in semi-continuous flow mode, however, the quantities of *sul1* genes declined by almost an order of magnitude, whereas the quantities of *intI1* were similar in the treated and in the untreated residual solids. Similarly, the rate by which *sul1* declined in batch operating mode was significantly faster than the rate by which *intI1* declined ($P = 0.01$; **Table 4**). This suggests that the coupling of the *sul1* gene to class 1 integrons is not universal; additional research is needed to better understand the relationship between class 1 integrons and *sul1* genes in the unit operations used to treat wastewater solids.

The most significant limitation of our research is the use of real-time quantitative PCR targeting various ARGs as a surrogate

Table 4 | Values of *P* for comparing the relative statistical significance of different kinetic coefficients determined using Welch's *t*-test for unequal *n* and unequal sample variance.

Gene	16S rRNA gene	*Bacteroides* spp.	Human-specific *Bacteroides* spp.	*erm*(B)	*intI1*	*sul1*	*tet*(A)	*tet*(W)	*tet*(X)
16S rRNA gene	1	3×10^{-5}	0.6	0.02	0.3	0.06	0.03	7×10^{-4}	0.8
Bacteroides spp.		1	2×10^{-4}	1×10^{-4}	2×10^{-5}	5×10^{-5}	6×10^{-5}	6×10^{-4}	3×10^{-5}
Human-specific *Bacteroides* spp.			1	0.4	0.4	1	0.9	0.1	0.6
erm(B)				1	9×10^{-3}	0.1	0.2	0.1	0.02
intI1					1	0.01	5×10^{-3}	2×10^{-4}	0.3
sul1						1	0.6	4×10^{-3}	0.02
tet(A)							1	6×10^{-3}	0.01
tet(W)								1	6×10^{-4}
tet(X)									1

for ARB. The genes quantified here could be present in dead but intact bacteria or in bacteria in which the gene is non-functional. Similarly, the identity of the ARB harboring the ARGs detected in this study, and their clinical significance, remain unknown. Finally, only a select group of ARGs were targeted; even though these genes represent several important classes of antibiotics and all three known molecular mechanisms of resistance to tetracyline, they cover a relatively small cross-section of possible resistance gene targets.

In conclusion, aerobic digestion can be used to eliminate ARGs in untreated wastewater solids, but rates can vary substantially depending on the reactor design and the specific ARG examined. This information represents a critical step toward our long-term goal of applying wastewater treatment technologies to mitigate the spread of antibiotic resistance. This knowledge is particularly useful to wastewater treatment engineers as they compare the relative merits of alternative residual solids treatment technologies and for designing specific unit operations to eliminate ARGs. Specifically, aerobic digestion technology, which is used by numerous full-scale municipal wastewater treatment facilities, appears less effective at eliminating ARGs than both conventional and high temperature anaerobic digestion.

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Chronological change of resistance to β-lactams in *Salmonella enterica* serovar Infantis isolated from broilers in Japan

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Epidemiologic surveillance study was conducted in southern Japan to determine the antimicrobial resistance phenotypes and characterize the β-lactamase genes and the plasmids harboring these genes in *Salmonella enterica* serovar Infantis (*S.* Infantis) isolates from broilers. Between January, 2007 and December, 2008, a total of 1,472 fecal samples were collected and examined at the Laboratory of Veterinary Public Health, Kagoshima University, Japan. In 93 (6.3%) isolates recovered, 33 (35.5%) isolates showed resistance to cefotaxime, an extended-spectrum cephalosporin (ESC), conferred by TEM-20, TEM-52 and CTX-M-25 extended-spectrum β-lactamases (ESBLs). In addition to ESC-resistance, eight (8.6%) isolates exhibited resistance to cefoxitin mediated by CMY-2 AmpC β-lactamase. Plasmid analysis and polymerase chain reaction replicon typing revealed the bla_{TEM-20} and bla_{CMY-2} genes were associated with IncP plasmids, bla_{TEM-52} was linked with a non-typable plasmid and $bla_{CTX-M-25}$ was carried by an IncA/C plasmid. Non-β-lactam resistance to streptomycin, sulfamethoxazole, and oxytetracycline encoded by the *aadA1*, *sul1*, and *tet*(A) genes, respectively, was found in 86 (92.5%) isolates. Resistance to kanamycin and ofloxacin was exhibited in 12 (12.9%) and 11 (11.8%) isolates, respectively, the former was mediated by *aphA1-lab*. These data indicate that *S.* Infantis isolates producing ESBLs and AmpC β-lactamase have spread among broiler farms in Japan. These data demonstrated that the incidence of ESC-resistant S. Infantis carrying bla_{TEM-52} remarkably increased and *S.* Infantis strains harboring bla_{CMY-2}, bla_{TEM-20}, or $bla_{CTX-M-25}$ genes emerged from broilers in Japan for the first time in 2007 and 2008.

Keywords: antimicrobial resistance, β-lactamase gene, broiler, extended-spectrum cephalosporin, *Salmonella enterica* serovar Infantis, plasmids

INTRODUCTION

Non-typhoidal *Salmonella enterica* serovars (*Salmonella*) are a major cause of bacterial food-borne diseases world-wide (Majowicz et al., 2010). The food poisoning statistics in Japan show that bacterial food poisoning patients during the year 2008 numbered at 10,331 and *Salmonella* were the leading etiological agents accounting for 24.7% of the cases [http://idsc.nih.go.jp/iasr/29/342/graph/t3421.gif]. Poultry products are important vehicles in the transmission, and have been incriminated in several *Salmonella* outbreaks (Kimura et al., 2004; Chittick et al., 2006). Since the late 1990s, *Salmonella enterica* serovar Infantis (*S.* Infantis) has been the commonest serovar of *Salmonella* isolated from both broiler flocks and retail chicken meat in Japan (Asai et al., 2007b; Iwabuchi et al., 2011).

The emergence of multidrug-resistant *Salmonella* has become a serious global health problem because antimicrobial treatment is lifesaving for invasive infections, particularly in neonates of <1 year of age. Preventive antimicrobial treatment is also generally given to patients suffering from immunosuppressive or other predisposing conditions (Hohmann, 2001). The options of first-line therapy for *Salmonella* infection include ampicillin (AMP), sulfamethoxazole-trimethoprim, fluoroquinolones, and extended-spectrum cephalosporins (ESCs). ESCs are among the preferred drugs because of resistance to the other aforementioned drugs is relatively frequent in *Salmonella* isolates (Hohmann, 2001). At present, ESC-resistance in *Salmonella* is mainly attributed to the acquisition of plasmid-mediated extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases (Bonnet, 2004; Arlet et al., 2006).

Salmonella carrying the bla_{CMY-2} gene were recently recovered from bovine and porcine salmonellosis cases (Dahshan et al., 2010; Sugawara et al., 2011). Besides, *S.* Infantis isolates harboring the bla_{TEM-52} gene were reported from broilers for the first time in the year 2004 (Shahada et al., 2010a). Thus, a major concern has been increased prevalence of resistance to ESCs noted in *S.* Infantis isolates. This study was conducted to determine the antimicrobial

resistance phenotypes and characterize the β-lactamase genes and the plasmids harboring these genes in *S.* Infantis isolated from broiler flocks.

MATERIALS AND METHODS

BACTERIAL ISOLATES

Between January, 2007 and December, 2008, a total of 1,472 cecal specimens derived from 92 broiler flocks (ca. 10,000 birds per flock) were collected from a poultry processing plant located in the southern part of Japan. Usually, 16 samples per flock were randomly selected fortnightly. The isolation, identification and serotyping of S. Infantis isolates were performed at the Laboratory of Veterinary Public Health, Kagoshima University, Japan as previously described (Shahada et al., 2008).

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS

Antimicrobial susceptibility testing was assayed by the agar dilution method on Mueller-Hinton (MH) agar (Oxoid Ltd., Basingstoke, Hampshire, England) plates according to the National Committee for Clinical and Laboratory Standards guidelines (National Committee for Clinical Laboratory Standards, 2001). *S.* Infantis isolates were tested for sensitivities to AMP, cefotaxime (CTX), cefoxitin (FOX), chloramphenicol (CHL), streptomycin (STR), sulfamethoxazole (SUL), oxytetracycline (TET), kanamycin (KAN), and ofloxacin (OFX). The MIC range was set at 0.125–512 μg/ml for all tested antimicrobial agents. The MIC breakpoints were interpreted according to the new criteria established by the Clinical and Laboratory Standards Institute (2012). *Escherichia coli* (*E. coli*) ATCC 25922 and *Staphylococcus aureus* ATCC29213 were used as quality control strains.

DOUBLE-DISK SYNERGY ASSAY

The double-disk synergy testing was conducted to screen for ESBLs and AmpC β-lactamases as previously described (Shahada et al., 2010a). This test was performed as a standard Kirby-Bauer disk diffusion assay on MH agar (Oxoid) plates.

DETECTION OF RESISTANCE DETERMINANTS

All DNA templates were prepared using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA, USA). Detection of resistance genes was performed by polymerase chain reaction (PCR). The amplification reactions were carried out using primers and conditions as previously described (Dahshan et al., 2010; Shahada et al., 2010b). Briefly, the targets were as follows: bla_{TEM}, bla_{OXA}, bla_{PSE}, bla_{SHV}, $bla_{CTX-M-1}$ group, $bla_{CTX-M-2}$ group, $bla_{CTX-M-25}$, and *Toho-1* encoding for penicillinases; bla_{CIT}, bla_{CMY-2}, bla_{DHA}, bla_{FOX}, bla_{MOX}, bla_{ACC}, and bla_{EBC} encoding for cephalosporinases; *tet*(A), *tet*(B), and *tet*(G) mediating tetracycline efflux proteins; *aadA1* and *aadA2* encoding for resistance to STR and spectinomycin; *sul1* conferring resistance to SUL; and *aphA1-Iab* encoding for resistance to KAN.

When β-lactamase-encoding genes were positive following PCR amplification, obtained products were directly sequenced using a BioDye Terminator version 3.1 Ready Reaction sequencing kit and ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The DNA alignments and deduced amino acid sequences were examined using the BLAST program (National Center for Biotechnology Information, USA).

PLASMID ANALYSIS

All *S.* Infantis isolates were examined for the carriage of plasmids by employing the alkaline lysis method as previously described (Kado and Liu, 1981). The molecular size of plasmids was determined by using the standard *Salmonella enterica* serovar Choleraesuis ATCC 7001 (50 kbp) and *S.* Typhimurium DT104 strain 300-98 (90 kbp). The plasmid size was estimated by graphing the molecular size of standard strains versus the distance traveled from the wells using logistic graph paper. Conjugation experiments were performed as described previously (Shahada et al., 2010a). In brief, *S.* Infantis donor isolates and rifampicin-resistant *E. coli* DH5α recipient derivatives were used for conjugal mating. Conjugants were selected onto deoxycholate hydrogen sulfide lactose agar containing 128 μg/ml rifampicin and 128 μg/ml AMP, followed by antimicrobial testing and the detection of transferred resistance genes as above. Plasmids were characterized by the PCR-based replicon typing method as described previously (Carattoli et al., 2005) to detect the plasmid types: IncI1, IncA/C, IncHI1, IncHI2, IncN, IncX, IncW, IncY, IncP, IncT, IncFIIs, IncL/M, IncFIA, IncFIB, IncFIC, IncFrepB, IncK/B, and IncB/O.

RESULTS

ANTIMICROBIAL RESISTANCE

S. Infantis isolates were recovered in 54 (58.7%) of 92 flocks surveyed and 93 (6.3%) of 1,472 samples examined. Antimicrobial susceptibility profiles of the isolates are summarized in **Table 1**. Of 93 *S.* Infantis isolates recovered, 34 (36.5%) exhibited resistance to AMP (MIC, 256 to >512 μg/ml), 33 (35.5%) showed resistance to CTX (MIC, 4-256 μg/ml) and eight (8.6%) demonstrated resistance to FOX (MIC, 32–128 μg/ml). From the 93 isolates and with regard to non-β-lactam antibiotics, 86 (92.5%) isolates exhibited resistance to STR (MIC, 16–512 μg/ml, conferred by *aadA1*), SUL (MIC >512 μg/ml, conferred by *sul1*) and TET (MIC, 64–512 μg/ml, conferred by *tetA*); 12 (12.9%) isolates showed resistance to KAN (MIC, ≥512 μg/ml, conferred by *aphA1*); and 11 (11.8%) isolates demonstrated resistance to OFX (MIC, 2–8 μg/ml). All 93 isolates were fully susceptible to CHL. On the basis of resistance patterns, 11 phenotypes (A to K) were demonstrated as depicted in **Table 2**. The most frequent pattern was STR/SUL/TET (43 isolates) followed by AMP/CTX/STR/SUL/TET (14 isolates).

ESBL AND AMPC CHARACTERIZATION

Double-disk synergy testing revealed 25 ESBL- and eight AmpC β-lactamase-producing *S.* Infantis isolates. TEM-type ESBL was identified in 23 (24.7%) isolates, CTX-M-type ESBL was demonstrated in one isolate and CMY-type AmpC β-lactamase was detected in eight isolates. Nucleotide sequencing of PCR products confirmed that the amplicons were bla_{TEM-52} (22 isolates), bla_{TEM-20} (one isolate), bla_{CTX-25} (one isolate in combination with bla_{TEM-52}), and bla_{CMY-2} (8 isolates). Of 54 S. Infantis-positive flocks, As indicated in **Table 3**, *S.* Infantis isolates harboring bla_{TEM-1} were not detected during the present study; but, they were found during 1998–2003 (one isolate) and 2004–2006 (17 isolates). On the other hand, isolates harboring bla_{TEM-52} were identified during 2004–2006 (11 isolates) and 2007–2008 (22 isolates). Isolates carrying the bla_{TEM-20}, bla_{CTX-25}, and

Table 1 | Antimicrobial susceptibility among 93 *Salmonella* Infantis isolates detected in this study

Antimicrobial agent	MIC breakpoint (μg/m/)	No. of resistant isolates (%)	
		This study	Previous study
		2007–2008	2004–2006[a]
AMP	≥32	34 (36.5)	29 (24.2)
CTX	≥4	33 (35.5)	11 (9.1)
FOX	≥32	8 (8.6)	0 (0)
CHL	≥32	0 (0)	0 (0)
STR	≥16	86 (92.5)	120 (100)
SUL	≥512	86 (92.5)	120 (100)
TET	≥16	86 (92.5)	120 (100)
KAN	≥64	12(12.9)	9 (7.5)
OFX	≥2	11(11.8)	25 (20.8)

CTX, cefotaxime; FOX, cefoxitin; CHL, chloramphenicol; STR, streptomycin; SUL, sulfamethoxazole; TET, oxytetracycline; KAN, kanamycin; OFX, ofloxacin. (a) Cited from the previous study (Shahada et al., 2010b)

bla_{CMY-2} genes were demonstrated during 2007–2008 study period.

PLASMID CHARACTERIZATION

Three plasmid profiles were defined among *S.* Infantis isolates. Seventy isolates harbored ca. 180-kbp plasmids (profile I), 22 isolates carried two plasmids of ca. 50 and 180 kbp (profile II), and one isolate possessed three plasmids of ca. 50, 125, and 180 kbp (profile III; **Figure 1**). Isolates exhibiting resistance to ESCs displayed diverse profiles: Of 34 ESC-resistant *S.* Infantis isolates, 22 belonged to profile II, 11 were classified into profile I and one isolate was categorized into profile III (**Table 2**). PCR-based replicon typing revealed three kinds of plasmids. Large-size plasmids (ca.180 kbp) were typed as IncP, intermediate-size plasmid (ca. 125 kbp) was typed as IncA/C and small plasmids (ca. 50 kbp) were non-typable. Large-, intermediate- and small-size plasmids were self-transmissible to *E. coli* recipient strain by conjugation. The bla_{TEM-20} and bla_{CMY-2} genes were associated with large IncP plasmids, $bla_{CTX-M-25}$ was affiliated with intermediate IncA/C plasmid and the bla_{TEM-52} gene was linked to small non-typable plasmids.

DISCUSSION

The results obtained in this study show that resistance to ESCs has increased to 35.5% from the previous 9.2% reported during an earlier investigation (Shahada et al., 2010b). Other new findings include the detection of *S.* Infantis isolates harboring the bla_{TEM-20}, $bla_{CTX-M-25}$, and bla_{CMY-2} genes. Demonstration of these resistance traits in *S.* Infantis serovar is a rare phenomenon. This is the first report describing these mechanisms of ESC-resistance exhibited by *S.* Infantis isolates derived from broilers. Previous reports indicate that β-lactam resistance in broilers was once mediated by bla_{TEM-1}, a narrow-spectrum β-lactamase gene that was demonstrated in one *S.* Infantis isolate recovered during 1998–2003 (Shahada et al., 2006) and 14.2% of the isolates obtained in 2004–2006 (Shahada et al., 2010a). Besides, during the period of 2004–2006, resistance to ESCs mediated by bla_{TEM-52} was reported for the first time from the broiler industry in Japan (Shahada et al., 2010a).

Table 2 | Distribution of resistance phenotypes, plasmid profiles and β-lactamase phenotypes in *Salmonella* Infantis isolates

Resistance pattern	Resistance phenotype	Plasmid			No. of isolates	β-lactamase phenotype
		profile	size (ca. kbp)	replicon type		
A	AMP/CTX/FOX/STR/SUL/TET/KAN	I	180	IncP	1	AmpC
B	AMP/CTX/FOX/STR/SUL/TET	I	180	IncP	7	AmpC
C	AMP/CTX/STR/SUL/TET/OFX	II	180, 50	IncP, Non-typable	4	ESBL
D	AMP/CTX/STR/SUL/TET/KAN	II	180,50	IncP, Non-typable	2	ESBL
E	AMP/CTX/STR/SUL/TET	I	180	IncP	2	ESBL
	AMP/CTX/STR/SUL/TET	II	180, 50	IncP, Non-typable	11	ESBL
	AMP/CTX/STR/SUL/TET	III	180, 125, 50	IncP, IncA/C, Non-typable	1	ESBL
F	AMP/CTX	II	180, 50	IncP, Non-typable	5	ESBL
G	AMP/STR/SUL/TET	I	180	IncP	1	-
H	STR/SUL/TET/OFX	I	180	IncP	7	-
I	STR/SUL/TET/KAN	I	180	IncP	7	-
J	STR/SUL/TET	I	180	IncP	43	-
K	KAN	I	180	IncP	2	_

AMP, ampicillin; CTX, cefotaxime; FOX, cefoxitin; CHL, chloramphenicol; STR, streptomycin; SUL, sulfamethoxazole; TET, oxytetracycline; KAN, kanamycin; OFX, ofloxacin.

Table 3 | Chronological change of β-lactamase genes in serovar Infantis

β-lactamase gene *(bla)*	No. of isolates during the isolation period		
	1998–2003[a] (178 isolates)	2004–2006[b] (120 isolates)	2007–2008[c] (93 isolates)
TEM-1	1 (0.7%)	17 (14.2%)	0 (0%)
TEM-52	0 (0%)	11 (9.2)	22 (24.4%)[d]
TEM-20	0 (0%)	0 (0%)	1 (1.8%)
CTX-M-25	0 (0%)	0 (0%)	1 (1.8%)
CMY-2	0 (0%)	0 (0%)	8 (8.6%)

[a] *Cited from the previous study (Shahada et al., 2006).*
[b] *Cited from the previous study (Shahada et al., 2010b).*
[c] *This study.*
[d] *CTX-M-25 and TEM-52 were simultaneously identified in one isolate.*

In the present study, we identified one *S.* Infantis isolate harboring the bla_{TEM-20} gene while other isolates had bla_{TEM-52}. It's worth noting that the wild-type bla_{TEM-1} gene was not detected during this study suggesting the likelihood of occurrence of point mutations which led to the emergence of observed variants, bla_{TEM-20} and bla_{TEM-52}. This hypothesis is supported by deduced amino acid sequence analysis which revealed that the bla_{TEM-20} gene differs from bla_{TEM-1} by two substitutions, Met182→Thr and Gly238→Ser; whereas the bla_{TEM-52} gene differs from bla_{TEM-1} by three substitutions, Glu104 Lys, Met182→Thr, and Gly238→Ser (Arlet et al., 1999; Weill et al., 2004). This phenomenon has the implications for bacterial adaptation mechanism because bla_{TEM-1} seems to have lost its fitness as a potential resistance trait necessary for survival of *Salmonella*. Thus, point mutations have taken place as the evolutionary adaptation mechanism crucial for successful colonization of the broiler chicken intestinal tract.

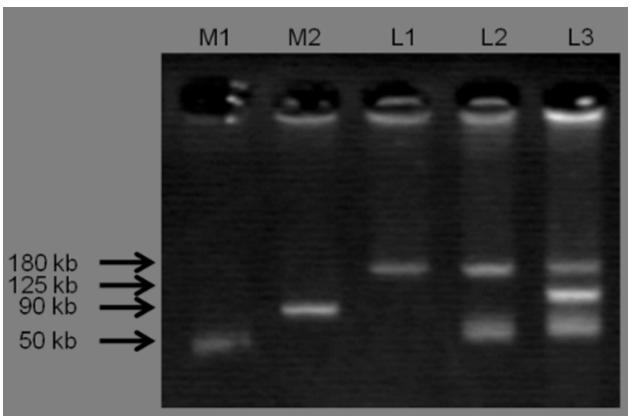

FIGURE 1 | Three plasmid profiles obtained from *S.* Infantis isolates. Lane M1 and M2 shows standard sizes of plasmids extracted from *S. Choleraesuis* ATCC 7001 (50 kbp) and *S.* Typhimurium DT104 strain 300-98 (90 kbp), respectively. L1: IncP (ca. 180 kbp), L2: IncP (ca. 180 kbp) and Untypable plasmid (ca. 50 kbp), L3: IncP (ca. 180 kbp), IncA/C (ca. 125 kbp), and Untypable plasmid (ca. 50 kbp).

In Japan, CTX-M-type ESBL-producing *Enterobacteriaceae* are important nosocomial infectious agents raising considerable concern in the public health community. Similarly, the detection of CTX-M-2 and CTX-M-25 ESBL-producing *E. coli* isolates from chickens affected with colibacillosis has raised concerns in the veterinary public health community (Kojima et al., 2005; Asai et al., 2011). We report for the first time one *S.* Infantis isolate harboring $bla_{CTX-M-25}$ on IncA/C plasmid and bla_{TEM-52} on non-typable plasmid. Because $bla_{CTX-M-25}$ was initially demonstrated in *E. coli* (Asai et al., 2011) a comprehensive molecular study is required to determine the likely source and elucidate mechanisms involved in collecting antimicrobial resistance traits and mobilizing them across taxonomical borders.

To date, several reports have described the emergence of AmpC β-lactamase-producing *Salmonella* derived from farm animals affected with salmonellosis. One of the studies involving *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) identified bla_{CMY-2} associated with self-transmissible IncI1-Iγ and A/C plasmids (Sugawara et al., 2011). Another study revealed a novel chromosomally integrated multi-drug resistance genomic island harboring bla_{CMY-2} among clonally related *S.* Typhimurium isolates (Shahada et al., 2011). Consequently, the detection of bla_{CMY-2} in several *S.* Infantis isolates from broilers poses another challenge in the veterinary public health community. Carriers of bla_{CMY-2} harbored IncP plasmids initially demonstrated in *Pseudomonas* bacteria (Shintani et al., 2011).

Antimicrobial susceptibility data indicate a gradual decrease of OFX resistance to 11.8 from 20.8% previously reported (Shahada et al., 2010b). Fluoroquinolones (e.g., OFX) are broad-spectrum antimicrobial agents widely used in clinical medicine. Emergence of fluoroquinolone resistance was attributed to overuse of this group of drugs in domestic animals either therapeutically or for the purpose of growth promotion (Asai et al., 2007a). Since 1991, the Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF) approved this class of antimicrobials in veterinary medicine for therapeutic purposes and prohibited its use as feed additives. Fluoroquinolones might have been prescribed prudently among antimicrobial agents used in broiler farms in Japan. This likely has contributed to the decline of resistance to OFX observed in the present study.

Taken together, these results show increased resistance against ESCs mediated by both ESBLs and AmpC β-lactamases. It seems these resistance traits have spread among farm animals in Japan while their likely source remains largely unknown. The probability that resistance to ESCs may continue to spread among members of the *Enterobacteriaceae* family poses another public health challenge because ESBLs and AmpC β-lactamases limit the effectiveness of cephalosporin therapy.

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Anaerobic expression of the *gadE-mdtEF* multidrug efflux operon is primarily regulated by the two-component system ArcBA through antagonizing the H-NS mediated repression

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The *gadE-mdtEF* operon encodes a central acid resistance regulator GadE and two multidrug efflux proteins MdtEF. Although transcriptional regulation of *gadE* in the context of acid resistance under the aerobic growth environment of *Escherichia coli* has been extensively studied, regulation of the operon under the physiologically relevant environment of anaerobic growth and its effect on the expression of the multidrug efflux proteins MdtEF in the operon has not been disclosed. Our previous study revealed that anaerobic induction of the operon was dependent on ArcA, the response regulator of the ArcBA two-component system, in the M9 glucose minimal medium. However, the detailed regulatory mechanism remains unknown. In this study, we showed that anaerobic activation of *mdtEF* was driven by the 798 bp unusually long *gadE* promoter. Deletion of *evgA*, *ydeO*, *rpoS*, and *gadX* which has been shown to activate the *gadE* expression during acid stresses under aerobic condition did not have a significant effect on the anaerobic activation of the operon. Rather, anaerobic activation of the operon was largely dependent on the global regulator ArcA and a GTPase MnmE. Under aerobic condition, transcription of *gadE* was repressed by the global DNA silencer H-NS in M9 minimal medium. Interestingly, under anaerobic condition, while Δ*arcA* almost completely abolished transcription of *gadE-mdtEF*, further deletion of *hns* in Δ*arcA* mutant restored the transcription of the full-length P*gadE-lacZ*, and P1- and P3-*lacZ* fusions, suggesting an antagonistic effect of ArcA on the H-NS mediated repression. Taken together, we conclude that the anaerobic activation of the *gadE-mdtEF* was primarily mediated by the two-component system ArcBA through antagonizing the H-NS mediated repression.

Keywords: multidrug efflux pump, acid resistance, anaerobic adaptation, H-NS, ArcBA two-component system, antagonization

INTRODUCTION

Drug efflux constitutes an important mechanism in bacterial drug resistance. Efflux activity is mediated by a class of membrane protein transporters called multidrug efflux pumps, which actively extrude a variety of cytotoxic substances including antibiotics out of bacterial cells (Li and Nikaido, 2009; Allen et al., 2010). Based on phylogenetic analysis and functional properties, drug efflux pumps of bacteria are classified into five families: the ATP-binding cassette (ABC), the major facilitator (MFS), the multidrug and toxic compound extrusion (MATE), the small multidrug resistance (SMR), and the resistance nodulation division (RND) family (Piddock, 2006). Among them, the tripartite RND pumps are particularly noteworthy in gram-negative bacteria owing to their capability of exporting a broad range of structurally diverse drugs directly to the outside of bacterial cells (Poole, 2008). The three components of RND efflux pumps include cytoplasmic membrane transporter component of RND family, outer membrane factor (OMF) component, and periplasmic component belonging to the membrane fusion protein (Murakami et al., 2006; Piddock, 2006). The constitutive activity of these efflux systems, such as the AcrAB-TolC pump in *Escherichia coli* and the MexAB-OprM and MexXY-OprM systems in *Pseudomonas aeruginosa*, renders the bacteria a low level, intrinsic resistance to a wide range of toxic substances (Poole, 2005).

Previous genome wide studies revealed that there are 20 efflux systems encoded in the *E. coli* K-12 genome (Nishino and Yamaguchi, 2001). However, except for the housekeeping AcrAB-TolC pump, the expression of other 19 pumps is largely inactive under ordinary laboratory growth condition, i.e., at 37°C in rich medium with aeration (Nishino and Yamaguchi, 2001). Increasing evidence suggests that bacterial stress response, i.e., bacterial growth in the adverse environment of their natural ecological niches and human host, induces the expression of specific efflux pumps (Piddock, 2006; Li and Nikaido, 2009; Poole, 2012a). Indeed, our previous studies revealed that MdtEF (previously known as YhiUV), an RND family pump, is activated during the anaerobic growth of *E. coli*, a signature environment of human gut where the bacterium primarily colonizes (Zhang

et al., 2011). The pump was shown to expel the cytotoxic indole nitrosative compounds from *E. coli* cells accumulated during the anaerobic respiration of nitrate and thus protect the bacterium from nitrosative stress under this physiological condition (Zhang et al., 2011). Activation of MdtEF under this condition was shown to be dependent on ArcA, the response regulator of the ArcBA two-component system, which is a global regulatory system dedicated to the anaerobic adaptation of *E. coli* (Green and Paget, 2004), but the detailed regulatory mechanism remains unknown.

MdtEF forms an RND type multidrug efflux system with the common outer membrane channel TolC in which MdtF forms the cytoplasmic membrane transporter and is connected to TolC by the periplasmic protein MdtE (Nishino and Yamaguchi, 2002). Over-expression of the pump from a multicopy plasmid has been shown to confer resistance to a broad range of antimicrobial agents such as some β-lactams (e.g., oxacillin, cloxacillin, and nafcillin), macrolide antibiotic erythromycin, as well as doxorubicin, crystal violet, ethidium bromide, rhodamine 6G, tetraphenylphosphonium bromide (TPP), benzalkonium, SDS, deoxycholate, suggesting that MdtEF is a multidrug resistance determinant (Nishino and Yamaguchi, 2001; Nishino et al., 2003; Lennen et al., 2013). Recently, MdtEF was also found to be involved in the efflux of physiological substance fatty acids (Lennen et al., 2013), and their expression can be induced by various of environmental and physiological signals, such as the entry of the stationary growth phase, N-acetyl-glucosomine, indole, as well as the combined environmental challenges of oxygen limitation and acid stress (Hirakawa et al., 2005, 2006; Hayes et al., 2006; Kobayashi et al., 2006), highlighting the physiological relevance of the MdtEF-TolC efflux pump.

MdtEF genes are located in the *gadE-mdtEF* operon where *mdtE* is 339 bp downstream of the *gadE* gene (Keseler et al., 2011). Interestingly, the *gadE* gene in this operon encodes a key regulator of the major acid resistance system in *E. coli* which is composed of the glutamate decarboxylase isoenzymes GadA and GadB, and a dedicated glutamic acid/γ-aminobutyrate (GABA) antiporter GadC (Hommais et al., 2004). During bacterial response to acid stresses, GadA and GadB catalyze the decarboxylation of glutamic acid, yielding GABA, which is subsequently exported by GadC in exchange for another molecule of glutamic acid (Foster, 2004). Since the reaction consumes a proton and releases CO_2, it effectively limits the intracellular acidification during acid stresses, thus plays an important role in the adaptation and survival of *E. coli* in certain host niches, such as the extremely low pH environment (pH = 2) of gastric acid (Foster, 2004). The expression of this system is primarily subject to the control of the acid resistance regulator GadE in the *gadE-mdtEF* operon (Masuda and Church, 2003).

Owing to its significant roles in acid resistance, transcriptional regulation from the *gadE* promoter has been extensively studied. It was demonstrated that the *gadE* promoter encompasses an unusually large 798 bp intergenic region between the *hdeD* and *gadE* gene (Ma et al., 2004), representing one of the eight similarly large intergenic regions in the entire *E. coli* genome (Tjaden et al., 2002). Transcription from at least four starting sites which are located at -21 (T), -124 (T1), -324/-317 (T2), and -566 (T3) relative to the *gadE* start codon have been identified (Ma et al., 2004; Itou et al., 2009; Sayed and Foster, 2009). More than ten transcription regulators including EvgA, YdeO, GadE, TorR, H-NS, PhoP, RpoS, CRP, MnmE, GadX, and GadW have been found to participate in the regulation of *gadE* during various circumstances of acid stresses through at least five different regulatory circuits (Ma et al., 2004; Sayed and Foster, 2009). Involvement of EvgA, YdeO, H-NS, CRP, GadX, as well as a small RNA DsrA in the regulation of the *mdtEF* gene in the same operon has also been reported (Nishino and Yamaguchi, 2002, 2004; Oshima et al., 2006; Nishino et al., 2008a,b, 2009, 2011). However, little is known about the regulation of the *gadE-mtEF* expression in the anaerobic physiological condition of *E. coli* which is the predominant environmental challenge encountered by the *Entericbacteria* family of bacteria when they are being transmitted from the status of free living to human intestinal tract, except for the involvement of the global transcription regulator ArcA as shown in our previous study (Zhang et al., 2011). Moreover, it remains unclear whether the various of regulatory circuits that activate the expression of *gadE* in the context of acid resistance also lead to the expression of the multidrug efflux genes *mdtEF* present in the same operon.

In the current study we set out to investigate the detailed regulatory circuits governing the anaerobic transcription from the *gadE* promoter in *E. coli* and examine its effect on the expression of the multidrug efflux proteins MdtEF present in the operon. We found that unlike the regulatory patterns occurred during aerobic acid stresses, anaerobic activation of the *gadE-mdtEF* operon is primarily mediated by ArcA through its antagonization of the H-NS mediated repression.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

The bacteria strains and plasmids used in this study are listed in **Table 1**. Gene deletion mutants were constructed either by P1 phage transduction from the Keio collection (Baba et al., 2006) or using the method described by Datsenko and Wanner (2000). All of the constructed strains were verified by colony PCR and DNA sequencing (BGI, Hong Kong). *E. coli* was cultured in Luria Bertani (LB) broth (USB) or M9 minimum medium supplemented with 0.2% glucose (USB), 0.2% casamino acids (CAA) (USB), 4 μg/ml thiamin (Sigma), 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml ferric ammonium citrate (Sigma), and 0.5 μM $(NH_4)_6Mo_7O_{24}$ (Sigma). Without specific indication, exponential phase cells were harvested when A_{600} of cultures reached around 0.3. Antibiotic concentrations used for bacterial culture or colony screening were 100 μg/ml ampicillin (USB), 20 μg/ml kanamycin (USB), or 25 μg/ml chloramphenicol (USB). All other chemicals without specification were purchased from USB.

CONSTRUCTION OF PROMOTER-*lacZ* TRANSCRIPTION REPORTER IN pNN387

Promoter-*lacZ* fusions were constructed based on the methods described previously (Zhang et al., 2011). Firstly, DNA fragments corresponding to the desired promoter regions were amplified using iProof High Fidelity DNA polymerase (Bio-Rad). The PCR condition and program is as follows: initial

Table 1 | Bacterial strains and plasmids used in this study.

Constructs	Genotype	Source
STRAIN		
MG1655	Wild type *Escherichia coli*	Mettert and Kiley, 2007
AY0416	MG1655 Δ*lacZ::kan*	Zhang et al., 2011
AY0433	MG1655 Δ*lacZ::kan* Δ*arcA::bla*	Zhang et al., 2011
AY0451	MG1655 Δ*arcA::cat mdtE-FLAG::kan*	Zhang et al., 2011
AY0452	MG1655 *mdtE-FLAG::kan*	Zhang et al., 2011
AY1543	MG1655 Δ*gadE* Δ*lacZ::Km*	This study
AY1603	MG1655 Δ*gadX::cat mdtE-FLAG::kan*	This study
AY1611	MG1655 Δ*rpoS mdtE-FLAG::kan*	This study
AY1612	MG1655 Δ*evgA mdtE-FLAG::kan*	This study
AY1613	MG1655 Δ*crp::cat, mdtE-FLAG::kan*	This study
AY2304	MG1655 Δ*mnmE mdtE-FLAG::kan*	This study
AY2305	MG1655 Δ*ydeO mdtE-FLAG::kan*	This study
AY2306	MG1655 Δ*ydeO* Δ*lacZ::kan*	This study
AY2311	MG1655 Δ*mnmE* Δ*lacZ::kan*	This study
AY2312	MG1655 Δ*evgA* Δ*lacZ::kan*	This study
AY2313	MG1655 Δ*rpoS* Δ*lacZ::kan*	This study
AY2400	MG1655 Δ*hns* Δ*lacZ::kan*	This study
AY2401	MG1655 Δ*crp* Δ*lacZ::kan*	This study
AY2420	MG1655 Δ*hns mdtE-FLAG::kan*	This study
AY2421	MG1655 P*icdA-lacZ* Δ*mnmE*	This study
AY2422	MG1655 P*lpdA-lacZ* Δ*mnmE*	This study
AY2426	MG1655 Δ*hns* Δ*arcA::bla* Δ*lacZ::kan*	This study
AY2442	MG1655 Δ*hns* Δ*arcA::cat* mdtE-FLAG::kan	This study
PK9460	MG1655 P*lpdA-lacZ*	Gift from Prof. Patricia J. Kiley (University of Wisconsin-Madison)
PK9463	MG1655 P*lpdA-lacZ* Δ*arcA*	Gift from Prof. Patricia J. Kiley
PK9483	MG1655 P*icdA-lacZ*	Gift from Prof. Patricia J. Kiley
PK9484	MG1655 P*icdA-lacZ* Δ*arcA*	Gift from Prof. Patricia J. Kiley
PLASMID		
pNN387	Single-copy vector, Cm^r, a NotI-HindIII cloning site upstream of promoterless *lacZ*	Kobayashi et al., 2006
pNN387mdtE	pNN387 (P*gadE-lacZ*)	Nishino and Yamaguchi, 2001
pAY1633	pNN387 (P*mdtE-lacZ*)	This study
pAY1655	pNN387 (P2/P4+P1+P-*lacZ*)	This study
pAY1656	pNN387 (P4+P1+P-*lacZ*)	This study
pAY1657	pNN387 (P1+P-*lacZ*)	This study
pAY1658	pNN387 (P-*lacZ*)	This study
pAY2401	pNN387 (P3-*lacZ*)	This study
pAY2402	pNN387 (P3+P2/P4-*lacZ*)	This study
pAY2403	pNN387 (P3+P2/P4+P1-*lacZ*)	This study
pAY2404	pNN387 (P2/P4-*lacZ*)	This study
pAY2405	pNN387 (P2/P4+P1-*lacZ*)	This study
pAY2406	pNN387 (P1-*lacZ*)	This study
pAY2409	pNN387 (P*mnmE-lacZ*)	This study

denaturation at 98°C for 30 s followed by 35 cycles of denaturation, annealing, and extension which are achieved by the program of: 98°C for 10 s, 60°C for 30 s, 72°C for 45 s, and the final extension at 72°C for 5 min. The pair of primers used in PCR includes protective bases (lowercase), restriction enzyme site (NotI or HidIII), followed by ∼20 bp homologous to the 5′- and 3′-end of the desirable promoter regions respectively (e.g., PgadE-F: 5′-aaggaaaaaaGCGGCCGCTTACCCCGGTTGTCACCCGGAT-3′; PgadE-R: 5′-cccAAGCTTAACTTGCTCCTTAGCCGTTATC-3′). Primers for construction of other *promoter-lacZ* fusions were designed similarly and the detailed sequences are available upon request. The PCR fragments were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE) followed by NotI/HindIII (NEB) digestion, and then were ligated into NotI/HindIII (NEB) digested single copy plasmid pNN387 using Quick Ligase (NEB). Colonies were screened by chloramphenicol resistance and then verified by colony PCR and DNA sequencing (BGI, Hong Kong).

CONSTRUCTION OF CHROMOSOMAL *mdtE-FLAG* BY P1 vir TRANSDUCTION

Construction of *chromosomal mdtE-FLAG* in various of deletion mutants was achieved by P1 phage transduction using the lysate from the strain AY0451 (Zhang et al., 2011). To prepare the lysate of the donor strain, AY0451 was grown in 2 ml LB medium till A_{600} achieved 0.1–0.2, and then 40 μl P1vir and 10 mM $CaCl_2$ was added to the culture. The mixture was shaken at 37°C for ∼3 h or till cells lysed. 2 drops of chloroform per ml of culture was then added to the lysate to diminish any chance of cell viability. After a vortex and centrifugation at 16,000 g for 2 min, the supernatant was transferred to a fresh tube and stored at 4°C in the presence of couple of drops of chloroform. Culture of the recipient strain was prepared as follows: the recipient strain was grown in LB medium till A_{600} exceeded 0.7. 10 mM $CaCl_2$ was then added followed by continuing growth for 15 min. 200 μl of the culture was then mixed with 100 μl lysate of the donor strain and incubated at 37°C for 20 min. Following the incubation, 100 μl of 1 M citrate was added to terminate the infection. Following the addition of 600 μl LB medium, the transductant was incubated at 37°C for 1 h before being spread onto LB agar plates containing kanamycin and 4 mM citrate. Desirable colonies were screened by colony PCR and were verified by DNA sequencing (BGI, Hong Kong).

β-GALACTOSIDASE ACTIVITY ASSAY

β-galactosidase activity assay was performed based on the method by Miller (1972) and detailed experimental procedure has been described previously (Zhang et al., 2011). Briefly, cells were grown anaerobically by inoculating a small number of cell cultures (initial cell density of 10^3 cells/ml) into screw-capped culture tubes filled with M9 medium, and subsequently cultured in 37°C water bath without aeration. When A_{600} of the cultures reached ∼0.3, tetracycline (10 μg/ml) was added to terminate protein synthesis and cell growth, and the cultures were placed on ice until assayed. The assays were performed in triplicate, and results

were presented as the mean in either Miller unit or the percentages relative to the activity of full length promoter-*lacZ* fusion in WT (wild type) strain. Error bars indicate the standard deviation.

TOTAL RNA EXTRACTION

8 ml anaerobically grown (the same condition as used in β-galactosidase activity assay) culture of *E. coli* MG1655 was mixed with 1.25 ml ice-cold ethnol/phenol stop solution (5% water-saturated phenol pH 4.5 in ethanol) and placed on ice for 10 min before being harvested by centrifugation at 4000 g for 9 min at 4°C. After removing supernatant, the cell pellet was frozen in liquid nitrogen and stored at −80°C to aid lysis. Cells were lysed by resuspending in 800 μl TE buffer (pH 8.0) containing 1.4 μl 36 kU μl^{-1} lysozyme (epicenter), and then placed in 64°C water bath for 2 min. After incubation, 88 μl 3M NaOAc (pH 5.2) was added to adjust the pH and ion strength of the lysate solution. Subsequently, acid-phenol/chloroform extraction followed by ethanol precipitation was performed to obtain the total RNA following the manufacturer's instruction. To remove trace amount of genomic DNA contamination, the extracted RNA was subject to DNase I treatment using the turbo DNA Free Kit (Amibion). Absence of genomic DNA contamination was confirmed by PCR suing the prepared RNA as template. The quantity of RNA was determined using NanoDrop 2000 (Thermo Scientific).

RNA LIGASE-MEDIATED RAPID AMPLIFICATION OF 5′ cDNA ENDS (5′ RLM-RACE)

1 μg of RNA which was treated with the turbo DNA Free Kit (Amibion) as above and shown as tight, no smearing band on the agrose gel (following the instructions of the FirstChoice RLM-RACE Kit (Ambion)) was treated with the Tobacco Acid Pyrophosphatase (TAP), ligated to the 5′ RACE adapter, and reverse transcripted with reagents supplied in the FirstChoice RLM-RACE Kit (Ambion) following the manufacturer's instruction. The obtained cDNA was subsequently utilized to perform outer (primer: PgadEout: 5′-TCCAGAAAT TTAATCGCTTCTTCATC-3′) and inner (primer: PgadEin: 5′-GTACTCGAGGTGATTATCTTTCAACTGCCAAAAGC-3′) PCR using Fastart Taq (Roche) DNA polymerase. The PCR products were gel band purified using PCR DNA and Gel Band Purification Kit (GE) and cloned into BamHI and XhoI sites of pPK7035 (Kang et al., 2005) followed by DNA sequencing. The first nucleotide being sequenced following the 5′ RACE adapter sequence was determined as the transcription start site.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOT

E. coli strains containing chromosomal FLAG tagged MdtE were grown anaerobically (the same condition as used in β-galactosidase activity assay) to log phase ($A_{600} = 0.3$) in M9 minimal medium supplemented with 0.2% glucose, 0.2% CAA, 4 μg/ml thiamin, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml ferric ammonium citrate, and 0.5 μM $(NH_4)_6Mo_7O_{24}$. Cells were harvested by centrifugation at 3800 g at 4°C for 10 min. Cell pellet was resuspended in lysis mix (BugBuster reagent (Merck) supplemented with 2.5 mg/mL lysozyme and 10 U/mL DNase I (Invitrogen)). Following the cell lysis, a small fraction of the lysis was subject to the *Dc* Protein Assay kit (BioRad) to measure the concentration of total proteins in the lysate. The volumes of the cell lysis loaded onto the SDS-PAGE were then determined based on the concentrations of each of the samples such that each of the lanes contains the same amount of total protein. The lysis was then heated at 55°C for 25 min prior to being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrically (Wet/Tank Blotting Systems, Bio-Rad; 110V) transferred to a nitrocellulose membrane (Bio-Rad) for 1.5 h. After blocking with 5% non-fat milk in 1% TBST (*T*ris *B*uffered *S*aline with *T*ween 20, 0.1 M Tris, 0.15 M NaCl, 0.1% Tween 20, and pH was adjusted with HCl to 7.6), the membrane was incubated with monoclonal anti-FLAG antibody (Sigma), and then secondary antibody (goat anti-mouse IgG HRP conjuate, Bio-Rad). After treatment of the membrane by the ECL Plus Western Blotting Detection Reagents (GE health), the protein bands were visualized by X-ray film development. Signals corresponding to MdtE-FLAG proteins were quantified using ImageJ (National Institutes of Health) and are presented as percentages relative to the level of MdtE-FLAG in the wild type strain.

EB STAINED ELECTROPHORESIS GEL MOBILITY SHIFT ASSAY (EMSA)

Purification and subsequent *in vitro* phosphorylation of His_6-ArcA protein was following the method described previously (Bekker et al., 2010). Briefly, *E. coli* strain BL21 transformed with pET-His_6ArcA were grown in LB to A_{600} of 0.4 before adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the overexpress of His_6-ArcA. After grown for additional 2.5 h, cells were harvested by centrifugation. Cell pellet was then resuspended in 4 ml of buffer A (0.5 MNaCl, 20 mMTris-HCl, pH 7.9) containing 1.3 mg/ml lysozyme, 30 g/ml DNase and RNase. Cells were then incubated at room temperature for 30 min followed by sonication to lyse the cells. Cell lysate was obtained by centrifugation at 15,000 g for 30 min and then was subjected to a 1.5-ml Ni-nitrilotriacetic acid–agarose column (Qiagen) equilibrated with buffer A. Following washing the column with 10 ml buffer A containing 10 mM imidazole, His_6-ArcA was eluted with buffer A containing 50 mM imidazole. Purified His_6-ArcA protein was phosphorylated by incubating the protein in TEGD buffer (50 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 10% glycerol) supplemented with 5 mM $MgCl_2$ and 50 mM (final concentration) carbamoyl phosphate. The mixture was incubated for 90 min at 25°C, and then the phosphorylated ArcA was used immediately for DNA-binding reaction. To initiate the DNA binding reaction, various concentration of DNA probes and phosphorylated proteins were mixed and subsequently incubated at 37°C for 20 min in EMSA binding buffer (pH 7.2, 20 mM Tris, 50 mM NaCl, 10 mM EDTA, 4 mM DTT, 5% glycerol, 0.5 mg/mL BSA). The reaction mixture was then subjected to 6% non-denaturing polyacrylamide gel electrophoresis in 0.5× TBE buffer. The polyacrylamide gel was visualized under UV light (254–366 nm) following the staining in 0.5× TBE buffer containing 0.5 μg/ml ethidium bromide (EB) for 10 min.

RESULTS

ANAEROBIC TRANSCRIPTION OF *mdtEF* IS INITIATED FROM THE *gadE* PROMOTER

MdtEF are located downstream of the *gadE* gene, which encodes a central regulator of the glutamic acid dependent acid resistance system in *E. coli* (Nishino and Yamaguchi, 2002). Previous RT-PCR analysis revealed that *mdtEF* were co-transcribed with *gadE*, thus were annotated in the operon of *gadE-mdtEF* (Hirakawa et al., 2006). However, an EMSA study by Nishino et al. showed that EvgA, one of the regulators that can activate the expression of *mdtEF*, could bind to the intergenic region between *gadE* and *mdtEF*, indicating that this region might serve as a potential promoter of *mdtEF* under certain conditions (Nishino and Yamaguchi, 2002). Thus, we first examined whether this intergenic region (namely P*mdtE*) is involved in the transcription of *mdtEF* under anaerobic condition. To address this, we constructed *lacZ* fusion of the intergenic region (denoted as P*mdtE-lacZ*) in the single copy plasmid pNN387 and performed β-galactosidase activity assay. It was shown that no transcription from P*mdtE-lacZ* was detected under either aerobic or anaerobic condition, while transcription from P*gadE-lacZ* constructed in the same vector was significantly induced under anaerobic condition (data not shown). These results suggested that anaerobic transcription of *mdtEF* was primarily initiated from the *gadE* promoter. Therefore we focused on the 798 bp P*gadE* region in the following investigation to examine its contribution to the expression of *mdtEF* under anaerobic condition.

TRANSCRIPTION FROM THE SAME FOUR STARTING SITES OF *gadE* WERE IDENTIFIED IN THE ANAEROBICALLY GROWN *E. coli*

In previous studies, three transcription starting sites, located at -124 (T1), -324/-317 (T2), and -566 (T3) relative to the *gadE* start codon respectively, have been identified in *E. coli* grown in LB glucose to early stationary phase ($A_{600} = 1.75$) (Sayed and Foster, 2009) and one transcription starting site located at the -21 position was detected from *E. coli* grown in the EG minimal buffer at pH 5.5 (Ma et al., 2004). To examine whether transcription from all these starting sites, and whether transcription from additional starting site, is active under anaerobic growth condition, we performed 5′ RLM-RACE using RNA isolated from *E. coli* grown anaerobically in M9 glucose medium which has been shown to significantly induce the expression of MdtEF in our previous study (Zhang et al., 2011). 5′-RACE revealed that transcription from the three starting sites identified previously was detected from several clones (data not shown). Transcription starting site of -21 was identified from one clone, but a weak -35 and -10 element was present upstream of this starting site. Two putative new transcription starting sites were also detected, however, since no recognizable -35 and -10 elements were present upstream of the sites, they are probably the digestive products from the established four transcripts. This result suggested that transcription from all four starting sites of *gadE-mdtEF* operon, -21 (T), -124 (T2/T4), -566 (T3), is active under anaerobic conditions. We next investigated the transcription regulatory mechanism of *gadE-mdtEF* from all these sites under anaerobic condition.

THE P3 REGION IS IMPORTANT IN THE ANAEROBIC ACTIVATION OF THE *gadE-mdtEF* OPERON

Following the designation of the P*gadE* region in previous studies (Sayed and Foster, 2009), the four transcription starting sites from 5′- to 3′-end of the 798 bp intergenic region between *hdeD* and *gadE* are designated as T3, T2, T4, T1, and T, respectively (**Figure 1A**) (Ma et al., 2004; Itou et al., 2009; Sayed and Foster, 2009), and the promoter regions immediately upstream of these transcription start sites are designated as P3, P2, P4, P1, and P accordingly. To examine which promoter regions are important for the anaerobic transcription of the *gadE-mdtEF* operon, we first constructed three P*gadE* truncations which lacked P3, P2/P4, P1 from 5′-end of the region successively, resulting in the promoter-*lacZ* fusion containing the intact P2/P4+P1+P, P1+P, and P regions, respectively (the upper four constructs in **Figure 1A**). Transcription activity assay showed that promoter-*lacZ* fusions lacking the P3 region, i.e., *lacZ* fusions of P2/P4+P1+P, and P1+P, displayed only ∼1/3 activity of that of the full length P*gadE-lacZ* fusion (**Figure 1B**), and that of P region showed almost no activity. These results suggested that the P3 region is important in anaerobic activation of the *gadE-mdtEF* operon and that transcription from the P promoter region is not active under this condition. This result also indicated that the -21 transcription starting site (driven by the P promoter) detected from the RACE analysis might be a digestive product from the transcripts initiated from the other three sites.

To dissect the contribution of each of these promoter regions, we performed another series of truncation on P*gadE*, resulting in *lacZ* fusions containing individual or combined promoters (the lower six constructs in **Figure 1A**). Transcription activities of these truncations showed that promoter-*lacZ* fusions containing the P3 region, i.e., P3, P3+P2/P4 and P3+P2/P4+P1, displayed similar or even higher activity than that of the full length, whereas promoter-*lacZ* fusions lacking the P3 region, i.e., P2/P4, P2/P4+P1, and P1, displayed very low activities (**Figure 1B**), confirming the important role of the P3 region in the up-regulation of *gadE-mdtEF* operon under anaerobic condition. This result indicated that a strong activator binding site may exist at the P3 region. Furthermore, transcription of *lacZ* fusion containing the P2/P4 promoter region was almost undetectable, suggesting that similar as the transcription starting site T which is driven by the individual P promoter region, transcription from the individual P2/P4 promoter is also inactive under anaerobic conditions. These results combined explained the observed very low activities of the promoter-*lacZ* fusions of P2/P4+P1, P1, P2/P4+P1+P and P1+P (**Figure 1B**).

INVOLVEMENT OF DIFFERENT REGULATORS IN THE ANAEROBIC EXPRESSION OF *mdtEF* FROM THAT OF AEROBIC CONDITION

Previous studies focusing on the expression of *gadE-mdtEF* operon during acid resistance have revealed that several regulators, such as EvgA, YdeO, MnmE, GadE, GadX, CRP, and H-NS, are involved in the regulation of *gadE-mdtEF* operon (Gong et al., 2004; Ma et al., 2004; Hirakawa et al., 2006; Tramonti et al., 2008). However, all those studies were carried out under aerobic condition and primarily focused on the transcription of the *gadE* gene. A previous study from our group focusing on the

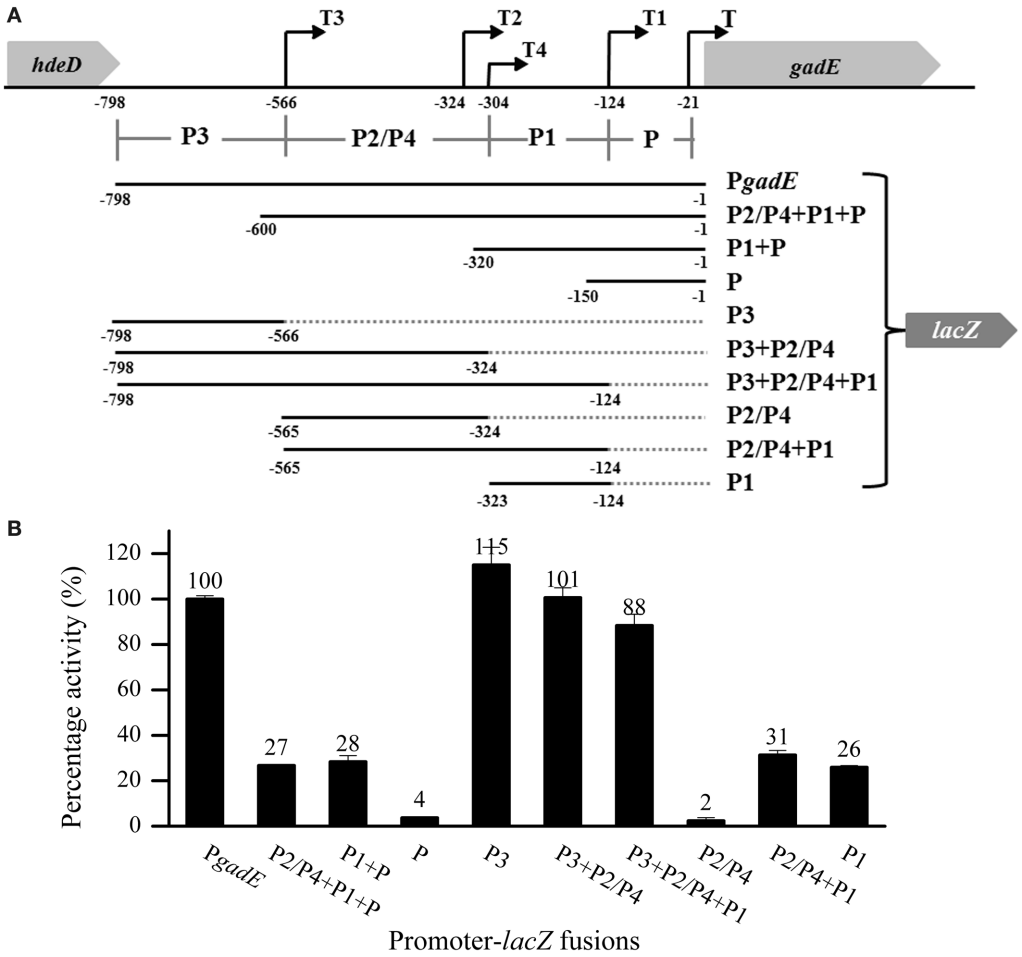

FIGURE 1 | The P3 region is important in the anaerobic up-regulation of *gadE-mdtEF*. (A) Schematic diagram of the constructed promoter-*lacZ* fusions. P*gadE* is defined as the 798 bp intergenic region between the *hdeD* and *gadE* genes. Labeled numbers indicate the nucleotides positions relative to the ATG start codon of *gadE*. Transcription starting sites are indicated as black arrows, and the regions upstream of each of the transcription starting sites are designated as its individual promoters, labeled as P3, P2/P4, P1, and P, respectively. **(B)** Promoter activity of all P*gadE* truncations determined by β-galactosidase activity of corresponding *lacZ* fusions. Cells were grown anaerobically to exponential phase ($A_{600} = 0.3$) in minimal M9 glucose medium. Results are presented as percentages relative to that of full length *gadE* promoter activity (100%), and error bars represent the standard errors of triplicate experiments ($n = 3$).

expression of the multidrug efflux genes *mdtEF* present in the same operon showed that under anaerobic condition the global regulator ArcA activates the expression of *mdtEF*, since Δ*arcA* caused significant decrease of both transcription and protein levels of *mdtE* and *mdtF* (Zhang et al., 2011). However, whether other regulators also participate in the regulation of *mdtEF* under this condition is unknown. Thus, in the present study, we first examined whether previously identified regulators, such as EvgA, YdeO, MnmE, GadE, GadX, and H-NS, also participate in the anaerobic activation of *mdtEF*. Since our previous finding on the anaerobic activation of *mdtEF* occurred in M9 glucose minimal medium in which the effect of CRP on gene expression is minimal, we focused on the effect of other regulators in the current study.

We constructed strains containing deletion of each of these genes and measured β-galactosidase activities of P*gadE-lacZ* in these strains. As shown in **Figure 2A**, deletion of *arcA* caused dramatic decrease of the β-galactosidase activity of P*gadE-lacZ*, confirming the role of ArcA in activating the *gadE-mdtEF* expression under anaerobic condition. Interestingly, except for *mnmE*, deletion of the regulators previously shown to participate in the regulation of *mdtEF* under aerobic condition, such as EvgA, YdeO, GadE, and GadX, caused no or only a moderate decrease (17–40%) of the transcription of P*gadE-lacZ*, and deletion of H-NS caused a moderate increase of *PgadE-lacZ* activity, suggesting that these regulators are not responsible for the activation of *mdtEF* under anaerobic condition. Interestingly, deletion of the GTPase MnmE caused a significant decrease of the transcription of *gadE-mdtEF*, suggesting that MnmE may also contribute to the activation of *gadE-mdtEF* under this condition. Since the current study focuses on the regulation of the multidrug efflux genes *mdtEF* present in the

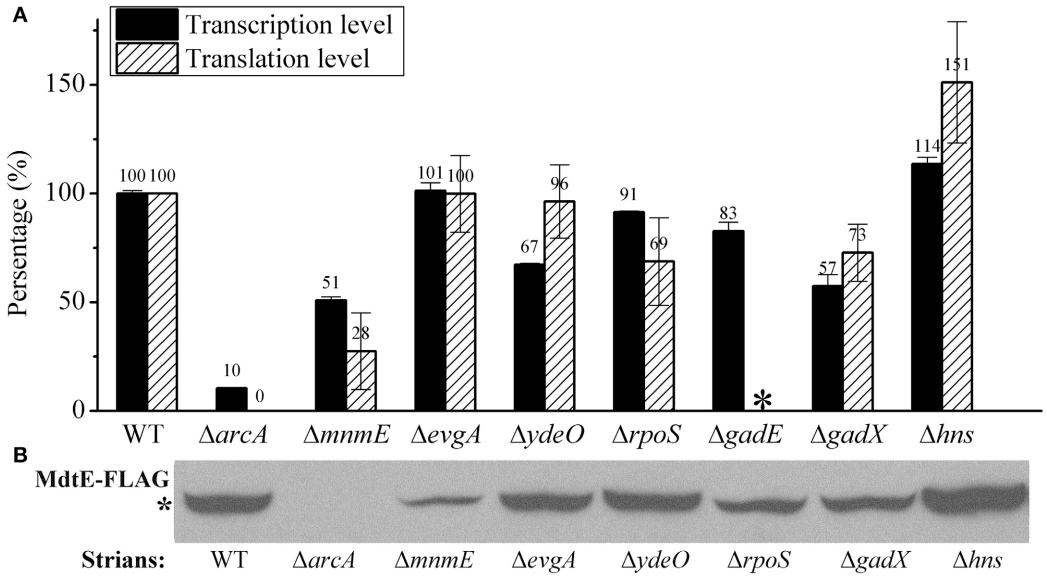

FIGURE 2 | *MdtEF* expression in various mutants at both transcription and translation level under anaerobic condition. (A) Transcription of *gadE-mdtEF* was determined by β-galactosidase activity of P*gadE-lacZ*, and translation of the gene is determined by Western blot to detect the production of MdtE-FLAG. **(B)** Production of chromosomal MdtE-FLAG in various mutants. Cells were grown anaerobically to exponential phase ($A_{600} = 0.3$) in minimal M9 glucose medium. All results were presented as percentages relative to the transcription of P*gadE-lacZ* or production of MdtE-FLAG in wild type strain. Error bars represent the standard errors of triplicate experiments ($n = 3$). **mdtE-flag* in Δ*gadE* mutant was not constructed because of the close proximity of *gadE* and *mdtEF* which is not applicable by the P1 phage transduction.

same operon, we next examined whether deletion of these genes caused alteration of MdtEF protein levels. We constructed chromosomal *mdtE-flag* in these strains (except for Δ*gadE*) and performed Western blot to examine the level of MdtE-FLAG. As shown in **Figure 2B**, production of MdtE-FLAG in the corresponding deletion mutants showed a similar pattern as that of β-galactosidase activity assay except Δ*mnmE*, which caused a more significant reduction of MdtE expression at translational level. Together these results confirmed that ArcA and MnmE are primarily responsible for the anaerobic activation of *mdtEF*.

As a control, we also measured the effect of deletion of these regulators on the aerobic expression of *gadE-mdtEF* in *E. coli* grown in M9 glucose medium at log phase. As shown in **Figure 3**, P*gadE-lacZ* activities in wild type strain, and in Δ*arcA*, Δ*mnmE*, Δ*evgA*, Δ*ydeO*, Δ*gadE*, Δ*gadX* mutants are very low (around 20 Miller units), consistent with the fact that ArcA is not active under this condition. However, in Δ*hns* mutants, the P*gadE-lacZ* activities increased more than 10-fold to about 250 Miller units, suggesting H-NS strongly represses the expression of *gadE-mdtEF* under aerobic condition in the M9 glucose minimal medium. This result is consistent with previous findings that H-NS represses the transcription from P*gadE* through its interaction with various sites in the *gadE* promoter region (Sayed and Foster, 2009). It is noted that deletion of *evgA*, *ydeO*, *gadE*, and *gadX* did not show obvious effect on the transcription of P*gadE-lacZ* in this assay. This is not unexpected as previous studies on the regulation of *gadE-mdtEF* by these regulators was found to be effective in the acidified minimal medium (pH 5.5) containing

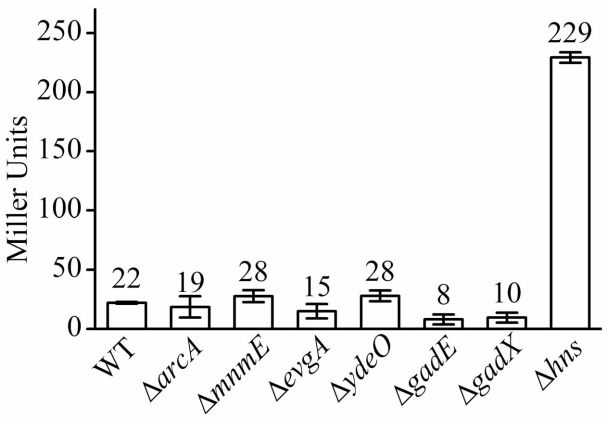

FIGURE 3 | β-galactosidase activity of P*gadE-lacZ* fusion in WT (MG1655 Δ*lacZ*), Δ*arcA*, Δ*mnmE*, Δ*evgA*, Δ*ydeO*, Δ*gadE*, Δ*gadX*, and Δ*hns* mutant strains under aerobic condition.

glucose or during stationary phase in rich medium (Ma et al., 2004), whereas the current study is carried out in the neutral M9 minimal medium with glucose, the identical culture medium as in our previous assay which showed increased expression of MdtEF under anaerobic condition. These results together suggested that the regulatory network of *mdtEF* expression under anaerobic condition is different from that under aerobic condition, and anaerobic activation of *mdtEF* is largely dependent on ArcA and MnmE.

DELETION OF EvgA, YdeO, H-NS DOES NOT HAVE OBVIOUS EFFECT ON THE TRANSCRIPTION FROM INDIVIDUAL PROMOTERS UNDER ANAEROBIC CONDITION

Our investigation above showed that ArcA and MnmE are the major activators responsible for the expression of *gadE-mdtEF* under anaerobic condition, whereas other regulators tested did not show obvious regulatory effects. However, since four promoter regions (P, P1, P2/P4, and P3) are involved in the transcription initiation of *gadE-mdtEF*, we cannot rule out the possibility that they might participate in the regulation of individual promoters contained in the complete 798 bp promoter of *gadE*. For example, it is possible that a certain regulator activates the transcription from one promoter region while represses transcription from another promoter, consequently cancels its effect on the transcription from the full promoter in our assay. To verify this, we examined the anaerobic transcription activity of those P*gadE* truncations in the deletion mutants constructed in this study except for GadX and GadW since they were indicated to be active in low pH, complex medium or stationary phase (Ma et al., 2003). It was shown that deletion of *evgA*, *ydeO*, and *hns* did not cause significant changes on the transcription activity from each of the individual promoters (data not shown), indicating that these regulators had no effect on the transcript from the individual promoters contained in the 798 bp full promoter of *gadE* either under anaerobic condition.

GadE AUTOREGULATES TRANSCRIPTION FROM P1 REGION UNDER ANAEROBIC CONDITION

Interestingly, deletion of *gadE* caused different effects on the transcription from different promoters contained in the full 798 bp P*gadE* region, which is dissimilar from that observed in the case of Δ*evgA*, Δ*ydeO*, and Δ*hns*. As shown in **Figure 4** (stripe bars), while *gadE* deletion had no significant effect on the transcription from the *lacZ* fusions of the full length, P3, P3+P2/P4, as well as P3+P2/P4+P1, deletion of *gadE* caused almost complete abolishment of transcription from the P2/P4+P1 and the P1 promoters. This observation combined with the results above which showed that individual P2/P4 was not active under anaerobic conditions suggested that GadE autoactivates the transcription of *gadE-mdtEF* from its P1 promoter. This result is consistent with a previous truncation study performed under aerobic condition, where deletion of *gadE* caused decrease of transcription from P2/P4+P1 and P1 promoters (Sayed and Foster, 2009), and the presence of a conserved GAD box to which GadE binds in the P1 region (Ma et al., 2004). Moreover, the fact that deletion of *acrA* and *mnmE* caused decrease of transcription from P1 at variable degrees whereas deletion of *gadE* almost completely abolished transcription from this promoter suggested that activation of P1 is dependent on the production of GadE which is transcribed from the ArcA and MnmE activated other promoters, such as P3, in the case of the full length, native promoter in the cell.

ArcA DIRECTLY BINDS TO MULTIPLE SITES AT THE *gadE* PROMOTER TO ACTIVATE THE EXPRESSION OF THE *gadE-mdtEF*

Since ArcA and MnmE were identified as transcription activators of the *gadE-mdtEF* operon, we next asked how these two regulators activate *gadE* expression, i.e., through which promoters these two regulators affect the transcription of the *gadE-mdtEF* operon under anaerobic conditions. To address this, we measured the transcription of promoter-*lacZ* fusions containing various lengths of the P*gadE* region in Δ*arcA* and Δ*mnmE* strains, respectively (**Figure 4**). Surprisingly, deletion of *arcA* (grid bars) or *mnmE* (gray bars) caused decrease of transcription from the full length and all the truncation mutants with variable promoter lengths, indicating ArcA and MnmE might affect transcription from multiple promoters, especially the P3 and P1 regions, since P2/P4 and P individual promoters were not active under this condition as shown above. Considering that the activation of the P1 promoter is dependent on GadE, it is possible that ArcA and MnmE primarily activate the P3 promoter.

To examine whether the ArcA dependent activation of P*gadE* is through its direct binding on the *gadE* promoter region, we

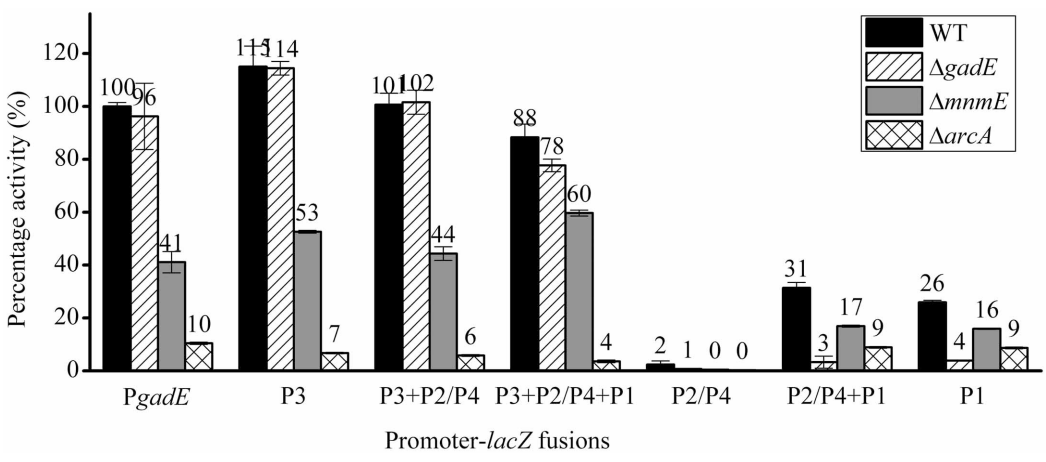

FIGURE 4 | β-galactosidase activity of P*gadE* truncations in WT (black bars), Δ*gadE* (stripe bars), Δ*mnmE* (gray bars) and Δ*arcA* (grid bars) strains under anaerobic growth condition.

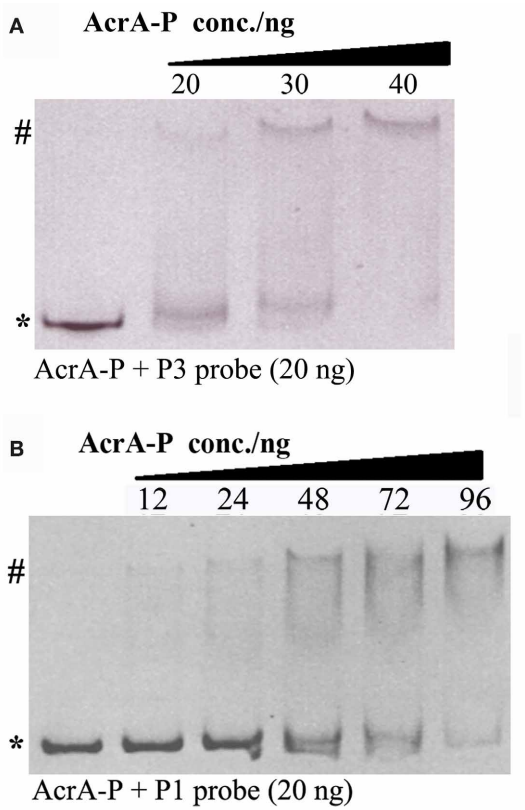

FIGURE 5 | Binding of ArcA-P to P1 and P3 promoters using EB stained EMSA assay. Various concentrations of AcrA-P were incubated with P3 **(A)** or P1 **(B)**. AcrA-P refers to phosphorylated His_6-ArcA protein. Asterisk indicates free DNA probe and pound indicates protein–DNA complex.

performed EMSA to measure the direct binding of ArcA-P (phosphorylated His_6-ArcA) to the P1 and P3 promoters. As shown in **Figure 5**, retarded bands corresponding to the DNA-protein complexes of ArcA-P with both P3 and P1 were observed, suggesting AcrA-P binds to multiple sites at the *gadE* promoter encompassed in the individual P1 and P3 promoters and activates the expression of *gadE* under anaerobic condition. Genome wide CHIP-chip (Chromatin immunoprecipitation with microarray) and CHIP-seq study which determines all the ArcA binding sites across the *E. coli* K-12 MG1655 genome under anaerobic conditions in glucose minimum media also identified two broad ArcA binding peaks in the *gadE* promoter. Furthermore, the presence of the ArcA peaks correlated with the presence of a strong σ^{70} peak comparing with the very low σ^{70} peak on this region under aerobic condition (Park and Kiley, et al., unpublished data, pers. communication), further confirming the ArcA dependent activation of the *gadE-mdtEF* operon through its direct binding of multiple sites on the *gadE* promoter.

ArcA ANTAGONIZES THE H-NS MEDIATED REPRESSION OF *gadE-mdtEF* DURING THE TRANSITION FROM AEROBIC TO ANAEROBIC GROWTH

Our results above showed that the expression of *gadE-mdtEF* is largely repressed by the DNA silencer H-NS under aerobic conditions in M9 glucose medium, whereas under anaerobic condition in the same medium its expression is activated primarily in an ArcA dependent manner. This led us to ask how the H-NS mediated repression is replaced by the ArcA dependent activation during the transition of the facultative bacterium *E. coli* from its aerobic to anaerobic growth. To answer this question, we first examined which individual promoter region is responsible for the H-NS mediated repression under aerobic condition. As shown in **Figure 6A**, in addition to the full length promoter P*gadE*, deletion of *hns* caused de-repression of transcription from the P1 and P3 promoters, but no effect on the transcription from the P2/P4 and P promoters, indicating that H-NS represses the transcription of P*gadE* from both the P1 and P3 promoters under aerobic condition in the M9 glucose medium. This pattern is similar to that of how ArcA achieves its activation of P*gadE* under anaerobic condition which is also through its interaction with the P3 and P1 promoters.

Two possibilities may explain the transition from H-NS mediated repression to ArcA mediated activation of *gadE-mdtEF* during the transition from aerobic to anaerobic growth. One possibility is that H-NS is inactive under anaerobic conditions and it disassembles from the *gadE* promoter, allowing ArcA which is active under this condition to bind the promoter of *gadE* and initiate gene transcription. Alternatively, under anaerobic conditions H-NS can still bind to the *gadE* promoter, however, its binding is antagonized by the activated ArcA, resulting in the recruitment of RNAP and transcript initiation of the operon under this condition. To differentiate these two possibilities, we constructed Δ*arcA* Δ*hns* double deletion and examined transcription of P*gadE-lacZ* in this strain. If H-NS is inactive under anaerobic condition and is disassembled from the *gadE* promoter, then transcription of P*gadE-lacZ* in Δ*arcA* Δ*hns* should be similar to that of Δ*arcA* alone. However, if H-NS was not inactivated, rather, it can still bind to the promoter but its binding was antagonized by ArcA, then its binding to the *gadE* promoter should be resumed when ArcA is absent and an increase of P*gadE-lacZ* activity should be observed in Δ*hns* Δ*arcA* than Δ*arcA* single deletion strain. As shown in **Figure 6B**, transcription of P*gadE-lacZ* in Δ*arcA* Δ*hns* double deletion did display significantly higher activity than that in Δ*arcA* strain, suggesting that the anaerobically up-regulated *gadE-mdtEF* expression was through the antagonization of H-NS mediated repression by ArcA. Western blot analysis of chromosomal MdtE-FLAG showed the same pattern as that of transcription assay (**Figure 6C**). More interestingly, the increased transcription of *lacZ* fusions in Δ*arcA* Δ*hns* double deletion strain than that in Δ*arcA* single deletion strain was also observed in the case of individual P3 and P1 promoters, suggesting that ArcA antagonization of H-NS takes place at multiple sites in the P*gadE* promoter, consistent with the notion that both H-NS and ArcA can oligomerize and bind to a broad region in its regulated gene promoters (Green and Paget, 2004; Dorman, 2006). CHIP-chip data of H-NS and ArcA are also consistent with this proposal, as characterized by a broad peak of H-NS binding to PgadE under aerobic conditions and the replacement of this broad peak by that of ArcA under anaerobic conditions (Park and Kiley et al., unpublished data, pers. communication).

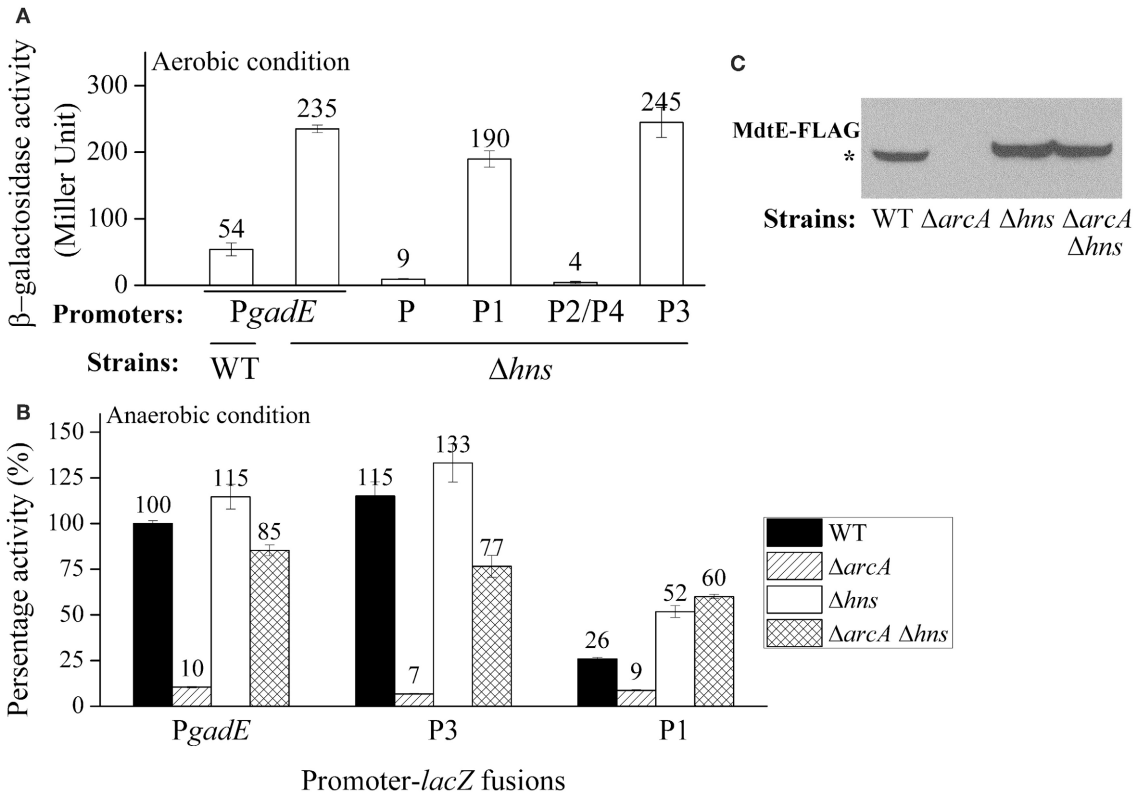

FIGURE 6 | ArcA antagonizes the H-NS mediated repression of *gadE* under anaerobic growth. (A) Promoter activity of P*gadE* and its truncations under aerobic condition determined by β-galactosidase activity of corresponding *lacZ* fusions in WT and Δ*hns* strains; **(B)** Promoter activity of P*gadE* and its truncations under anaerobic condition determined by β-galactosidase activity of corresponding *lacZ* fusions in WT, Δ*arcA*, Δ*hns*, and Δ*arcA* Δ*hns* strains; **(C)** Protein level of MdtE-FLAG in WT, Δ*arcA*, Δ*hns* and Δ*arcA* Δ*hns* strains determined by Western blot. Cells used for all assays were grown anaerobically in M9 minimal glucose medium to exponential phase ($A_{600} = 0.3$). Results of β-galactosidase activity assay are presented in Miller unit or percentage activity to that of wild type strain, error bars represent the standard errors of triplicate experiments ($n = 3$). * indicates the band of MdtE-FLAG.

THE EFFECT OF MnmE ON THE ANAEROBIC ACTIVATION OF *gadE-mdtEF* IS NOT THROUGH ArcA

As shown in **Figure 4**, Δ*mnmE* caused a very similar pattern on the transcription activities of the various P*gadE* truncations as that caused by Δ*arcA*, only to a less extent. This, combined with the fact that MnmE itself does not have DNA binding property whereas ArcA was shown to directly bind to P*gadE* and activate its expression, led us to speculate that the effect of Δ*mnmE* on *gadE-mdtEF* expression may be indirect, i.e., MnmE may affect the translation and/or activity of ArcA (such as through affecting its phosphorylation). To address this, we tested the effect of Δ*mnmE* on the transcription of two ArcA dependent promoters, P*lpdA* and the mutated P*icdA*. Transcription of *lpdA* gene has been shown to be mediated by the repression of ArcA under anaerobic conditions. In the case of *icdA*, it contains two known promoters, P_I and P_{II}, in which P_I is the primary promoter and is repressed only by ArcA. In the mutated P*icdA-lacZ*, transcription from P_{II} was eliminated by removal of its −10 element such that transcription repression of this P*icdA-lacZ* is exclusively ArcA dependent (Park and Kiley et al., unpublished work, pers. communication). If MnmE affects either the production or phosphorylation of ArcA (which affects the regulatory activity of ArcA), Δ*mnmE* should cause full or partial de-repression of these two promoters under anaerobic condition. As shown in **Figure 7**, as expected, transcription of P*icdA-lacZ* and P*lpdA-lacZ* fusions were significantly repressed by ArcA under anaerobic condition, since Δ*arcA* caused de-repression of their transcription under anaerobic condition in M9 glucose medium, the same condition used in this study. However, Δ*mnmE* had little or no effect on the transcription of these two promoters, suggesting that MnmE does not affect the production or phosphorylation of ArcA *in vivo*. Therefore, the observed MnmE mediated anaerobic activation of *gadE-mdtEF* was not due to its potential effects on ArcA.

DISCUSSION

DIFFERENT REGULATORY CIRCUITS ON THE EXPRESSION OF THE *gadE-mdtEF* OPERON UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS

Owing to the important roles of GadE and MdtEF in acid resistance and drug efflux respectively in *E. coli*, transcriptional regulation of the *gadE-mdtEF* operon under various of growth phases and conditions has been extensively investigated. While previous

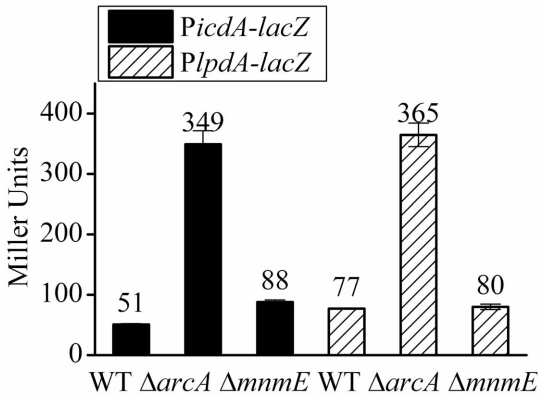

FIGURE 7 | MnmE has no effect on the transcription of P*icdA-lacZ* and P*lpdA-lacZ*, two ArcA dependent promoters. β-galactosidase activities of chromosomal P*icdA-lacZ* (black bars) and P*lpdA-lacZ* (stripe bars) fusions were measured from cells grown anaerobically to exponential phase ($A_{600} = 0.3$) in minimal M9 glucose medium. Results are presented in Miller unit and error bars represent the standard errors of triplicate experiments ($n = 3$).

studies largely focused on its relevance in bacterial acid resistance under aerobic growth environment, the current study aimed to dissect the up-regulation of the operon under the physiologically relevant environment of anaerobic growth and its effect on the production of the multidrug efflux protein MdtEF.

Several well-characterized regulatory circuits that activate *gadE* expression in the context of acid resistance have been disclosed. These include the EvgA-YdeO-GadE feed forward regulatory loop which is effective in minimal glucose medium under aerobic condition (Ma et al., 2004), the conditional activation of the operon in a MnmE dependent manner in LB containing glucose (LBG) (Gong et al., 2004), as well as the GadX or GadW dependent activation which functions primarily in rich media in stationary growth phase (Sayed et al., 2007). Here we show that the anaerobic activation of the operon, particularly the expression of the multidrug efflux protein MdtEF, is also dependent on the 798 bp *gadE* promoter and the activation is primarily dependent on the anaerobic global regulator ArcA, adding yet another regulatory circuit to the existing complex regulatory loops of the operon. These results reinforced the important role of the promoter region of *gadE* in integrating various of environmental and physiological signals to facilitate the adaptation of *E. coli*.

We also measured the transcription of *PgadE-lacZ* under anaerobic condition in stationary phase and examined whether the previously identified regulators, such as RpoS and GadE, participate in the regulation of the *gadE-mdtEF* expression under this growth phase anaerobically. Surprisingly, while a slightly higher transcription of *PgadE-lacZ* in stationary phase than in log phase (∼700 Miller units vs. ∼480 Miller units in log phase) was observed, deletion of *rpoS* or *gadE* did not have any effect on the transcription of *PgadE-lacZ* in this growth phase (data not shown), whereas Δ*arcA* still caused significant decrease of the *gadE-mdtEF* expression in the same growth phase. These results suggested that anaerobic expression of the *gadE-mdtEF* operon is primarily regulated by ArcA in both log phase and stationary phase under anaerobic condition. It is noted that previously a putative ArcA binding site located at the P2 region was proposed (Zhang et al., 2011), whereas in the current study, the P3 and P1 region of the *gadE* promoter was shown to be bound by ArcA and they play an important role in the anaerobic activation of the operon. This inconsistency is likely due to the inaccuracy of the bioinformatic search which utilized a sequence motif of "(5′ [A/T]GTTAATTA[A/T] 3′)" as the template and the motif was proposed as the ArcA binding site by Lynch and Lin (1996) following the DNA foot-printing analysis of a limited number of ArcA regulated promoters. Indeed, a genome-wide search of all the ArcA regulated promoters and subsequent characterization of them suggest a motif (Kiley et al., unpublished work, pers. communication) that deviates from the previously proposed ArcA binding site.

Interestingly, we demonstrated that the ArcA dependent activation of the operon is through its antagonistic effect on the H-NS mediated repression of the operon. This regulatory model explains the dynamic process taking place during the transition of *E. coli* from its aerobic to anaerobic growth, which mimics the transition of the bacterium from free living status to the anaerobic environment of the human host. H-NS mediated repression of the operon has also been implicated in previous studies (Ma et al., 2004; Sayed and Foster, 2009). However, the inter-relationship of this repression and gene activation mediated by other regulators has not been revealed. Our findings demonstrating that ArcA activates the expression of the operon through antagonizing the H-NS mediated repression probably provide a common model that underlies the dynamic and coordinated regulation of *gadE-mdtEF* in response to different environmental signals. Perhaps under unstressed conditions when the expression of the operon is unnecessary, the promoter of *gadE* is primarily occupied by H-NS and transcription from the various promoters in this region is repressed. Upon the activation of different transcription activators in response to different environmental or physiological conditions that presage acid stresses or efflux of certain metabolic by-products, the activators then displace H-NS binding on the promoter, resulting in the activation of the operon. Several lines of evidence support this proposal: first, bioinformatics have suggested the presence of an efficient DNA helix locking mechanism by H-NS in the promoter of *gadE* (Hommais et al., 2004); Second, H-NS mediated repression has been found to be common among acquired gene clusters which is consistent with the functions of these gene clusters in conferring the survival and adaptation of bacteria in various adverse growth environments (Oshima et al., 2006). The acid resistant island including the *gadE-mdtEF* operon indeed is one of the acquired gene clusters in *E. coli* with different GC content from the rest of *E. coli* genome; Third, the interplay between the H-NS mediated repression and ArcA mediated antagonizing of the repression is also observed in the case of another gene *cydAB* which is also involved in adapting *E. coli* to the microaerobic and anaerobic growth (Govantes et al., 2000). Thus, anaerobic activation of *gadE-mdtEF* through antagonizing the H-NS mediated repression by ArcA provides a regulatory model that is relevant to the dynamic lifestyle of the facultative bacterium *E. coli*. A model to explain this dynamic regulation is presented in **Figure 8**.

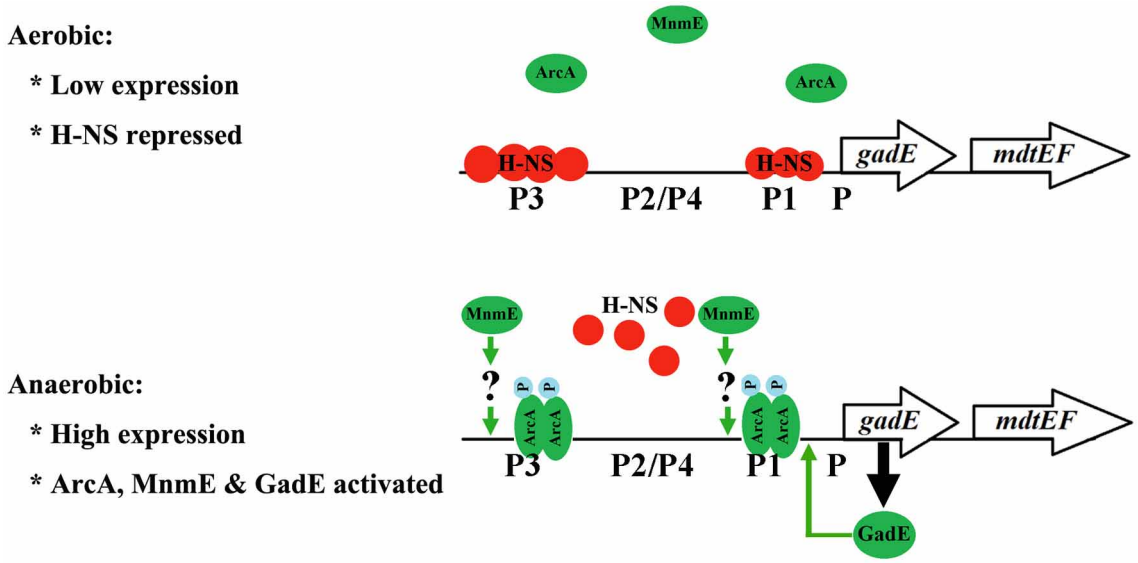

FIGURE 8 | A regulatory model for the transcriptional regulation of *gadE-mdtEF* operon during the transition from aerobic to anaerobic growth in minimal M9 glucose medium. During aerobic growth, the *gadE-mdtEF* is expressed at a very low level due to the strong repression by H-NS. Upon transition to anaerobiosis, the response regulator ArcA is phosphorylated and activated by its sensor kinase ArcB. Phosphorylated ArcA (ArcA-P) antagonizes H-NS binding to *gadE* promoter, resulting in the relief of repression. ArcA-P also directly binds to the *gadE* promoter and activates the expression of *gadE-mdtEF*. ArcA-P activated *gadE* expression produces GadE, which activates the transcription from the P1 promoter and further up-regulate the *gadE-mdtEF* expression. MnmE also participates in the anaerobic activation of *gadE-mdtEF*, but in a yet uncharacterized manner.

ROLES OF THE *gadE-mdtEF* OPERON IN BOTH ACID RESISTANCE AND DRUG EFFLUX

It is interesting that the drug efflux genes *mdtEF* exist in the same operon with the central acid resistance regulator *gadE*, and their expression is driven by the same unusually long 798 bp promoter region upstream of the *gadE* gene. While functions of GadE in controlling the expression of the major acid resistant system GadA and GadBC is well established, the relevance of MdtEF to acid resistance has not been fully recognized. Our previous studies showed that MdtEF functions to expel indole nitrosative by-products accumulated during nitrate respiration under anaerobic condition (Zhang et al., 2011). It is known that anaerobic metabolism often causes pH reduction in growth medium, especially during glucose fermentation by *E. coli* which leads to the production of various organic acids. Although anaerobic induction of *gadE-mdtEF* occurs at a fairly neutral pH (6.9 at the log phase culture when the cells were harvested for transcription and Western blot analyses), significant accumulation of the indole nitrosative by-product occurred when pH dropped to 5.8 or below (Weiss, 2006). Thus, it seems that MdtEF does contribute to the detoxification of acid induced metabolic by-products. Yet, its activation does not necessarily depend on the occurrence of acid stresses. This is consistent with the findings from previous studies which propose that the *gadE-mdtEF* operon is rigged to be induced under many different circumstances that could presage an encounter with acid stresses (Hommais et al., 2004).

This raises the interesting question of whether the housekeeping pump AcrAB is also involved in the nitrosative and/or acid resistance by expelling metabolic by-products under anaerobic conditions. Although the broad substrate specificity of AcrAB-TolC was established in terms of its capability of exporting antibiotics, we speculate that the same degree of broad substrate specificity may not apply in terms of its capability of exporting physiological substances, since it has been well recognized that expression of certain efflux pump genes other than the housekeeping AcrAB pump is activated by specific environmental or physiological conditions (Piddock, 2006; Li and Nikaido, 2009; Poole, 2012b). We recently performed metabolic profiling of the WT vs. Δ*mdtEF* under the growth condition of anaerobic nitrate respiration and it was shown that Δ*mdtEF* cells accumulated more indole nitrosative by-products than the WT (data not shown) in the presence of the *acrAB* genes, suggesting that the MdtEF pump exhibits at least certain degree of substrate specificity even in the presence of the housekeeping pump AcrAB. It is highly likely that MdtEF also expels other metabolic by-products accumulated during acid stresses. If that is the case, there are probably more extensive relevance between MdtEF mediated drug efflux and the physiology of *E. coli* during acid resistance. This relevance probably can be disclosed through metabolic profiling by comparing the total metabolites in Δ*mdtEF* and WT strain grown during acid stresses.

THE UNRESOLVED ROLE OF MnmE IN THE UP-REGULATION OF *mdtEF* EXPRESSION

MnmE is a GTP binding protein that is involved in tRNA modification (Cabedo et al., 1999). This protein was found to be in cytoplasm but can partially associate with the inner membrane (Cabedo et al., 1999). Its involvement in the conditional

activation of *gadE* at exponential growth in LB medium with glucose (LBG) or stationary growth of *E. coli* in unbuffered LBG under aerobic condition has been reported (Gong et al., 2004). However, the detailed regulatory mechanism remains elusive. Since MnmE itself does not have DNA binding property, it was speculated that MnmE affects the *gadE* expression by controlling an unknown regulator which can bind to *gadE* promoter and mediate its transcription. Since Δ*mnmE* resulted in a similar pattern of decreased transcription from both full length and various truncation promoter fusions as in Δ*arcA* strain except to a less extent, and ArcA was shown to directly bind to P*gadE*, we had speculated that MnmE regulation of the *gadE* expression was through its effect on ArcA, i.e., through its mediation of the translation and/or phosphorylation of ArcA. However, our β-galactosidase activity results showing that Δ*mnmE* does not have any effect on two different ArcA dependent promoters (P*icdA* and P*lpdA*) suggested that MnmE does not affect the production and/or activity of ArcA under anaerobic conditions, and thus the observed reduction of *gadE-mdtEF* expression in Δ*mnmE* strain was not due to its potential effect on ArcA.

Studies also indicated that MnmE dependent conditional expression of *gadE* mainly depends on the P2 promoter region (Sayed and Foster, 2009). However, our study showed that the P2 region is not active under anaerobic condition at log growth phase; instead, the MnmE dependent *gadE* activation was likely through its effect on the P3 region, which is indispensable in the anaerobic activation of *gadE*. Furthermore, we found that MnmE dependent activation of the *gadE-mdtEF* expression also occurred in non-glucose media, such as LB and glycerol supplemented with nitrate (data not shown). This observation is not in agreement with a previous finding which suggested that MnmE dependent *gadE* expression was induced only in the presence of glucose. Thus, although several studies support the notion that the GTPase MnmE participates in the transcriptional activation of the *gadE-mdtEF* operon under certain growth conditions and phases, how exactly MnmE is involved in the process is still ambiguous. This is largely due to the fact that the exact biological function of MnmE itself is still elusive. Obviously, further studies of MnmE protein is needed before we can reveal its role in the up-regulation of *gadE-mdtEF* under the physiologically relevant conditions.

It is noteworthy that the 798 bp sensory integration region of the *gadE-mdtEF* promoter is highly conserved among the pathogenic *E. coli* strains, including pathogenic strains O157:H7, 2369, RS128 and CFT073 and Shigella (96–99% identity), and GadE has been demonstrated to be indispensable for the survival of *E. coli* O157::H7 in a simulated gastric environment (Kailasan Vanaja et al., 2009), underlying the importance of the *gadE-mdtEF* operon to the physiology of *E. coli*. Since both low pH, such as that of gastric acid, and oxygen limitation (anaerobic environment) activates the expression of the *gadE-mdtEF* operon, it is conceivable that synergetic activation of the system by both regulatory circuits may occur during the transition of *E. coli* to human host, particularly the passage of the gastrointestinal tract, thus greatly facilitate the adaptation and survival of this neutrophilic bacterium in its host niches.

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Reclaimed water as a reservoir of antibiotic resistance genes: distribution system and irrigation implications

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Treated wastewater is increasingly being reused to achieve sustainable water management in arid regions. The objective of this study was to quantify the distribution of antibiotic resistance genes (ARGs) in recycled water, particularly after it has passed through the distribution system, and to consider point-of-use implications for soil irrigation. Three separate reclaimed wastewater distribution systems in the western U.S. were examined. Quantitative polymerase chain reaction (qPCR) was used to quantify ARGs corresponding to resistance to sulfonamides (*sul*1, *sul*2), macrolides (*erm*F), tetracycline [*tet*(A), *tet*(O)], glycopeptides (*van*A), and methicillin (*mec*A), in addition to genes present in waterborne pathogens *Legionella pneumophila* (L*mip*), *Escherichia coli* (*gad*AB), and *Pseudomonas aeruginosa* (*ecf*x, *gyr*B). In a parallel lab study, the effect of irrigating an agricultural soil with secondary, chlorinated, or dechlorinated wastewater effluent was examined in batch microcosms. A broader range of ARGs were detected after the reclaimed water passed through the distribution systems, highlighting the importance of considering bacterial re-growth and the overall water quality at the point of use (POU). Screening for pathogens with qPCR indicated presence of L*mip* and *gad*AB genes, but not *ecf*x or *gyr*B. In the lab study, chlorination was observed to reduce 16S rRNA and *sul*2 gene copies in the wastewater effluent, while dechlorination had no apparent effect. ARGs levels did not change with time in soil slurries incubated after a single irrigation event with any of the effluents. However, when irrigated repeatedly with secondary wastewater effluent (not chlorinated or dechlorinated), elevated levels of *sul*1 and *sul*2 were observed. This study suggests that reclaimed water may be an important reservoir of ARGs, especially at the POU, and that attention should be directed toward the fate of ARGs in irrigation water and the implications for human health.

Keywords: antibiotic resistance genes, water reuse, reclaimed water distribution systems, irrigation

INTRODUCTION

Water reuse is an increasingly common sustainable water management practice motivated by climate change, urbanization, energy efficiency, and environmental protection (US Environmental Protection Agency [USEPA], 2012). Reclaimed or recycled wastewater is treated by municipalities for a variety of purposes, including non-potable urban reuse (Grant et al., 2012; USEPA, 2012). In the United States Environmental Protection Agency (USEPA) guidelines on water reuse, the presence of antibiotics as trace organic contaminants in wastewater is noted and a need for more information is acknowledged to reduce the proliferation of antibiotic resistance and protect public health (USEPA, 2012).

Antibiotic resistance proliferation is currently outpacing the development of novel antibiotics, calling for effective strategies to mitigate the spread of antibiotic resistance (Carlet et al., 2012). Bacterial resistance to antibiotics is partially conferred through antibiotic resistance genes (ARGs), which code for specific antimicrobial functions such as efflux pumps (Webber and Piddock, 2003). ARG contamination has been quantified in a variety of environmentally relevant matrices, including wastewater treatment plant (WWTP) effluent, which is known to contribute to ARG loadings in surface waters (Pruden et al., 2013; Storteboom et al., 2010; LaPara et al., 2011). Some states require chlorine or UV disinfection for reused water (USEPA, 2012) and certain disinfectants (free chlorine, O_3, and UV) are capable of reacting with nucleic acids during treatment and therefore may potentially reduce ARGs, as recently reviewed by Dodd (2012). However, McKinney and Pruden (2012) recently demonstrated in a controlled lab study that typical UV doses applied at WWTPs are capable of reducing antibiotic resistant strains of bacteria, but not ARGs. Others have noted little reduction in ARGs following UV effluent treatment in full-scale WWTPs (Auerbach et al., 2007; Kim et al., 2010).

While WWTPs are now well-established as a reservoir of ARGs (Auerbach et al., 2007; Kim et al., 2010; Czekalski et al., 2012), and some have considered effect of irrigation with reclaimed water (McLain and Williams, 2012; Negreanu et al., 2012), there is a void of studies focused on the potential for re-growth in treated wastewater distribution systems ("purple" pipes). In one study examining soil irrigated with treated wastewater, no differences in the microbiome or ARG levels were observed compared to soil irrigated with fresh water (Negreanu et al., 2012). In fact, in Llobregat (NE Spain), reclaimed water emitted to a river

had lower concentrations of indicator organisms than the stream water (Rubiano et al., 2012). In contrast, re-growth of indicator organisms has been observed between the point of entry (POE) and point of use (POU) in reclaimed water systems (Ryu et al., 2005), raising the question of whether ARGs can also increase during distribution. In a study examining drinking water distribution systems, antibiotic resistant bacteria were shown to decrease between POE and POU, but ARGs were observed to increase (Xi et al., 2009).

In this study, ARG occurrence patterns were evaluated in the POU water in three arid western U.S. recycled water distribution systems using quantitative polymerase chain reaction (qPCR). Depending on access, POE, POU biofilm, and soil irrigated with recycled water were also examined. Samples were also screened by qPCR for the potential presence of known waterborne pathogenic bacteria and indicators, *Legionella pneumophila*, *Escherichia coli*, and *Pseudomonas aeruginosa*. To simulate the effect of reused water for irrigation, a series of batch laboratory soil microcosm studies were performed to compare irrigation with secondary, chlorinated, and dechlorinated effluent from a representative conventional WWTP.

MATERIALS AND METHODS

WATER REUSE SYSTEMS

Samples were collected from three non-potable reclaimed wastewater distribution systems in the western U.S., together served by four WWTPs (**Table 1**). Water samples (-POE or -POU) were collected in sterile centrifuge tubes. Biofilm (-F) was collected with a sterile swab, and packed in a sterile centrifuge tube. A soil sample was collected from a field irrigated with reclaimed wastewater (-S), in a sterile centrifuge tube. All samples were shipped overnight on ice and stored frozen until extraction. Water samples were freeze-dried prior to DNA extraction (FreeZone Plus, Labconco, Kansas City, MO, USA).

BATCH MICROCOSMS

Aerobic, batch microcosms were prepared to investigate two water reuse scenarios on historically manured soil: (1) a single irrigation event ("batch irrigation") and (2) repeated irrigation events ("periodic irrigation"). Soil was collected (upper 7.5–10 cm) in winter from historically manured corn fields near Virginia Tech campus using a soil probe. Soil was air dried and sieved (2 mm), and an aliquot was preserved for DNA extraction. For each study, microcosms were prepared in 250 mL flasks in triplicate with 50 g of soil and incubated at room temperature on a shaking table to maintain aerobic conditions. Slurry samples were collected weekly without sacrifice. Secondary, chlorinated, and dechlorinated WWTP effluents were collected before each irrigation treatment event from a representative 4.5 Mgd Domestic WWTP.

Batch irrigation soil was initially treated with 80 mL of freshly collected WWTP effluent fractions. Slurry samples (~0.4 g wet) were collected and an equal volume of deionized (DI) water was added to each flask to maintain soil moisture. WWTP effluent fractions (2–4 L) were filtered through 0.22 μm membrane and total DNA was extracted from the filter, as described below. The periodic irrigation soil was initially treated with 100 mL of freshly collected WWTP effluent fractions. Slurry samples (10 mL) were collected, centrifuged at 3,300 × *g* for 5 min, and 0.4 g of the pellet was used for DNA extraction. Fresh WWTP effluent fractions were added to the flasks to replace the volume removed during each sampling. WWTP effluent fractions (60 mL) were freeze-dried, as above, and DNA was extracted from the residuals.

Table 1 | Summary of reclaimed WWTP tertiary treatment characteristics.

	Aa[1]	Ab[1]	B	C
Capacity (Mgd)	6	4	1.1	0.6
Filtration	Media	Dual media	Carbon filter	Sand + activated carbon
Disinfection	Chlorine	UV	Chlorine	Chlorine

All WWTPs studied use conventional primary and secondary treatment processes.

[1]*Aa and Ab are separate WWTPs that emit water to a commingled distribution system.*

MOLECULAR TECHNIQUES

DNA was extracted from freeze-dried water/slurry, 0.4 g soil, or swabs using a FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) and diluted 1:50 or 1:100 for the water reuse field study and 1:30 for the irrigation studies prior to downstream analysis. qPCR was performed to quantify 16S rRNA (Suzuki et al., 2000), *sul*1 (Aminov et al., 2001), *sul*2 (Aminov et al., 2001), *tet*(A) (Aminov et al., 2002), *tet*(O) (Aminov et al., 2001), *erm*F (Chen et al., 2007), *van*A (Dutka-Malen et al., 1995), *mec*A (McKinney and Pruden, 2012), *L. pneumophila*-specific *mip* (Nazarian et al., 2008), *E. coli*-specific *gad*AB (Chen et al., 2006), and *P. aeruginosa*-specific *ecf*X/*gyr*B (Anuj et al., 2009) genes for the water reuse field study. Reaction matrix and PCR protocols were as previously described (Ma et al., 2011; Wang et al., 2012). For the irrigation study, 16S rRNA, *sul*1, *sul*2, *tet*(O), and *tet*(W) genes were monitored. All standard curves of qPCR were constructed from serial dilutions of cloned genes ranging from 10^8 to 10^2 gene copies/μL. Samples were analyzed in triplicate with a standard curve and negative control included in each run. Limits of quantification with respect to sample volume varied depending on the volume processed and the dilution of DNA extract, ranging from −1.4 to 0.6 $\log_{10}$ gene copies/mL, 3.1–5.1 $\log_{10}$ gene copies per swab, and 0.5–2.5 $\log_{10}$ gene copies/g of soil. Additionally, cloning and sequencing of qPCR product was performed for assays that had not been validated previously (*van*A) to demonstrate specificity of PCR product (GenBank accession number KC792557–KC792573).

STATISTICS

Cluster analysis was performed on transformed (square root) 16S rRNA gene normalized ARG profiles from the reclaimed wastewater systems and significance testing was carried out using the SimProf test in PrimerE (Plymouth, UK). To compare between wastewater treatment effluent fractions and treatments in the irrigation study, data was Box–Cox transformed. Transformed data were compared using ANOVA, and significant differences

($p < 0.05$) were determined using Tukey's honest significance test, as implemented in R (http://www.r-project.org/). Multiple comparisons for the distribution system and irrigation studies were performed on Box–Cox transformed data using least square means comparison with a Satterthwaite estimation of degrees of freedom in SAS, again using Tukey adjustment for multiple comparisons. Linear modeling for the irrigation study data was performed in Microsoft Excel.

RESULTS

ARG OCCURRENCE PATTERNS IN RECYCLED WATER

Antibiotic resistance genes were detected in all water reuse samples (**Figures 1** and **2**). The most frequently detected ARGs were *van*A, *erm*F, and *sul*2 with frequencies of detection of 100, 73, and 65% ($n = 23$), respectively. *tet*(O) had the lowest frequency of detection (17%, $n = 23$) other than *mec*A, which was not detected in any of the samples. For System A, significant differences in ARG

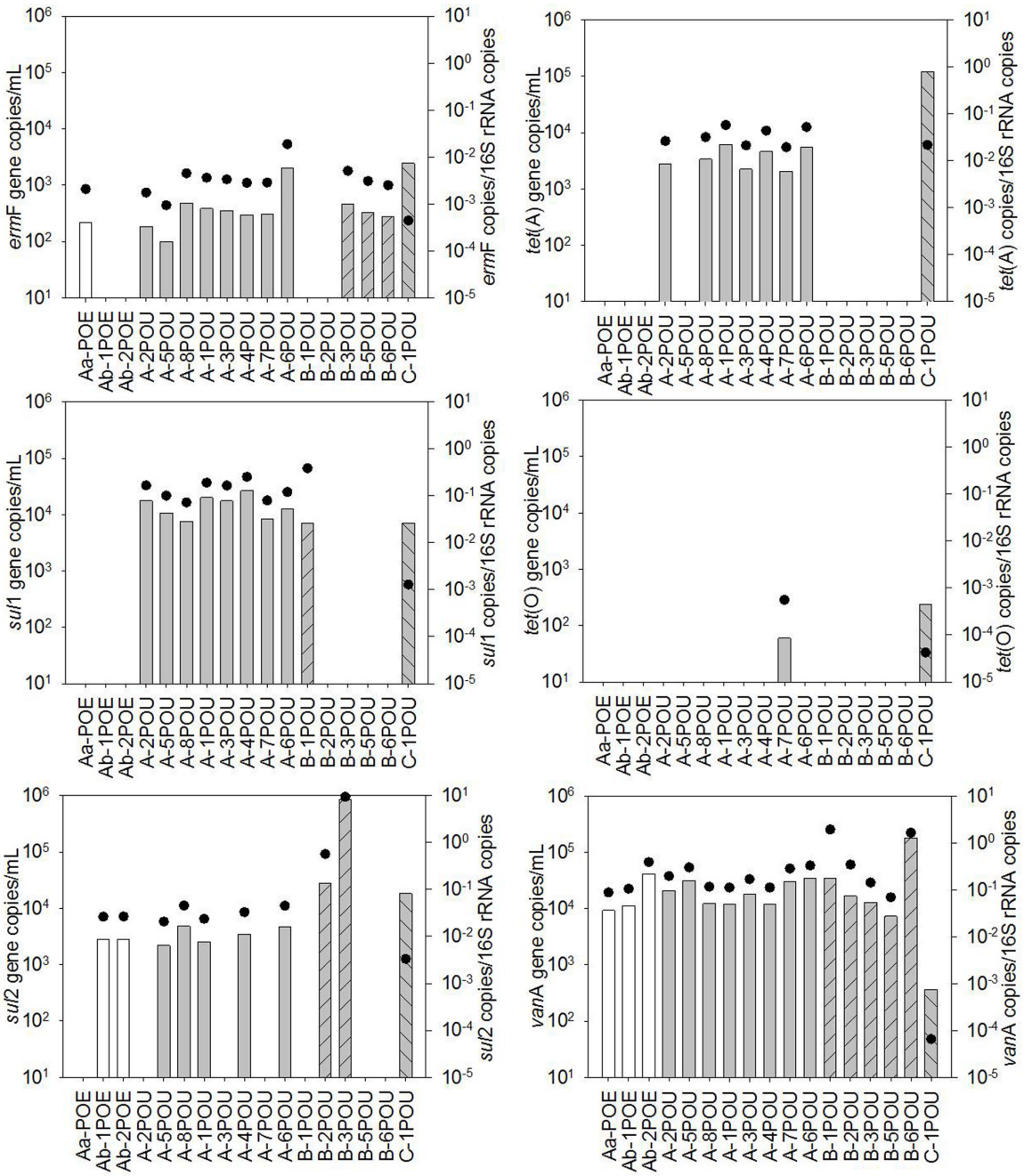

FIGURE 1 | Absolute (bars) and 16S rRNA gene-normalized (symbols) levels of ARGs in water samples collected at point of entry (POE) for WWTPs Aa and Ab and at point of use (POU) for systems A, B, and C, numbers differentiate between sample locations.

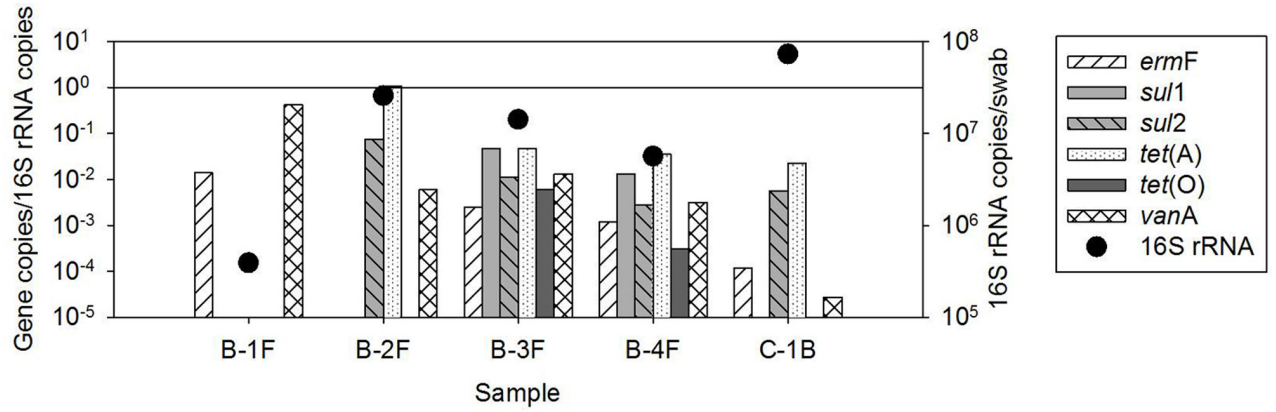

FIGURE 2 | Quantification of ARGs in biofilm samples available from recycled water distribution systems B and C. Bars represent absolute ARG copies per swab, symbols represent 16S rRNA gene copies per swab.

concentrations were observed between POE and POU for *sul*1 and *tet*(A), $p < 0.001$ for both. Concentrations of *van*A, *erm*F, and *sul*2, 16S rRNA, and *tet*(O) were not significantly different between POE and POU ($p = 0.33$–0.99).

In the irrigated soil sample (B-7S), 9.5, 7.3, 7.2, and 5.6 $\log_{10}$ gene copies/g of soil were quantified for 16S rRNA, *sul*1, *sul*2, and *van*A, respectively; *erm*F, *tet*(A), and *tet*(O) were below detection. Normalizing ARGs to 16S rRNA gene copy numbers indicated that *sul*1 and *sul*2 were one to two orders of magnitude lower and *van*A three to five orders of magnitude lower in the soil sample than observed in water and biofilm samples.

The occurrence of ARGs varied among the five biofilm samples examined, ranging from two to six classes of ARGs (**Figure 2**). However, individual ARG levels in the biofilm did not correlate with corresponding bulk water ARGs among the available paired samples (**Figure 3**). For example, the most abundant ARG, *tet*(A), in B-2F was below detection in B-2POU.

Cluster analysis performed on 16S rRNA gene normalized ARG profiles considered both the kinds of ARGs detected and their frequencies and resulted in several significantly different sample clusters (**Figure 4**). Generally, three distinct clusters were formed primarily by POE, WWTP-A POU, and WWTP-C POU samples, while the WWTP-B POU samples were interspersed among the three clusters, and a fourth cluster consisting only of WWTP-B samples. The three WWTP-A POE samples formed a significantly different cluster from POU samples in its corresponding distribution system (50% similarity). POU samples from System A ($n = 7$) formed a cluster with 78–96% similarity, except A-5POU which was significantly different (71%) and clustered with some samples from System B ($n = 5$). No patterns were observed in the biofilm and water clustering patterns for paired samples ($n = 4$). Biofilms did not cluster with one another nor did biofilm samples cluster with paired water sample with similarities ranging from 16% for F2 and F3 (significantly different) to 100% for F1 (no difference). The soil sample (B-7S) clustered with other B system biofilm and water samples, which were only 20% similar to the remainder of the samples selected.

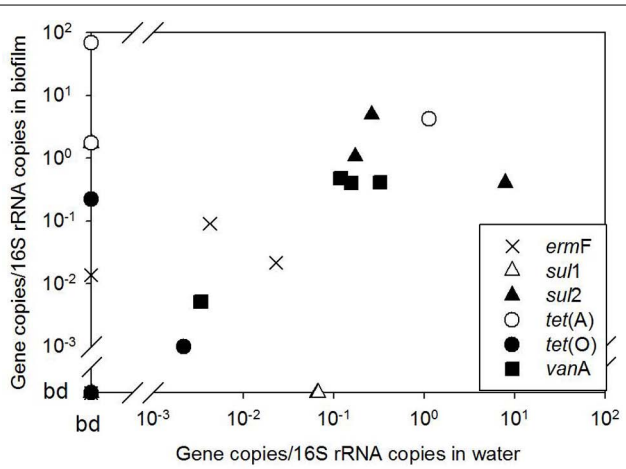

FIGURE 3 | Biofilm versus bulk water ARG copies normalized to 16S rRNA gene copies in available paired biofilm and water samples. ARGs were below detection in several samples, indicated on the *x* and *y* axes as bd.

WATERBORNE PATHOGEN AND FECAL INDICATOR SCREENING

Quantitative PCR screening for *E. coli* and *L. pneumophila* through *gad*AB and *mip* resulted in positive detections for 48% of samples: 35 and 17% for *gad*AB and *mip*, respectively (**Table 2**). *ecrf*X/*gyr*B, corresponding to *P. aeruginosa*, were below detection in all samples.

ARGs DURING SIMULATED LAND APPLICATION

WWTP effluent fraction ARG loads

Levels of ARGs in secondary, chlorinated, and dechlorinated WWTP effluent are compared in **Figure 5**. *sul*1 was the most frequently detected ARG (100%), followed by *sul*2 (71%), *tet*(W) (71%), and *tet*(O) (61%; $n = 21$). Comparing the ARGs and 16S rRNA gene levels across effluent fractions, secondary effluent was significantly higher than chlorinated and dechlorinated (both $p < 0.0001$) effluents, which were not significantly different from each other ($p = 0.45$). Comparing by gene across effluent fractions,

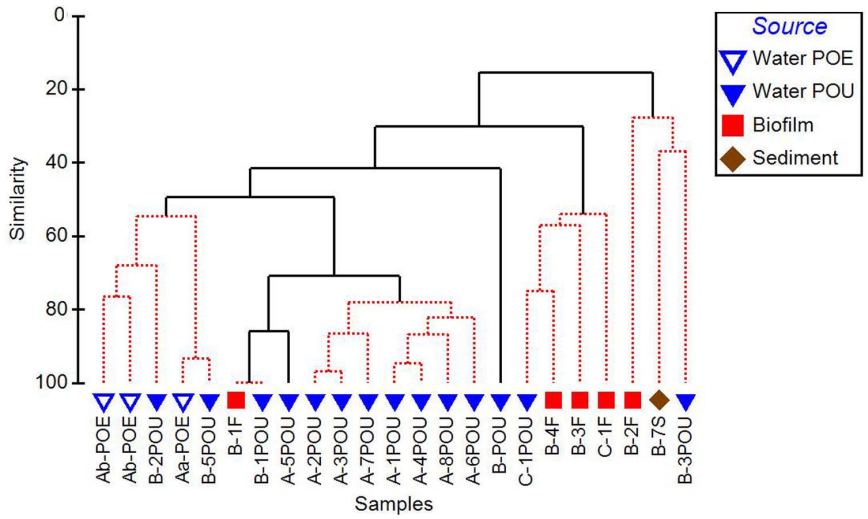

FIGURE 4 | Cluster analysis of 16S rRNA gene normalized ARG copy numbers, considerate of class and relative abundance of each ARG measured in the distribution systems (Aa, Ab, B, C) and across environmental matrices (POE, point of entry water; POU, point of use water; F, biofilm; S, soil). Solid branches indicate significantly different clusters ($p < 0.05$).

Table 2 | Log copies of *E. coli* (*gad*AB) and *L. pneumophila* (*mip*) specific genes per milliliter (POE and POU's), swab (F's), or gram soil (S).

Sample	*gad*AB	*mip*
Ab-POE	3.7	bd*
A-1POU	4.2	bd
A-2POU	5.1	1.8
A-3POU	4.4	bd
A-8POU	5.3	bd
B-1POU	bd	1.4
C-1POU	6.6	bd
B-1F	bd	4.6
B-2F	6.0	bd
C-1F	6.9	bd
B-7S	bd	5.6

*bd, below detection.

secondary effluent and chlorinated effluent were significantly different in terms of 16S rRNA and *sul*2 gene copies ($p < 0.0001$ for both). Interestingly, when ARGs were normalized to 16S rRNA gene copies, significant differences were not observed among the secondary, chlorinated, or dechlorinated effluents ($p = 0.29$–0.91). This suggests no preferential destruction of specific gene types by chlorination.

Irrigation studies

Results of the batch irrigation study (**Figure 6**) indicated no difference between soil irrigated with secondary, chlorinated, or dechlorinated effluent, or DI water in terms of ARG concentration with time for *sul*1, *sul*2, *tet*(O), or *tet*(W) ($p > 0.405$ for all, except for *sul*2 deionized $p = 0.05$ and secondary effluent $p = 0.006$).

Soil periodically irrigated resulted in a significant increase in *sul*2 when receiving secondary effluent, compared to irrigation with the other water types (p ranging from 0.032 to $p < 0.0001$; **Figure 7**). Additionally, soil irrigated with secondary effluent had significantly higher *sul*1 copies than that irrigated with chlorinated effluent, dechlorinated effluent, or DI water (all $p < 0.0001$). No significant difference across time was observed among the irrigation water types for *tet*(O) ($p = 0.13$–1.0) or *tet*(W) ($p = 0.74$–1.0). Linear modeling of gene copies versus time revealed an increasing trend of *sul*2 copies with time in soil irrigated with secondary effluent ($R^2 = 0.92$).

DISCUSSION

This study explored the occurrence of several ARGs in three reclaimed water distribution systems in the U.S. Given limited access to such systems, only one sampling event was possible. Nonetheless, the results provide important baseline information to support future research, including insight into the kinds of ARGs, bacteria, and applications that may be of concern. To the knowledge of the authors, this is the first study specifically investigating the potential for ARGs to persist or amplify within the reclaimed water distribution pipes.

ARG OCCURRENCE IN RECYCLED WATER DISTRIBUTION SYSTEMS

Several ARGs were detected at the POU in this study, many of which were below detection at the POE. This highlights the need to consider microbiological processes occurring in reclaimed water distribution systems that may be contributing to ARG amplification and suggests that focus on the water quality at the POU may be the most appropriate for assessing risk. Re-growth is a well-known

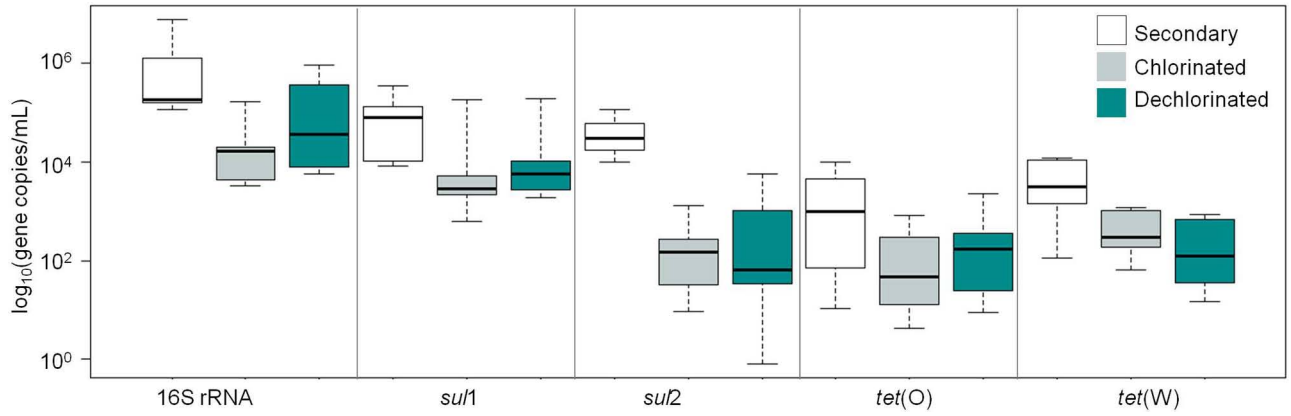

FIGURE 5 | Censored boxplot of 16S rRNA genes, *sul*1, *sul*2, *tet*(O), and *tet*(W) gene copies per milliliter in secondary (no fill), chlorinated (gray), and dechlorinated (blue) domestic WWTP effluents applied in the batch (2–4 L 0. 22 μm filtered) and periodic (60 mL freeze-dried) irrigation study ($n = 6$ *tet* genes, 7 *sul* and 16S rRNA genes).

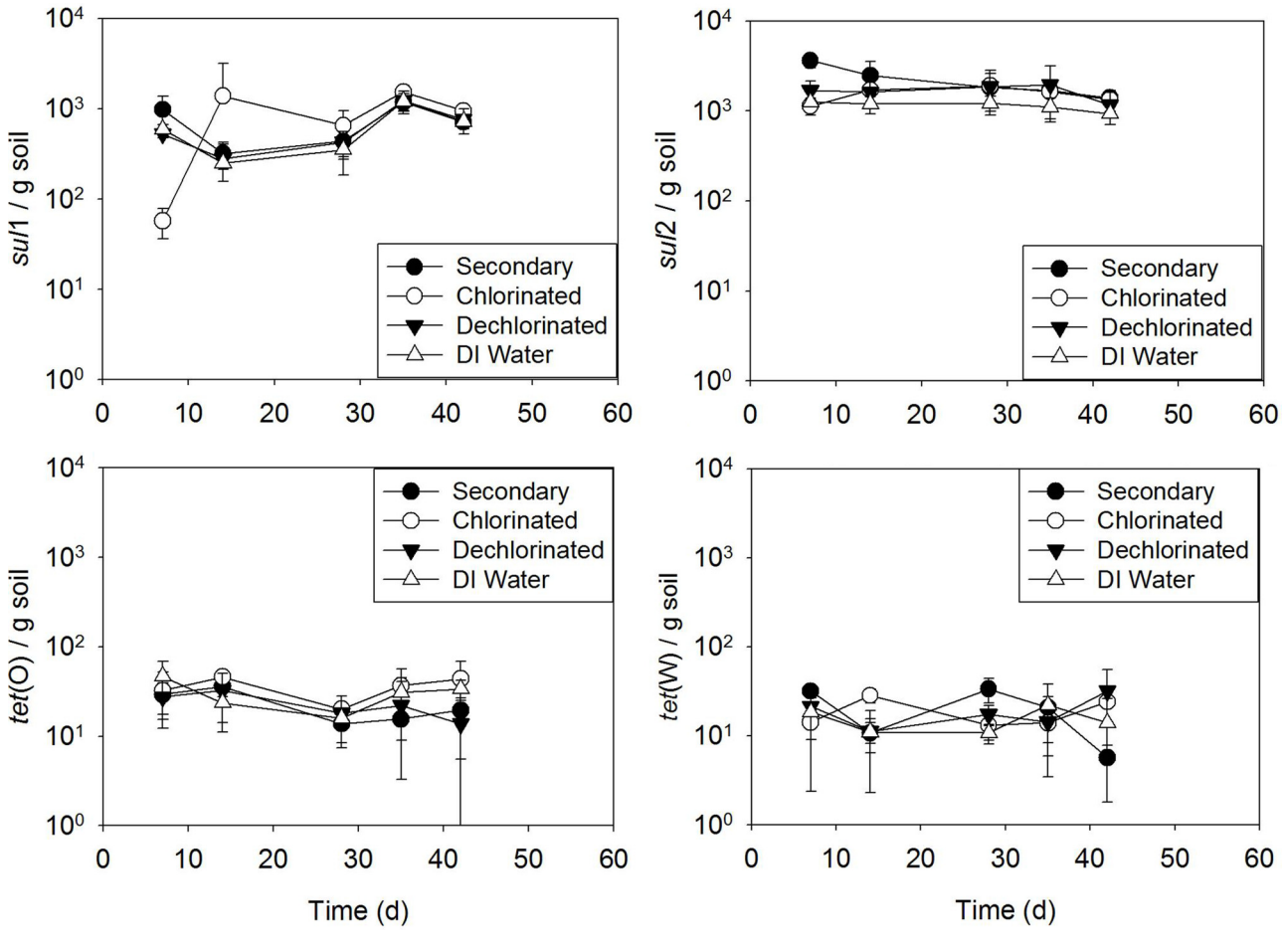

FIGURE 6 | *sul*1, *sul*2, *tet*(O), and *tet*(W) genes in soils subject to one time (batch) irrigation with secondary, chlorinated, dechlorinated domestic WWTP effluents and deionized (DI) water, per gram of soil (slurry). Error bars represent standard deviation of triplicate microcosms.

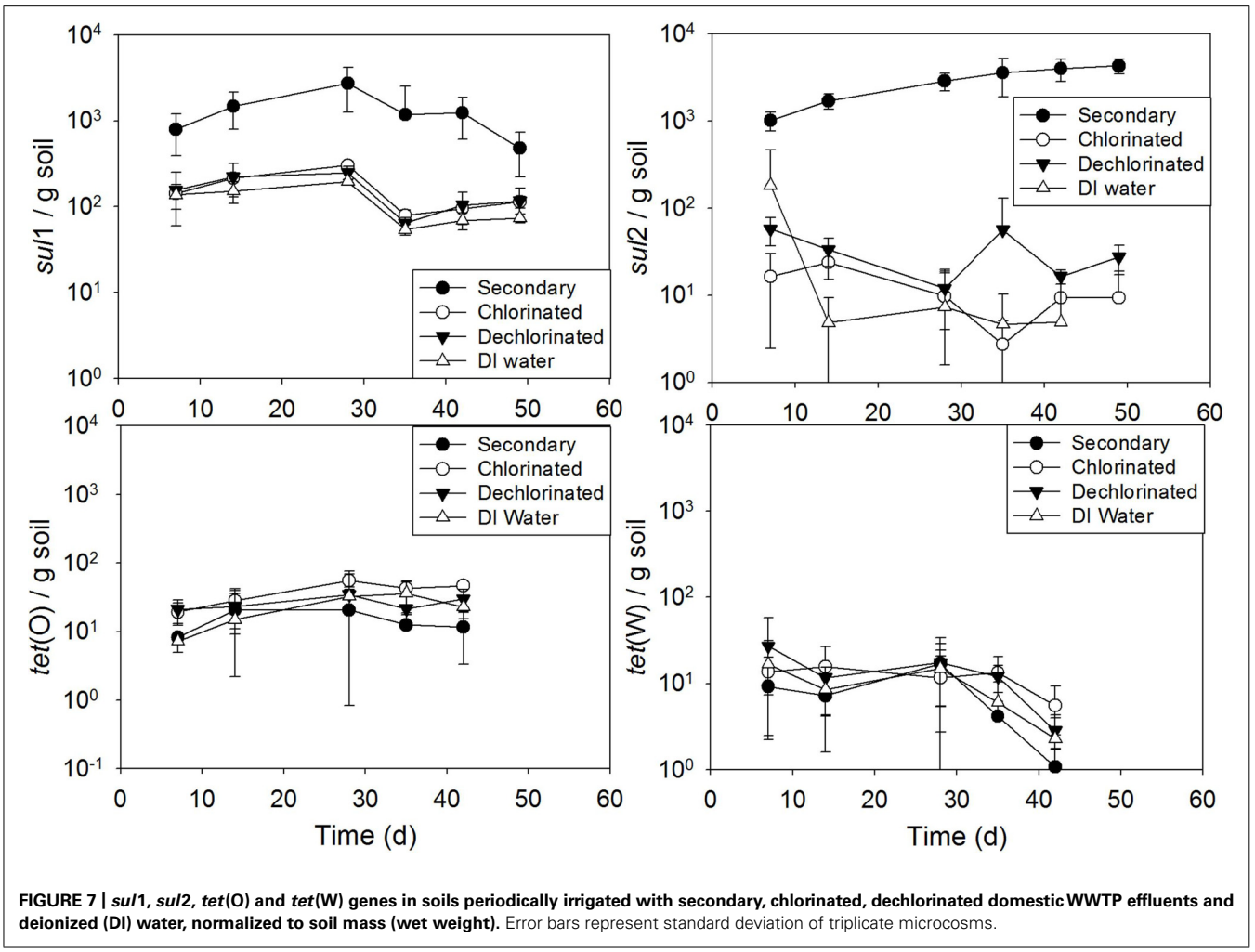

FIGURE 7 | *sul*1, *sul*2, *tet*(O) and *tet*(W) genes in soils periodically irrigated with secondary, chlorinated, dechlorinated domestic WWTP effluents and deionized (DI) water, normalized to soil mass (wet weight). Error bars represent standard deviation of triplicate microcosms.

phenomenon even in drinking water distribution systems (Xi et al., 2009), and has also recently been documented in a recycled water system (Ryu et al., 2005).

Several studies have documented levels of ARGs in WWTP effluents, providing a reference for comparison. Generally, *erm*F, *sul*1, *sul*2, *tet*(A), and *tet*(O) levels measured in the POE, POU, and WWTP effluent applied in the irrigation study were below or at the lower end of ranges reported by others in WWTP effluents (Kim et al., 2010; Czekalski et al., 2012; Negreanu et al., 2012). However, the irrigated soil in this study carried higher levels of *sul*1 (7.3 $\log_{10}$ copies/g) and *sul*2 (7.2 $\log_{10}$ copies/g) than reported by others in a field study of irrigation with recycled water (5.1–6.7 and 3.5–4.7 $\log_{10}$ copies/g, respectively; Negreanu et al., 2012).

The occurrence of *van*A is of particular interest given that it confers resistance to vancomycin, a last-resort life-saving antibiotic. Vancomycin is commonly prescribed to treat methicillin-resistant *Staphylococcus aureus* (MRSA) infections, but has been losing effectiveness due to increased resistance among staphylococci (Stevens, 2006). *van*A was detected in every sample in this study using primers targeting a 732-bp product. Interestingly, using *van*A primers designed for longer target products (1030 bp; Clark et al., 1993), *van*A was detectable at several POU sites, but not in any POE samples (data not shown). Detection differences between the two PCR primer sets may indicate that *van*A was partially damaged during disinfection, preventing amplification of longer PCR products. Long-amplicon PCR has recently been demonstrated to provide enhanced detection of DNA damage events (McKinney and Pruden, 2012). Few studies have reported detection of *van*A in environmental samples, providing little reference for comparison. However, *van*A has been reported in drinking water biofilms and wastewater (Schwartz et al., 2003), but was below detection in wastewater reclaimed for groundwater recharge environments (Böckelmann et al., 2009). To the knowledge of the authors, this is the first report of the presence of *van*A in distributed recycled water.

MOLECULAR DETECTION OF WATERBORNE PATHOGENS AND INDICATORS

Opportunistic pathogens residing in water systems, such as *L. pneumophila* and *P. aeruginosa*, are now the primary source of waterborne disease outbreak in developed countries (Brunkard et al., 2011). However, there is a need for epidemiological studies to better quantify the precise contributions of various water

systems to human disease (Pruden et al., 2013). Of interest to the present study was whether such organisms may be present in recycled water, which could be of special concern for bacterial pathogens because they are capable of developing antibiotic resistance. *P. aeruginosa* is an example of an opportunistic pathogen that colonizes taps and is prone to multi-antibiotic resistant forms (Trautmann et al., 2005). *E. coli* was also of interest as a fecal indicator.

The *mip* gene, specific to *L. pneumophila*, was detectable at levels comparable to those recently observed in chloraminated drinking water distribution systems (Wang et al., 2012). Thus, there could be concern for aerosolization of *L. pneumophila* during spray irrigation. Future research is suggested to more closely examine this potential transmission pathway. *gad*AB, specific to *E. coli*, detected at POU in this study, was comparable to levels previously observed in manure runoff (4.6–4.9 $\log_{10}$ copies/mL, assuming one gene copy per cell) and higher than previously observed in WWTP effluent (2.74 $\log_{10}$ copies/mL) (Grant et al., 2001). This combined with the observation of *gad*AB below detection at POE, but detectable at POU, is further evidence of bacterial re-growth within reclaimed water distribution systems.

EFFECT OF DISINFECTION ON ARGs

Chlorination is commonly applied to WWTP effluents during warm seasons, in which case the effluent must be dechlorinated prior to discharge. Chlorination had a significant impact on 16S rRNA and *sul*2 gene copies, which is consistent with the expectation that chlorination would have a moderate reactivity with nucleic acids (Dodd, 2012). Although occasionally ARGs detected in dechlorinated effluent were not detected in chlorinated effluent, the levels of detected genes were not significantly different. The levels of ARGs in secondary, chlorinated, and dechlorinated WWTP effluent fractions were comparable to those detected at the POU in the field study (**Figure 5**).

ARG FATE DURING WWTP EFFLUENT LAND APPLICATION

In the lab study, periodic irrigation with secondary effluent increased the prevalence of *sul*1 and *sul*2 in historically manured soil compared to soil irrigated with chlorinated, dechlorinated, or DI water. This could be due to direct inputs of extracellular ARGs, intracellular ARGs, or horizontal gene transfer to native soil bacteria (Dodd, 2012). Interestingly, *tet*(O) or *tet*(W) levels in the soil slurry were not affected by irrigation. This highlights that different ARGs have different environmental fates, as has been observed recently with respect to *sul*1 and *tet*(W) in a watershed-scale study (Storteboom et al., 2010; Pruden et al., 2012). Differences in ARG fate likely relate to host properties and their overall propensity for horizontal gene transfer. Because 16S rRNA gene copy levels were relatively consistent with time across the soil irrigation treatments (data not shown), changes in total bacterial population sizes were not likely a factor in the observed differences.

A recent field study carried out in Israel suggested that irrigation does not significantly affect the soil microbiome, and increases were not observed in *sul*1, *sul*2, *tet*(O), *erm*F, or *erm*B in soil subjected to long-term (6–12 years) irrigation with secondary effluent compared to freshwater irrigation (Negreanu et al., 2012). Given the difference in controls, irrigation frequency, soil type, climate, and wastewater chemistry, direct comparison between the studies is difficult. It is suspected that the difference in soil moisture and incubation time may likely be an important difference. Negreanu et al. (2012) irrigated with 4 L of water/m^2 soil/day which experienced a combination of infiltration, run off, and evaporation. In this study, soil-water slurries were used with irrigation waters that incubated with soil rather than infiltrating, allowing for greater contact time. Therefore, the microcosm results presented here may indicate that irrigating at high rates with secondary effluent may still result in amplified soil ARGs. The present study is consistent with the observation of increased ARG copies directly under irrigation drippers compared to soils 50 m from drippers (Negreanu et al., 2012). Further, soil type has been noted to be a critical factor in determining the level of impact of land application of biosolids containing ARGs (Munir and Xagoraraki, 2011), and may also affect the fate of ARGs applied by irrigation.

Understanding land use scenarios that affect soil ARGs is of utmost importance given that the resistome of multi-drug resistant soil bacteria was recently shown to match that from diverse human pathogens (Forsberg et al., 2012). Heavy irrigation with secondary effluent is shown here to be capable of increasing soil ARGs. In practice, given that ARG prevalence can increase between POE and POU within distribution systems, increased ARG levels in irrigation waters and therefore soils are expected. Given that spray irrigation of recreational fields with treated wastewater is common practice, there is considerable potential for human contact with aerosols and soil.

CONCLUSION

This study brings to light the occurrence of ARGs at the POU in recycled water irrigation systems, including *van*A, which is of significant concern to human health. Differences between POE and POU ARG occurrences underscore the need to take into consideration re-growth that occurs in the distribution system when estimating overall exposure and risk. Based on the lab microcosm study, amplified levels of ARGs in soil irrigated with recycled water is possible. Molecular data in this study also indicates the potential presence of waterborne bacterial pathogens, such as *L. pneumophila*. As ARGs are emerging contaminants, risk assessment is in its infancy and no guidance yet exists on safe levels (Pruden, 2011). In addition to direct contact with water and aerosols during recreational activity, a greater concern may be overall contribution to the global pool of antibiotic resistance and ultimately reducing the effectiveness of available antibiotics for treating human disease.

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Resistance in tuberculosis: what do we know and where can we go?

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Tuberculosis (TB) has become a worldwide threat, mainly due to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (*Mtb*). This mini-review is focused on the various mechanisms of resistance to the currently available anti-TB drugs and provides perspective on novel strategies and lead scaffolds/compounds aimed at inhibiting/overcoming these resistance mechanisms.

Keywords: antibiotics, inhibitors, mechanisms of resistance, new drug targets, synergistic drug therapy

INTRODUCTION

Approximately 1.4 million deaths were attributed to *Mtb* infections in 2012. When compared to other infectious agents, only HIV claims more lives (WHO, 2012). While recent efforts have resulted in a global decline in TB incidence and mortality (WHO, 2011), the number of individuals infected with drug-resistant isolates continues to increase, presenting a serious global health threat. Generally, drug-susceptible *Mtb* infections are treated with a combination of four compounds; rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA), and isoniazid (INH) for 2 months, followed by treatment with RIF and INH for 4 additional months (Lienhardt et al., 2012) (**Figure 1A**). By definition, MDR-TB is resistant to the most potent first-line drugs, RIF and INH. The WHO reports that ∼60,000 cases of MDR-TB were diagnosed in 2011, likely an underestimate. To treat MDR-TB, second-line drugs including fluoroquinolones (FQs), amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) are employed. These drugs are administered for ∼20 months and can be toxic, poorly tolerated, and difficult to procure. Approximately 95% of MDR-TB is XDR-TB, having additional resistance to at least one FQ and one injectable drug (AMK, KAN, or CAP), and for which treatment options are limited. Therefore, new therapeutics are critically needed to overcome drug resistance and to eliminate TB as a public health threat.

Herein we briefly discuss the drugs currently used to treat TB and the respective mechanisms of resistance followed by a review of approaches aimed toward overcoming TB drug resistance.

CURRENT DRUGS AND RESISTANCE

Mtb is intrinsically resistant to many antibiotics due to the low permeability of its mycolic acid-rich waxy cell envelope, the action of efflux pumps (Banerjee et al., 1996; Silva et al., 2001; Singh et al., 2011), and the presence of chromosomally encoded resistance genes. Drug resistance in *Mtb* has been emerging due to the accumulation of chromosomal mutations and not acquisition of mobile genetic elements. The major mechanisms of acquired drug resistance in *Mtb* can be broken down into several categories: (1) mutations or modifications (e.g., RNA methylation) of the drug targets (RIF, EMB, KAN, AMK, CAP, and the FQs), (2) the inability to activate a prodrug [INH, PZA, ethionamide (ETH)] due to mutations leading to a loss of function, and (3) enzymatic inactivation of the drug (KAN).

MUTATIONS OR MODIFICATIONS OF THE DRUG TARGETS

Mutations

Antibiotics target cellular processes that are vital in bacteria by binding their targets at a specific site, often directly interacting with key functional residues of the target. The most common mechanism of resistance in *Mtb* is the alteration of the target's binding site through the accumulation of mutations. These mutations decrease the binding affinity of the drug to its target and typically occur in a very defined region of the gene termed the resistance-determining region. This mechanism is used by *Mtb* to confer resistance to RIF, EMB, and FQs by altering the binding site of their respective targets: the β-subunit of RNA polymerase (Campbell et al., 2001), a glycosyltransferase (Telenti et al., 1997), and DNA gyrase (Takiff et al., 1994). Similarly, ribosomal mutations (e.g., A1401G) in the 16S rRNA have been found to confer resistance to AMK, KAN, and CAP (Wachino et al., 2010).

Modifications

An alternative mode of resistance in *Mtb* is the inactivation of rRNA methyltransferase enzymes. Mutations in TlyA hindering the methylation activity of rRNA 2′-*O*-methyltransferase at nucleotides C1409 of 16S rRNA and C1920 of 23S rRNA have been linked to resistance to the ribosome-targeting drugs CAP and viomycin (VIO) (Johansen et al., 2006). Mutations in a putative 16S rRNA methyltransferase, GidB, were found to confer low-level resistance to the aminoglycoside (AG) streptomycin (STR) (Spies et al., 2011; Wong et al., 2011).

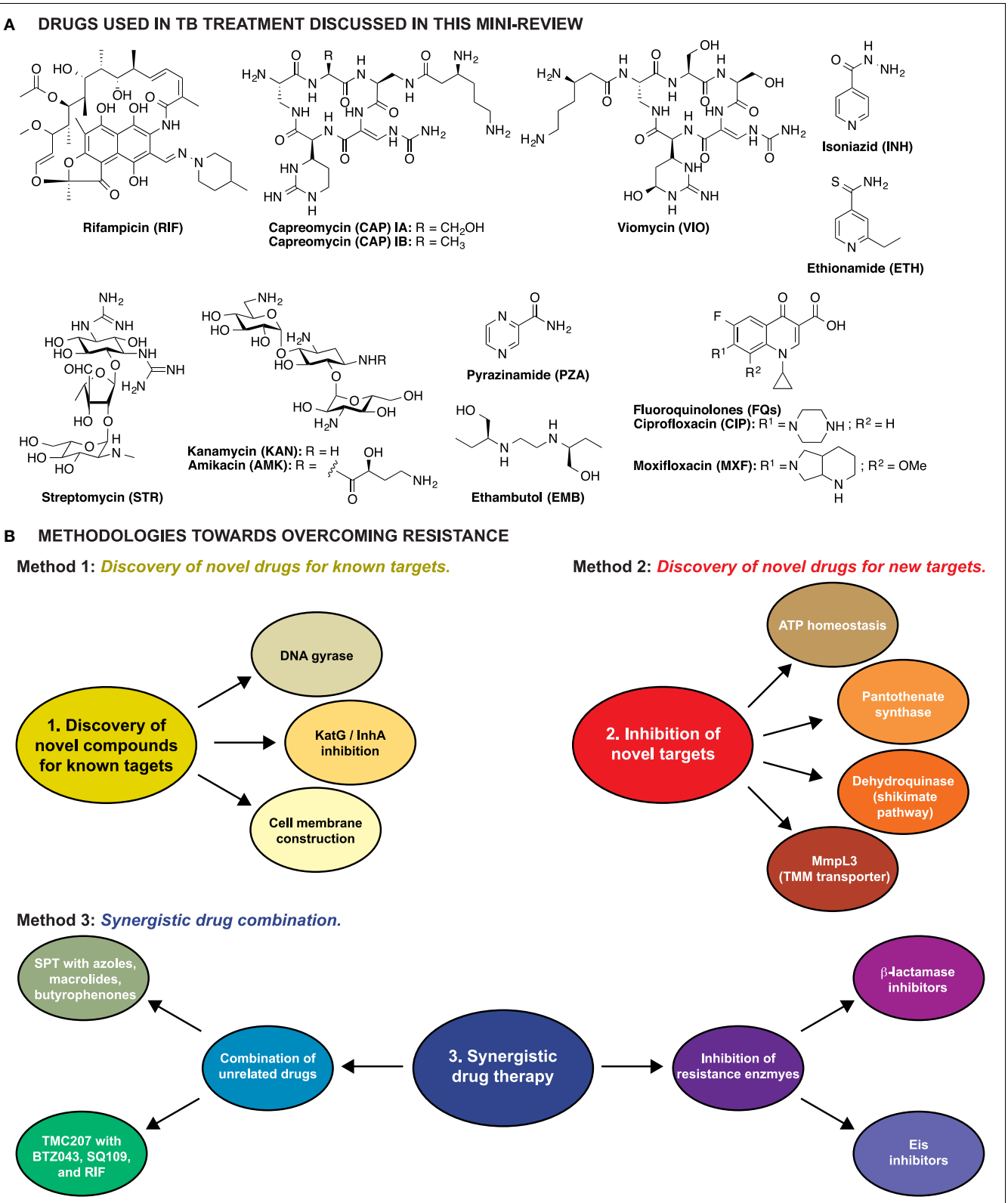

FIGURE 1 | (A) Structures of the anti-TB drugs discussed in this mini-review. **(B)** Schematic representation of the three methodologies explored to overcome resistance in *Mtb*.

INABILITY TO ACTIVATE A PRODRUG

Three first-line drugs, INH, PZA, and ETH, are prodrugs that must be metabolized to be active against *Mtb*. INH is activated by the catalase-peroxidase, KatG, and produces isonicotinic-acyl radicals that react with NADH to form an INH-NADH adduct. The adduct binds its main target, enoyl-acyl carrier protein reductase (InhA), and inhibits mycolic acid biosynthesis. ETH is a structural analog of INH and is activated by the monooxygenase EthA, which oxidizes ETH to its active form, 2-ethyl-4-amidopyridine. This activated compound also targets InhA and blocks mycolic acid biosynthesis similarly to INH. PZA is activated by pyrazinamidase/nicotinamidase, PncA, to pyrazinoic acid (POA). The proposed targets of POA range from cell membrane energy to fatty acid synthesis, but the exact killing mechanism of POA remains unclear. Resistance to any of the prodrugs can arise due to reduced metabolism by their corresponding activator. Structural mutations that lower or abolish the enzymatic activity of KatG (Ramaswamy et al., 2003; Hazbon et al., 2006), EthA (Baulard et al., 2000; Debarber et al., 2000; Morlock et al., 2003), and PncA (Stoffels et al., 2012; Rajendran and Sethumadhavan, 2013) were found to lead to INH, ETH, and PZA resistance, respectively. Mutations in InhA causing overexpression of the target (Baulard et al., 2000; Debarber et al., 2000; Larsen et al., 2002) or preventing binding of the active form of INH and ETH to the target (Banerjee et al., 1994; Vilcheze et al., 2008) also confer cross-resistance to these drugs.

INACTIVATION OF THE DRUG

Drug modification is perhaps the most prevalent and well-studied resistance mechanism employed by bacteria. Many bacteria either degrade or modify the offending compound thereby generating an inactive molecule. Resistance to penicillin is mediated by a class of enzymes known as the β-lactamases, which inactivate the antibiotic by destroying the β-lactam ring. *Mtb* naturally harbors a chromosomally encoded class A β-lactamase, BlaC, which is constitutively expressed providing intrinsic resistance to penicillin (Hugonnet and Blanchard, 2007). One mechanism of acquired resistance to AGs, such as KAN and AMK, is their modification and inactivation by a family of enzymes known as AG-modifying enzymes (AMEs) (Ramirez and Tolmasky, 2010; Labby and Garneau-Tsodikova, 2013). *Mtb* expresses two AMEs, the AG 2′-*N*-acetyltransferace AAC(2′)-Ic (Hegde et al., 2001) and the enhanced intracellular survival (Eis) protein (Zaunbrecher et al., 2009). Both enzymes acetylate AGs, reducing the ability of these drugs to inhibit the ribosome. In the case of Eis, increased expression due to mutations in the *eis* promoter or the 5′-untranslated region of the transcriptional activator WhiB7 leads to clinically relevant, low-level resistance to KAN (Zaunbrecher et al., 2009; Reeves et al., 2013). A unique feature of Eis resides in its ability to modify AGs at multiple sites *in vitro*, completely inactivating these compounds (Chen et al., 2011). A recent report has shown that Eis also acetylates CAP at its β-lysine side chain *in vitro*, but the clinical relevance of this finding remains unclear (Houghton et al., 2013).

METHODOLOGIES TO OVERCOME RESISTANCE

Three major strategies have been explored to overcome resistance in TB: (1) identification of novel drugs for well-established targets, (2) identification and characterization of novel compounds that target unexplored vital cell processes or enzymes, thus killing the cell by preventing regular metabolism, and (3) identification of compounds that behave synergistically with current anti-TB drugs (**Figure 1B**).

DISCOVERY OF NOVEL DRUGS FOR KNOWN TARGETS

DNA gyrase

Among the known *Mtb* drug targets, DNA gyrase remains attractive for development of inhibitors interacting with either GyrA or GyrB subunits. To overcome the FQ resistance of DNA gyrase mutants, a series of 30 ofloxacin derivatives with various substituents *ortho* to the fluoro moiety of the FQ scaffold were synthesized (Dinakaran et al., 2008). These compounds displayed MIC_{99} values $<10\,\mu M$ and retained anti-mycobacterial activity against MDR-*Mtb* strains. While ofloxacin displayed a 17-fold lower activity against MDR-*Mtb* relative to susceptible *Mtb* strains, its derivatives displayed at most a 4-fold lower activity and, in some cases, higher activity against MDR-*Mtb*. The toxicity of selected compounds in mammalian Vero cells displayed a range of IC_{50} values (60 to $>150\,\mu M$), 6-fold higher than the highest MIC_{99} value. When tested in culture, the compound with the best MIC_{99} value showed reduction in *Mtb* colonies similar to that observed with ofloxacin.

While many efforts have been directed toward the development of FQ analogs to target the GyrA subunit of DNA gyrase, development of inhibitors targeting GyrB has also been pursued. A series of aminopyrazinamides were found to target the ATPase site of GyrB and inhibit the growth of *Mtb* in the low micromolar/high nanomolar range (Shirude et al., 2013). A variety of naphthoquinones were also discovered to inhibit the ATPase domain of the GyrB subunit of *Mtb* DNA gyrase with IC_{50} values between 15 and $>200\,\mu M$ (Karkare et al., 2013). The most promising naphthoquinone, diospyrin, was tested against DNA gyrase from other bacterial species and found to be a broad spectrum DNA gyrase inhibitor. The aminobenzimidazole AB-1 was also investigated for its inhibitory properties of GyrB and compared to the well-characterized GyrB inhibitor novobiocin (Chopra et al., 2012). AB-1 displayed lower MIC values than novobiocin with *Mtb* strains resistant to STR, INH, CIP, and *p*-aminosalicylic acid. AB-1 also displayed improved activity in aerobic and anaerobic conditions while not interfering with RIF or INH mechanisms.

KatG/InhA inhibition

To overcome mutations of KatG and other mechanisms of INH resistance, analogs of INH have been synthesized. Early generations of INH analogs incorporated aryloxyacetonitriles onto the hydrazide of the INH scaffold (Bukowski et al., 1999). These compounds were then evolved into 1,2,3-triazole derivatives with MIC values slightly higher than those of INH (Boechat et al., 2011).

As an alternative strategy to overcoming KatG mutations or deletion, InhA inhibitors, not needing KatG activation, have been investigated. Triclosan (Parikh et al., 2000) served as a lead compound for the design of a series of 5-alkyl-diphenyl ethers (Sullivan et al., 2006). These ethers displayed IC_{50} values for InhA ranging from 5 nM to $2\,\mu M$, displayed higher bactericidal activity than the parent triclosan, and were further optimized to

slow the release time from the InhA active site (Luckner et al., 2010). More recently, from screening 300 molecules, CD117 was identified as an InhA inhibitor that likely targets other enzymes (Vilcheze et al., 2011). CD117 was active against hypoxic *Mtb* and yielded 80% Vero cell viability at the highest concentration tested. This year, virtual screening efforts led to the identification of 7 potential InhA inhibitors (Kinjo et al., 2013). In model *Mycobacterium* species the top hit compounds displayed similar growth inhibition to that of INH and directly inhibited *Mtb* InhA.

Cell membrane construction

The inhibition of cell membrane construction also remains a target of choice for the development of new drugs. Nitroimidazoles such as PA-824 and OPC-67683 have been found to inhibit the synthesis of membrane proteins and lipids (Mukherjee and Boshoff, 2011). Much like INH and PZA, PA-824 is a prodrug that requires activation by reduction of its nitro group (Stover et al., 2000). PA-824 and OPC-67683 have demonstrated activity against both susceptible and resistant-*Mtb* strains (Stover et al., 2000; Rivers and Mancera, 2008a,b). PA-824 showed activity in the high ng/mL range for both susceptible and resistant-*Mtb* (Ginsberg et al., 2009) and OPC-67683 displayed better MIC values in the low ng/mL range (Matsumoto et al., 2006).

Benzothiazinones (BTZs) (Makarov et al., 2006), such as BTZ043, have also been shown to interfere with cell membrane construction. Like EMB, these compounds disrupt arabinan's biosynthesis by targeting decaprenylphosphoryl-β-D-ribose 2′-epimerase (DprE) (Makarov et al., 2009). In infected macrophages, BTZ043 showed a faster decrease in percentage of infected cells and bacterial load compared to INH or RIF. BTZ043 has also been tested in 240 clinical isolates of *Mtb* from European hospitals and was active against all strains (Pasca et al., 2010).

DISCOVERY OF NOVEL DRUGS FOR NEW TARGETS

ATP homeostasis

One of the challenges in treating TB is the organism's extended period(s) of dormancy. One key difference in the states of growing and hypoxic non-growing *Mtb* is a reduced, but significant, ATP pool in the non-growing organism (Rao et al., 2008). A plausible way to attack the non-growing bacilli is to affect their ATP storage by inactivating ATP synthase to deplete the ATP and eventually kill the bacilli (Andries et al., 2005). This strategy led to the identification of the ATP synthase inhibitor TMC207 (bedaquiline or sirturo), which was approved by the FDA in December 2012 for TB treatment (Diacon et al., 2009; Avorn, 2013). TMC207 is the first FDA-approved anti-TB drug in 40 years (Mahajan, 2013). TMC207 targets the C-subunit of ATP synthase proton pump and mutations are known that are resistant to this novel compound (Segala et al., 2012). There are also some mycobacterial strains that are naturally resistant to TMC207. Because of the potential risks associated with this drug, it is recommended that it be utilized only in patients for which other treatment options have failed. A high-throughput screen (HTS) to identify additional compounds that block ATP synthesis and kill dormant *Mtb* resulted in 0.5% hit rate from a library of 600,000 compounds (Mak et al., 2012).

Pantothenate synthetase

Pantothenate synthetase has also been explored as an antibiotic target of *Mtb*. Actinomycin D was identified *via* HTS as an inhibitor of this *de novo* synthesis enzyme (Yang et al., 2011). Futher *in silico* HTS led to the discovery of two compounds that inhibit the panthothenate synthesis 10× more efficiently than actinomycin D does. These compounds displayed identical MIC values against susceptible and MDR-*Mtb* strains. Analogs of the pantothenate synthetase reaction intermediate were shown to bind the enzyme more tightly than the nucleotide substrate (Ciulli et al., 2008). Another essential enzyme, pantothenate kinase (CoaA) has also been considered as a target. Three classes of compounds were found to target CoaA: triazoles, quinoline carboxamides, and biaryl acetic acids (Venkatraman et al., 2012). All compounds inhibited CoaA with IC_{50} values ranging from 70 nM to 8.4 μM with various modes of inhibition.

Dehydroquinase of the shikimate pathway

The essential shikimate pathway, responsible for the biosynthesis of aromatic amino acids, folate, and vitamins E/K, is also an antibacterial target (Gonzalez-Bello and Castedo, 2007; Payne et al., 2007a,b; Tran et al., 2011). Recently, inhibitors of the dehydroquinase enzyme of the shikimate pathway have been optimized against *Mtb* and a series of 3,4-dihydroxyacetophenone and acetonide cores with MIC values in the low-micromolar range were identified (Tran et al., 2012). Similarly, a series of anhydroquinones were synthesized and found to be low-micromolar to high-nanomolar *Mtb* dehydroquinase inhibitors (Payne et al., 2007a; Prazeres et al., 2007). Other studies have focused on generating dehydroquinic acid derivatives (Lence et al., 2013) or 3-alkyl enol mimics (Blanco et al., 2012) to inhibit dehydroquinase and disrupt the shikimate pathway. Dehydroquinic acid analogs showed IC_{50} values in the mid-nanomolar range (26–100 nM), while the 3-alkyl enol mimics had a broader nanomolar range (28–780 nM).

MmpL3 (TMM transporter)

SQ109 is an EMB analog with an adamantyl moiety that targets cell well-biosynthesis. SQ109 was discovered in a screen of ∼63,000 compounds and showed high ng/mL MIC values (Sacksteder et al., 2012). Recent studies revealed that SQ109 acts on MmpL3, the trehalose monomycolate (TMM) transporter of *Mtb* (Tahlan et al., 2012). Adamantyl ureas, discovered by high-throughput screening, also displayed anti-tubercular activity (Brown et al., 2011) by targeting MmpL3 (Grzegorzewicz et al., 2012). Since their discovery, the structure of organic ureas has undergone several optimizations for solubility (Scherman et al., 2012) and pharmacokinetic properties (North et al., 2013). Additional scaffolds based on the structure of the pyrrole derivative BM212 have also recently emerged as MmpL3 inhibitors (Poce et al., 2013).

SYNERGISTIC DRUG COMBINATION

Combination of unrelated drugs

In an effort to reduce emerging resistance, combinations of small molecules have been explored. In a search for compounds that would work synergistically with spectinomycin (SPT), a high-throughput synergy screen (HTSS) was performed with a library

of molecules with known pharmacological properties. Three structural cores were found to enhance the antibiotic activity of SPT: the macrolides, azoles, and butyrophenones (Ramon-Garcia et al., 2011). Bromperidol displayed bactericidal activity, was effective against *Mtb* in a macrophage model, and displayed similar enhancement of the activities of RIF, STR, clofazimine, and clarithromycin. In addition to its anti-TB activity, TMC207 has also been shown to speed up treatment with the first-line anti-TB drugs (Diacon et al., 2009). Lechartier et al. combined BTZ043 with known and experimental TB treatments (Lechartier et al., 2012). In most cases additive interactions of BTZ043 with the second TB treatment were observed. However, BTZ043 was shown to act synergistically with TMC207. Both drugs used at one-quarter of the MIC concentration showed more significant growth retardation than TMC207 at its MIC value alone. The authors suggest that BTZ043 inhibits DprE1 leading to weaker cell walls allowing transmission of TMC207 across the cell membrane. In a recent study, SQ109 acted synergistically with RIF and TMC207 (Reddy et al., 2010).

Inhibition of resistance enzymes

The use of inhibitors of drug-modifying enzymes along with currently approved anti-TB drugs complements the drug combination strategy described above. Perhaps the best-known combination of this type is a β-lactam and a β-lactamase inhibitor. A combination of clavulanate and meropenem proved to be active against both susceptible and XDR-*Mtb* strains (Hugonnet et al., 2009) A novel β-lactamase inhibitor, NXL104, was recently reported and found to bind BlaC in a 1:1 molar ratio (Xu et al., 2012). Recently, inhibitors of the AG-resistance enzyme Eis were discovered (Green et al., 2012). These compounds encompass a wide variety of chemical structures and offer a plethora of scaffolds to be studied for development of novel anti-TB drug adjuvants. While these compounds have not yet been tested in AG-resistant *Mtb* strains, combinations of inhibitors with AGs are predicted to restore the antibacterial activity of AGs in cells.

CONCLUSION

Even though current TB treatments are still broadly employed, the rapid spread of MDR-TB and XDR-TB is a rising threat. Based on our current understanding of the resistance mechanisms, contemporary strategies are focused on generating compounds that will avoid or overcome the defenses of *Mtb*. Research is being carried out to find novel compounds that will disrupt DNA, cell membrane biosynthesis, and general cellular metabolism. Other lines of inquiry are focusing on pathways currently not used to treat TB: cellular energetics, cellular transport, and other aspects of cellular metabolism. A third facet relies on current TB treatment and combines drugs to achieve synergistic effects, either blocking two separate pathways or inhibiting a drug-modifying enzyme. While many of the compounds discussed herein are far from being approved as drugs, the recent approval of TMC207 and the multitude of efforts in searching for novel antibiotics give hope for the discovery of novel TB treatments.

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Interference of bacterial cell-to-cell communication: a new concept of antimicrobial chemotherapy breaks antibiotic resistance

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Bacteria use a cell-to-cell communication activity termed "quorum sensing" to coordinate group behaviors in a cell density dependent manner. Quorum sensing influences the expression profile of diverse genes, including antibiotic tolerance and virulence determinants, via specific chemical compounds called "autoinducers". During quorum sensing, Gram-negative bacteria typically use an acylated homoserine lactone (AHL) called autoinducer 1. Since the first discovery of quorum sensing in a marine bacterium, it has been recognized that more than 100 species possess this mechanism of cell-to-cell communication. In addition to being of interest from a biological standpoint, quorum sensing is a potential target for antimicrobial chemotherapy. This unique concept of antimicrobial control relies on reducing the burden of virulence rather than killing the bacteria. It is believed that this approach will not only suppress the development of antibiotic resistance, but will also improve the treatment of refractory infections triggered by multi-drug resistant pathogens. In this paper, we review and track recent progress in studies on AHL inhibitors/modulators from a biological standpoint. It has been discovered that both natural and synthetic compounds can disrupt quorum sensing by a variety of means, such as jamming signal transduction, inhibition of signal production and break-down and trapping of signal compounds. We also focus on the regulatory elements that attenuate quorum sensing activities and discuss their unique properties. Understanding the biological roles of regulatory elements might be useful in developing inhibitor applications and understanding how quorum sensing is controlled.

Keywords: quorum sensing, antagonist, inhibitor, antibiotic resistance, virulence control, autoinducer, homoserine lactone

OUTLINE OF "QUORUM SENSING" IN GRAM-NEGATIVE BACTERIA

The development of antibiotics originated with penicillin, and this approach to the treatment of bacterial infection has been an enormous success. However, the widespread use of antibiotics has resulted in bacteria acquiring resistance in addition to their innate tolerance derived from mechanisms such as biofilm formation and drug efflux. Since the discovery of bacterial quorum sensing in a marine bacterium 40 years-ago, similar systems have been discovered in many organisms, including animal and plant pathogens, and these systems have been characterized along with virulence and drug tolerance determinants. Thus, quorum sensing is now regarded as a potential target for the development of antibacterial agents. In the last 20 years, various quorum sensing inhibitors have been isolated and characterized from natural and chemically synthesized libraries. Some animal and plant infection models have demonstrated the antibacterial efficacy of these agents against quorum sensing pathogens. In this paper, we focus on quorum sensing inhibitors as a novel type of antibacterial agent and also provide an update on recent progress in quorum sensing studies. In the first section, we will review the background and literature relating to bacterial cell-to-cell communication, which is currently termed "quorum sensing."

DISCOVERY AND HISTORY

Bacteria are single cell organisms, however, they conduct a bacterial "cell-to-cell" communication activity with the same and/or different species via diffusible chemical compounds, and exhibit group behaviors similar to eukaryotic cells. This concept of social activity between bacteria has been termed "sociomicrobiology" (Parsek and Greenberg, 2005).

Sociomicrobiology was first described in a study carried out in the early 1970s on the bioluminescence phenomenon found in *Vibrio fischeri*, a marine bacterium associated with Hawaiian squid, (Nealson et al., 1970). When the bacteria were grown in shake flasks, expression of the luminescence gene (*lux*) was shown to be relatively low during early exponential growth, but was then followed by a rapid increase in expression during the late exponential and early stationary phases. The luminescence gene in exponential phase cultures can be activated by the addition of cell-free fluid extracts from stationary phase cultures. These observations implied that *Vibrio fischeri* has an environmental sensing system

to monitor its own population density, and a signaling substance termed "autoinducer," which was later shown to be 3-oxohexanoyl-homoserine lactone, activates *lux* expression in high cell density cultures (Eberhard et al., 1981). Currently, over 100 species of bacteria are known to produce autoinducer molecules in a cell density dependent manner similar to *Vibrio fischeri* and this signaling mechanism is now termed "quorum sensing" (Fuqua et al., 1994). Bacteria use three classes of autoinducer for quorum sensing. Acyl-homoserine lactone (AHL) is the most common class of autoinducer used by Gram-negative bacteria, whereas oligopeptide is the major class of autoinducer in Gram-positive bacteria (Dunny and Leonard, 1997). Most of these signals are highly specific and are produced and recognized by a single species. The other class of autoinducer is a 4,5-dihydroxy-2,3-pentanedione (DPD) derivative termed autoinducer-2 (AI-2; Bassler, 2002). It has been suggested that AI-2 is a non-species specific signal which mediates interspecies communication among Gram-negative and Gram-positive bacteria. Although the activity of AI-2 signals has been demonstrated in over 100 species of bacteria, their structures remain largely unknown. Only a few structures of the AI-2 ligand-receptor complex (from *Vibrio harveyi*, *Salmonella* Typhimurium, *Sinorhizobium meliloti*, and *Yersinia pestis*) have been described (Chen et al., 2002; Miller et al., 2004; Pereira et al., 2008; Kavanaugh et al., 2011). In this review, we will focus on the AHL quorum sensing mechanism, as that is the most well defined system of the three quorum sensing families. We will also review progress in the study of effectors which disrupt or attenuate the AHLs-mediated quorum sensing as potential antimicrobial targets.

ELEMENTS AND REGULATORY SYSTEM OF AHLs QUORUM SENSING

The first AHL molecule to be described was 3-oxohexanoyl-homoserine lactone (typically abbreviated 3-oxo-C_6-HSL) from *Vibrio fischeri* (Eberhard et al., 1981). Two elements, a signal generator LuxI and the cognate receptor LuxR, regulate the quorum sensing mediated by 3-oxo-C_6-HSL in this bacterium. The 3-oxo-C_6-HSL is biosynthesized in a catalytic reaction mediated by LuxI (Engebrecht and Silverman, 1984). The molecule can diffuse into and out of cells, and once a threshold concentration is reached, the 3-oxo-C_6-HSL binds the cognate receptor, LuxR (Kaplan and Greenberg, 1985; Hanzelka and Greenberg, 1995). This results in a conformational change in LuxR which leads to the activation of the luciferase genes (*lux*) by binding to a specific DNA sequence on the gene promoter.

Since the discovery of the *luxIR* genes, homologous genes have been identified in more than 100 species of Gram-negative bacteria and in some species their quorum sensing activities have also been demonstrated. AHL molecules contain a common homoserine lactone moiety from *S*-adenosyl-methionine (abbreviated SAM) and a specific fatty acid side chain from the bacterial cellular pool. The side chain varies within different species. Therefore, the specificity for AHL signals is conferred by the length and modifications to the fatty acyl groups. Fatty acyl groups are usually 4–18 carbons in length, and some are modified by a 3-oxo or 3-hydroxy substituent, a terminal methyl branch or various degrees of unsaturation (**Figure 1**.). Recently, a new category of homoserine lactone signals that have non-fatty acid side chain substrates has been reported. They utilize phenyl-carbonic acids derived from plant metabolites, or a branched amino acid generated in the process of bacterial amino acid biosynthesis (Schaefer et al., 2008; Ahlgren et al., 2011; Lindemann et al., 2011).

The signal generator LuxI family protein is an enzyme catalyzing the generation of homoserine lactone molecules from SAM and a specific acyl group. However, this enzyme uses the acyl-carrier protein (ACP)-modified thioester form of carbonic acids as a side chain substrate rather than their free form (Schaefer et al., 1996; Parsek et al., 1999). They also have a low affinity for Coenzyme A (CoA)-modified carbonyl substrates.

The LuxR family protein has dual roles as an AHL receptor and a transcriptional regulator. The protein interacts with specific AHLs at an N-terminal signal receiver domain and forms hydrogen bonds between amino acids in the protein and the AHL molecule, which is then subjected to a conformational change in the C-terminal helix-turn-helix (HTH) domain, which enables it to bind to a conserved inverted repeat DNA sequence termed the "Lux-box" located upstream of the target gene's promoter. LuxR family proteins usually respond to AHLs produced by the corresponding synthase, however, there are some proteins that have a wide range of AHL-binding specificity. For example, CviR, a LuxR homolog from *Chromobacterium violaceum* is able to respond to AHLs with side chains that are C_4 to C_8 in length (Mcclean et al., 1997).

Quorum sensing is known to control a variety of bacterial genes involved in bioluminescence, plasmid transfer, virulence, the biosynthesis of secondary metabolites and antibiotics, and biofilm formation. Comprehensive transcriptome and computational promoter analyses have revealed quorum sensing-controlled genes in several organisms. For example, in *Vibrio fischeri*, only (0.6% of total) genes are controlled by LuxR-3-oxo-C_6-HSL (Antunes et al., 2007), whereas more than 300 genes (6% of total) are regulated during quorum sensing in the opportunistic pathogen *Pseudomonas aeruginosa* (Schuster et al., 2003).

QUORUM SENSING AND BACTERIAL INFECTIOUS DISEASES

Although quorum sensing was originally discovered in a bioluminescence study using a marine organism, extensive studies in this area have been performed with pathogenic bacteria. There is increasing evidence that bacteria use the quorum sensing mechanism to regulate their own virulence genes. Quorum sensing is considered to be a strategic tool enabling bacteria to accomplish their infection processes and survive in the host. The physiological benefit allows the bacterial cells to multiply without displaying overt virulent behavior until a certain threshold population density is reached. As a consequence, a coordinated immunological response by the host is only made when the bacterial population is high, which increases the likelihood that any defenses will be successfully overwhelmed, thereby enhancing the survival prospects of the bacteria. In this section, we will summarize the studies that have investigated the contribution of quorum sensing to bacterial virulence and infectious disease in *Pseudomonas aeruginosa*, which is the quorum sensing pathogen studied in the most detail.

Pseudomonas aeruginosa is an opportunistic pathogen, is commonly associated with nosocomial infections and is infectious in immune-compromised patients. This organism is also known

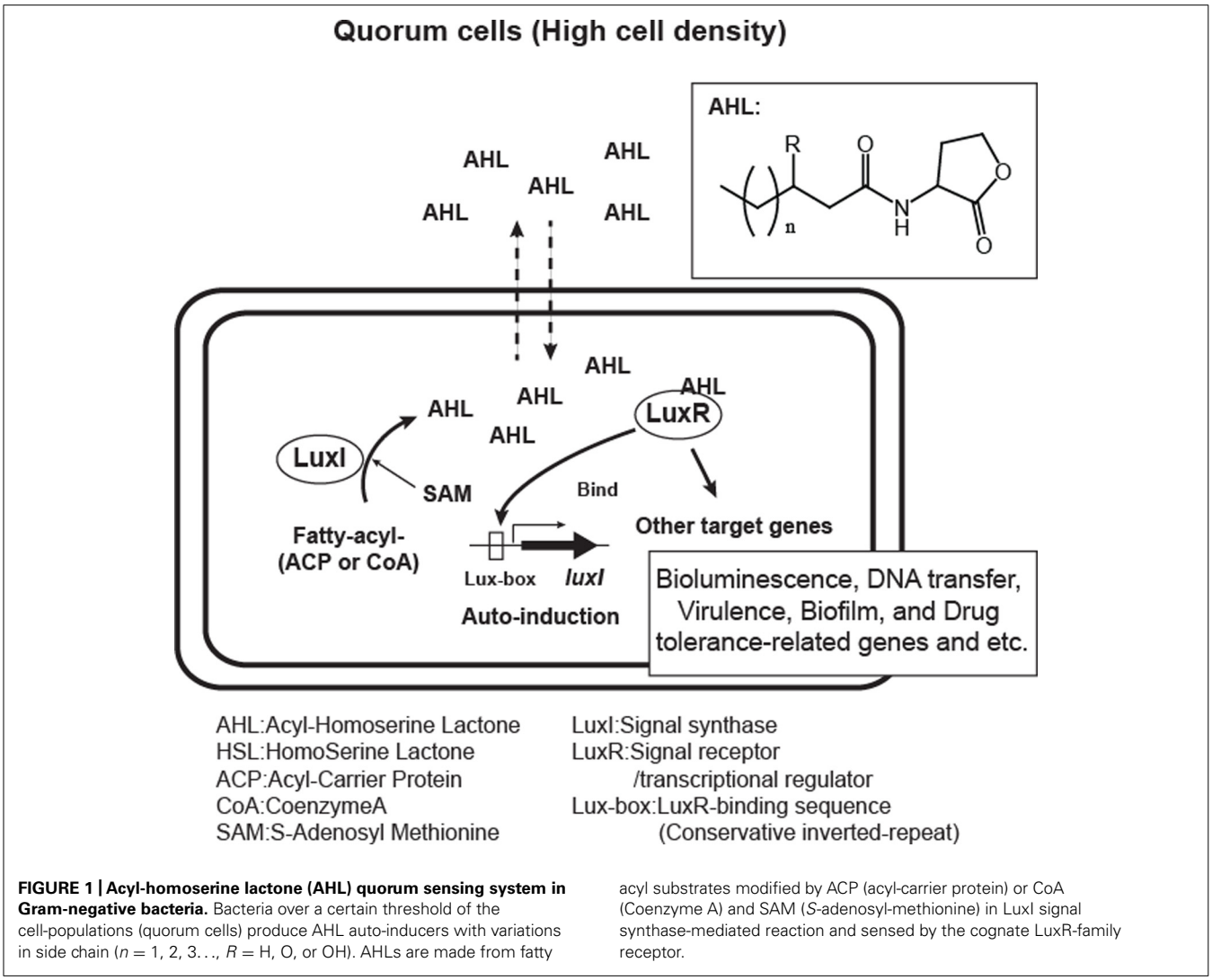

FIGURE 1 | Acyl-homoserine lactone (AHL) quorum sensing system in Gram-negative bacteria. Bacteria over a certain threshold of the cell-populations (quorum cells) produce AHL auto-inducers with variations in side chain ($n = 1, 2, 3\ldots$, R = H, O, or OH). AHLs are made from fatty acyl substrates modified by ACP (acyl-carrier protein) or CoA (Coenzyme A) and SAM (*S*-adenosyl-methionine) in LuxI signal synthase-mediated reaction and sensed by the cognate LuxR-family receptor.

for the chronic infection it causes in individuals with the genetic disease cystic fibrosis (CF) which can result in respiratory failure. In addition, this bacterium is regarded as a "model organism" in the quorum sensing field. The AHL signals produced by *Pseudomonas aeruginosa* are 3-oxododecanoyl-homoserine lactone (3-oxo-C_{12}-HSL; Pearson et al., 1994) and butanoyl-homoserine lactone (C_4-HSL) (Pearson et al., 1995). They are generated by AHL synthases called LasI and RhlI and subsequently bind to the cognate receptors called LasR and RhlR, respectively. This dual quorum sensing system is hierarchical. When the bacterial cell density reaches a particular threshold, the LasIR quorum sensing system is initiated. The 3-oxo-C_{12}-HSL-LasR complex activates *rhlI* expression as well as LasR-controlled genes including *lasI*, the cognate signal synthase, which then leads to activation of the RhlIR system. Either or both the *las* and *rhl* systems activate the production of virulence factors such as elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectines, and superoxide dismutase (Smith and Iglewski, 2003). In addition to LasR and RhlR, there is a third LuxR-family protein named QscR that is a homolog of LasR/RhlR, but does not have the cognate signal synthase (Chugani et al., 2001). QscR can bind with 3-oxo-C_{12}-HSL as well as LasR, and also with heterologous C_{12}, C_{10}, 3-oxo-C_{10}, and 3-oxo-C_6-HSLs (Lee et al., 2006; Oinuma and Greenberg, 2011). Apart from these AHLs, *Pseudomonas aeruginosa* also produces one non-AHL quorum sensing molecule termed "the *Pseudomonas* quinolone signal (PQS)" (Pesci et al., 1999). The PQS is synthesized by PqsABCD and PqsH from anthranilate that is an intermediate in the tryptophan biosynthetic pathway, and responded by PqsR (MvfR), a LysR-like protein. According to some studies, PQS and AHL-quorum sensing (*las* and *rhl*) are interlinked. The production of PQS is activated by *las* system and PQS influences the expression of C_4-HSL-regulated genes in *rhl*-dependent and –independent manners, suggesting that PQS could be also important for virulence of the organism. Highlight of PQS studies is reviewed elsewhere (Diggle et al., 2006; Huse and Whiteley, 2011)

Infection studies with mice have demonstrated the contribution of quorum sensing in the pathogenesis of *Pseudomonas aeruginosa*. *Pseudomonas* strains with mutations in quorum sensing-regulated genes induce less tissue destruction and pneumonia

and result in lower mortality compared with the wild-type (Rahme et al., 1995; Tang et al., 1996; Rumbaugh et al., 1999, 2009). Additional studies using alternative infection models with *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Dictyostelium discoideum* also have illustrated decreases in virulence with quorum sensing mutants (Rahme et al., 1995; Tan et al., 1999; Cosson et al., 2002). These model studies using an acute-infected animal host illustrate the contributions of the quorum sensing system to *Pseudomonas aeruginosa* infections. There are a few reports that *Pseudomonas aeruginosa* quorum sensing is also responsible for chronic lung infections. *Pseudomonas aeruginosa* with mutations of *lasI* and/or *rhlI* showed milder lung infections in mice and rat models (Wu et al., 2001; Imamura et al., 2005). However, the contribution of quorum sensing to chronic infections should be discussed in caution because accumulation of *lasR* mutants has been frequently observed in both many clinical isolates from CF patients and long-term laboratory cultures (Cabrol et al., 2003; Diggle et al., 2007). The *lasR* variants are considered social cheaters (Sandoz et al., 2007; Dandekar et al., 2012). LasR activates genes encoding for extracellular proteases which undertake proteolyses to cooperatively crop common metabolic/energy substrates, reasonably, the LasR-mediated quorum sensing can be promoted under nutrient-limited conditions in the presence of the particular protease substrates. The *lasR* cheaters exploit the social benefit provided by the cooperators (*lasR*-intact parent strain) saving biological costs to conduct their quorum sensing. It might be critical to answer about total quorum sensing activity of the bacterial population in infection sites and frequency of the mutant appearance.

Moreover, quorum sensing has also been shown to influence biofilm development. Biofilm is a biological architecture of aggregated microbes on a surface. It is closely associated with virulence because biofilm cells embedded within an extracellular matrix are less susceptible to antibacterial reagents than free floating cells (Nickel et al., 1985; Mah and O'Toole, 2001; Drenkard, 2003). As a result, biofilm infections tend to be chronic and difficult to eradicate. *Pseudomonas aeruginosa* is the principal pathogen in the lungs of patients with CF. The bacterium is known to exist there as a biofilm and produce significant amounts of quorum sensing molecules (Singh et al., 2000). There is a report that the *Pseudomonas aeruginosa* 3-oxo-C_{12}-HSL signal is involved in the maturation of a biofilm. A *lasI* mutant formed immature biofilms that, unlike wild-type biofilms, were sensitive to sodium dodecyl sulfate (SDS; Davies et al., 1998). Another report predicted that the *rhl* system is also important for biofilm development. The *rhl* defective *Pseudomonas aeruginosa* does not produce the rhamnolipid surfactants that are important for maintaining the biofilm architecture in the later stages (Davey et al., 2003). However, it should be noted that there is some conflict of opinion and confusion regarding the degree of involvement of quorum sensing in biofilm regulation. Biofilm architecture is easily influenced by growth conditions and wild-type, and the quorum sensing mutants might form identical biofilm under certain conditions (See reviews; Parsek and Greenberg, 2005; Parsek and Tolker-Nielsen, 2008). A number of animal infection studies carried out with different objectives have shown that quorum sensing is required for virulence, therefore in the context of virulence, it is generally believed that quorum sensing contributes to the formation of a functional biofilm. Studies of biofilm dynamics to define complexity and trace the development process have being developed over the last 10 years and these will provide insights into the actual role of quorum sensing on biofilm biology. In addition to biofilm development, quorum sensing induces the expression of the drug efflux system MexA-MexB-OprM and confers tolerance to a wide range of antimicrobial agents by extruding them from the cytoplasm (Poole, 2001; Maseda et al., 2004). Thus, quorum sensing is a key factor in determining the success of infection in host animals for *Pseudomonas aeruginosa*

QUORUM SENSING INTERFERENCE

Interfering with quorum sensing is expected to become a powerful strategy to control virulence and antibiotic tolerance in quorum sensing pathogens, and can be applied within antimicrobial chemotherapy to overcome bacterial infections. To date, methods that can be used to disrupt quorum sensing include (1) Antagonizing signal binding to LuxR-family receptor, (2) Inhibition of signal production, (3) Degrading signals, (4) Trapping signals, and (5) Suppression of synthase and receptor activities, stabilities or productions (**Figure 2**). A brief background and recent progress in these studies will be given in the following section.

ANTAGONIST FOR LuxR-FAMILY RECEPTOR

The initial step in quorum sensing is to bind a specific AHL signal to a LuxR protein. Thus, antagonists that interfere with the AHL-the receptor binding are potential quorum sensing inhibitors. Various natural and synthetic compounds have been tested for their antagonistic activity (Chemical structures of representative inhibitors are drawn in **Figure 3**.). In general, analogs are potential antagonists of the native AHL signal. In three early studies, analogs with alternations in the acyl side chain of 3-oxo-C_6-HSL for *Vibrio fischeri*, 3-oxo-C_{12}-HSL for *Pseudomonas aeruginosa* and 3-oxo-C_8-HSL for *Agrobacterium tumefaciens* were demonstrated to inhibit the binding of native AHLs (Passador et al., 1996; Schaefer et al., 1996; Zhu et al., 1998). These studies focused particularly on the length of the acyl side chains. These cognate receptors are able to bind some analogs at a higher affinity than native AHL ligands, but the analogs then inactivate gene expression, thus they are antagonists.

Following earlier studies, a library of synthetic analogs to the *Pseudomonas aeruginosa las* quorum sensing molecule 3-oxo-C_{12}-HSL was constructed by Smith et al. (2003a,b). The homoserine lactone moiety of their compounds was replaced with different alcohols, amines and/or a 5- or 6-membered ring. In their high throughput screening using a *lasI* promoter-fused *gfp* reporter strain, three compounds acted as antagonistics against 3-oxo-C_{12}-HSL-LasR-mediated quorum sensing. 3-Oxo-C_{12}-(2-aminophenol) and 3-oxo-C_{12}-(aminocyclopentanol) are able to inhibit the LasR activation attributed to the 3-oxo-C_{12}-HSL-LasR interaction. On the other hand, 3-oxo-C_{12}-(aminocyclohexanone) appeared to target not only LasR but also RhlR, which is the second quorum sensing receptor in *Pseudomonas aeruginosa*, although this antagonist still has a dodecanoyl (C_{12})-side chain. A synthetic analog of C_4-HSL, *N*-decanoyl cyclopentyl-amide (abbreviated C_{10}-CPA), has also been found to target both LasR and RhlR

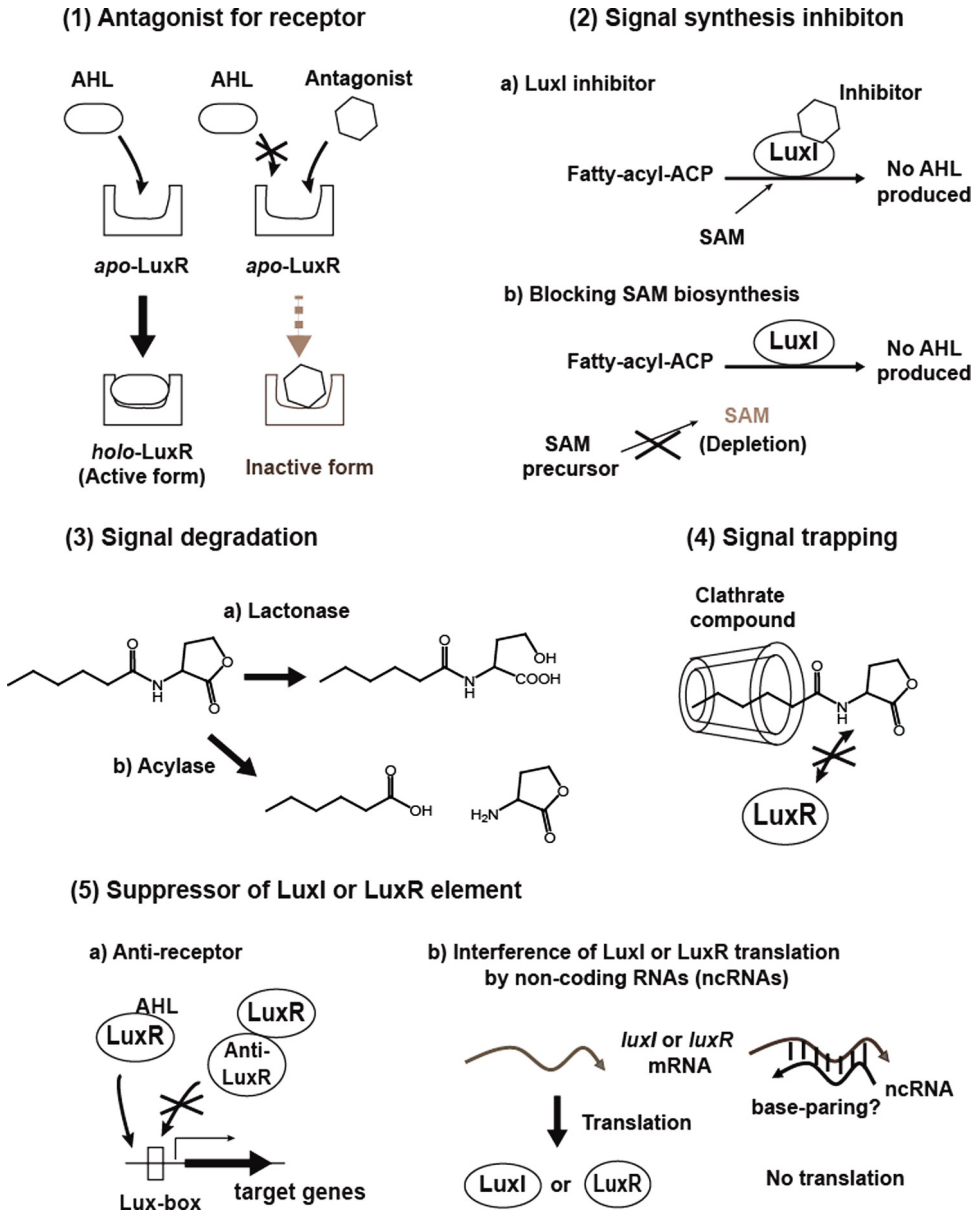

FIGURE 2 | Proposed methods for AHL quorum sensing interference.

proteins (Ishida et al., 2007). C_{10}-CPA inhibits *lasB* and *rhlA* gene activation by 3-oxo-C_{12}-HSL and C_4-HSL with IC50 (half-maximal inhibition) of 80–90 μM, respectively, and inhibition results in reductions in elastase, pyocyanin, and rhamnolipid levels and biofilm formation. Most of these analog compounds are modified with a homoserine lactone moiety and/or a side chain. Changes in the amide function bridging the lactone ring and the fatty acid also influences the AHL binding activity to receptor proteins, since the amide forms hydrogen bonds with a conserved tyrosine and aspartic acid in the AHL binding pocket (Vannini et al., 2002; Zhang et al., 2002). Changing the amide to a sulfonamide and/or a urea has been predicted to result in the formation of an additional hydrogen bond between a tyrosine residue in the ligand pocket and the sulfonamide, and to strengthen the hydrogen bond between the aspartic acid and the external NH of urea (Castang et al., 2004; Frezza et al., 2006, 2008). Compounds with either or both modifications have showed antagonistic behaviors with the *Vibrio fischeri* LuxR receptor. In recent studies, selective and broad-spectrum antagonists active across multiple species have also been developed. C_8-HSL, C_{10}-HSL, 4-bromophenylpropionyl-HSL and 4-iodophenylacetyl-HSL simultaneously antagonize the AHL-bindings to the receptor proteins of TraR in *Agrobacterium tumefaciens*, LuxR in *Vibrio fischeri* and LasR in *Pseudomonas aeruginosa*, while several other analogs work on two of these species (Geske et al., 2007).

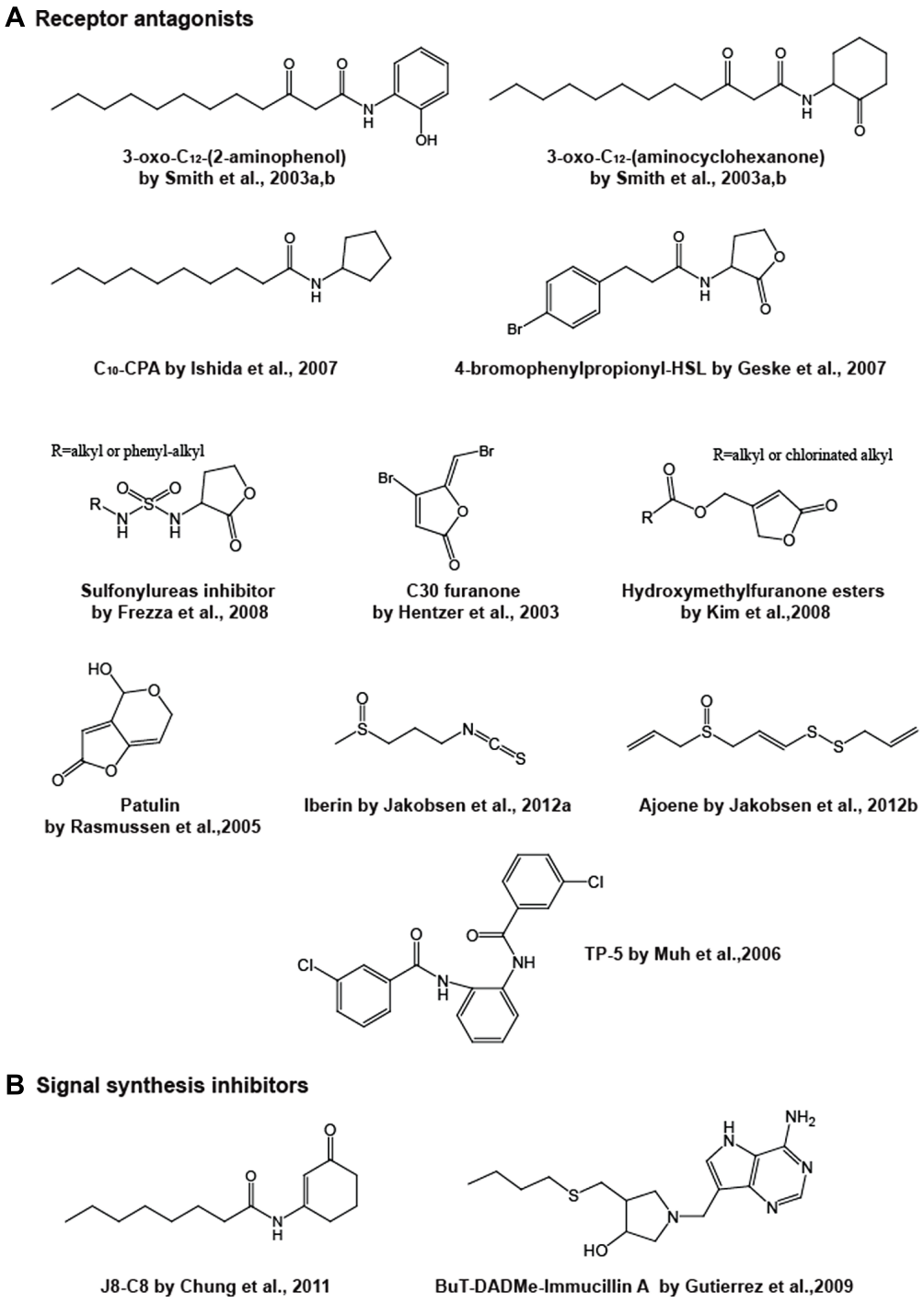

FIGURE 3 | Chemical structures of representative receptor antagonists (A) and signal synthesis inhibitors (B).

Some natural compounds have also been demonstrated to behave as antagonists. Halogenated acyl-furanones, which are structurally similar to AHLs, and are derived from the marine algae *Delisea pulchra* are one of the most-studied antagonist groups (Givskov et al., 1996). These naturally occurring compounds displace the 3-oxo-C_6-HSL signal from its cognate LuxR receptor protein, thus inhibiting the quorum sensing-mediated gene expression (Manefield et al., 1999). The SwaR receptor for C_4-HSL in *Serratia liquefaciens* is also a target for the antagonists, and the failure of C_4-HSL-SwaR interaction results in a reduction in the swarming motility linked to surface colonization and biofilm formation (Rasmussen et al., 2000). Gram et al. (1996) have reported

that a furanone isolated from the furanones pool of secondary metabolites produced by *Delisea pulchra* inhibits swarming motility in *Proteus mirabilis* without affecting cell growth and swimming motility, whereas other furanones have no inhibitory activity. However, the regulatory target in this system is not known. Although natural furanones have a limited inhibitory effect on the quorum sensing of *Pseudomonas aeruginosa*, the synthetic analogs "C30" and "C56," which lack the alkyl side chain, exhibit interference in the LasR-mediated *Pseudomonas aeruginosa lasB* gene expression (encoding the elastase that is associated with the virulence), increase the susceptibility to antimicrobial agents on biofilm cells, promote the bacterial clearance on the lung of infected mice and prolong the survival time of the mice (Hentzer et al., 2002, 2003; Wu et al., 2004; Christensen et al., 2012). Taha et al. (2006) have discovered three LasR antagonists by *in silico* screening with pharmacophore modeling utilizing the authentic furanone inhibitors C-30 and C-56 as leading compounds. These are phenyl compounds incorporating a mercury or lead atom bound by covalent bonds and have been shown to inhibit quorum sensing-driven pyocyanin and pyoverdin production. Subsequent studies have developed other furanone derivatives based on natural furanone core structures. A series of hydroxymethylfuranone esters condensed with fatty acids that have a modified carbon length or are chlorinated at the terminal has been synthesized (Kim et al., 2008). These compounds have been shown to repress the LasR-driven reporter expression in a *lasI-lacZ* fusion by competing with exogenous 3-oxo-C_{12}-HSL binding to a recombinant LasR in *Escherichia coli*, and they also inhibit biofilm formation on the flow cell system in *Pseudomonas aeruginosa*. The authors have built the inhibitor-LasR protein docking models. According to the *in silico* modeling analyses, the inhibitors are predicted to bind preferentially to the receptor rather than the natural ligand, but fail to change the conformation of LasR to the "active" form, which suggests they have antagonistic activities. In the same year, another group reported a series of furanone antagonists whose structures are closer to AHL. They replaced the homoserine lactone moiety of AHL with a furanone. These molecules dock with the LuxR protein at its binding pocket (Estephane et al., 2008). However, we should note a report that halogenated furanones destabilize LuxR receptors rather than antagonizing (Manefield et al., 2002). Therefore, as for exact action mode of this type of inhibitors, there is still some confusion. Remarkably, a very recent study has highlighted the possibility that furanone resistance might arise in *Pseudomonas aeruginosa* (Maeda et al., 2012). Maeda et al. (2012) have observed that a mutation in the *mexR* gene in *Pseudomonas aeruginosa* decreases its susceptibility to the synthetic C-30 furanone. As the *mexR* gene product functions as a repressor for the *mexAB-oprM* operon encoding a multidrug efflux system, the inactivation of *mexR* gene presumably leads to overexpression of MexAB-OprM, thereby enhancing the efflux of the furanone inhibitor.

In addition, other natural AHL inhibitors have been identified, such as patulin and penicillic acid produced by fungi (Rasmussen et al., 2005), iberin from horseradish extracts (Jakobsen et al., 2012a) and ajoene from garlic (Jakobsen et al., 2012b). These natural products inhibit *Pseudomonas aeruginosa* quorum sensing of either or both the *las* and *rhl* systems. Patulin and penicillic acid have structures reminiscent of the furanone compounds originally discovered in *Delisea pulchra,* whereas iberin and ajoene are structurally unrelated linear sulfide compounds, but can compete with *Pseudomonas aeruginosa* AHLs for binding to LasR or RhlR.

Aside from AHL structural analogs, a research group has identified three inhibitors for the LasR protein in combination studies by screening a library of 200,000 compounds and *in silico* structure modeling. A tetrazole and a phenyl ring compound with a common 12-carbon alkyl tail designated "PD12" and "V-06-018", respectively, were isolated from the library (Muh et al., 2006b). Both compounds inhibited LasR-controlled gene expression and expression of the virulence factors elastase and pyocyanin. A triphenyl compound designated "TP-5" was originally predicted to be an antagonist by the LasR-ligand docking model built from the 3D-structure of the ligand-bound TraR protein in *Agrobacterium tumefaciens* (Vannini et al., 2002; Zhang et al., 2002), which is highly homologous to LasR. Interestingly, it is more likely that TP-5 interacts with LasR by forming a hydrogen bond with Asp-73 of the polypeptide and exhibits LasR-inhibitory activity despite being structurally unrelated to the natural ligand. This compound was derived from a parent compound designated "TP-1" from their library of compounds. TP-1 mimics 3-oxo-C_{12}-HSL activity, and therefore acts as an agonist for LasR (Muh et al., 2006a).

Information on the 3D-structures of the LuxR-family receptors has enabled us to discover a variety of antagonists. However, this information is not enough. We need to know how the conformational change in the receptor occurs to distinguish between a "true ligand" (agonist) and "fake ligand" (antagonist). The crystal structure only provides a snapshot of the information required. Although the structures of receptor-AHL binding form are available for a few species, we need to obtain both structures; an antagonist-binding form and a ligand-free form. Recently, the crystal structure of CviR from *Chromobacterium violaceum* with an antagonist has been reported and so far this is the only example (Chen et al., 2011). On the other hand, analysis of the signal-free form is currently a difficult task because LuxR-family proteins are considered to be unstable in the absence of the cognate signal, and are eventually subjected to proteolysis (Zhu and Winans, 2001). However, although a major challenge, it is important to gain information about these structures.

SIGNAL SYNTHESIS INHIBITION

Interfering with AHL synthesis is another straightforward approach to the inhibition of quorum sensing. Simply, if no AHL is produced, no quorum sensing occurs. Early studies on quorum sensing inhibitors have focused on antagonists of AHL receptors. However, there have been few studies on inhibitors of AHL synthesis and data is very limited. In one of these studies, it was found that several analogs of SAM, which is the second substrate for LuxI synthases, inhibit the LuxI reaction (Parsek et al., 1999). However, two breakthrough studies have been recently carried out. The first study identified a C_8-HSL analog that binds to AHL synthase, thereby inhibiting its enzymatic activity (Chung et al., 2011). The second study described compounds targeting the reaction activity of 5′-methylthioadenosine nucleosidase (MTAN, alternatively abbreviated "Pfs") involved in SAM recycling (Gutierrez et al., 2009).

The compound in the first study is *N*-(3-oxocyclohex-1-enyl)octanamide (named J8-C8) (**Figure 3**), which was isolated from Smith's library (Smith et al., 2003a) and was characterized as an inhibitor for TofI, a C_8-HSL generator of the LuxI-family protein in *Burkholderia glumae* (Chung et al., 2011). J8-C8 significantly inhibited C_8-HSL production by TofI in a dose dependent manner, furthermore the effect was enhanced in the presence of MTA. Generally, the LuxI-family protein synthesizes a specific AHL from an acylated ACP and SAM, eventually AHL is released with holo-ACP and 5′-methylthioadenosine (MTA) as by-products from the protein. In a TofI/J8-C8/MTA ternary crystal structure, J8-C8 binds to TofI occupying the binding site for the acyl chain of the cognate substrate C_8-ACP. Simultaneously, a second substrate (MTA) binds to the binding site for SAM, which accounts for the synergistic effect of MTA.

The latter study describes AHL synthesis inhibitors that are transition state analogs of MTAN. The MTAN enzyme catalyses the hydrolytic deadenylation of MTA and *S*-adenosyl homocysteine (SAH) and produces 5′-methylthioribose (MTR) and *S*-ribosylhomocysteine (SRH) which are steps in SAM biosynthesis. MTAN enables AHL-producing bacteria to recycle SAM from the MTA released as a by-product after AHL syntheses. In addition, SRH also becomes a precursor for AI-2 generation (Xavier and Bassler, 2003). Thus, the MTAN inhibition provides a method of blocking not only AHL production, but also AI-2. Three analogs of the transition state during the reaction from the MTA substrate into MTR and adenine, have been designed and are named 5′-methylthio- (MT-), 5′-ethylthio- (EtT-), and 5′-butylthio- (BuT-) DADMe-ImmucillinAs (**Figure 3**; Gutierrez et al., 2009). According to a 3D-structural analysis of MTAN in *Vibrio cholerae* with BuT-DADMe-ImmucillinA, the inhibitor binds to the catalytic active site of the protein producing hydrophobic stacking interactions. These analogs, including BuT-DADMe-ImmucillinA, have been shown to inhibit MTAN activity with IC50 values at the nM level and reduce AI-2 production and biofilm formation in *Vibrio cholerae* and *Escherichia coli* O157. Although this study principally described the AI-2 effect, the researchers might investigate AHL quorum sensing in the near future.

DEGRADATION ENZYMES

In addition to small molecules which interfere with signal sensing or generation, signal breakdown by catalytic enzymes is an alternative strategy. Two classes of enzymes, lactonase and acylase, are known to perform this function. The former is a catalytic enzyme that cleaves the homoserine lactone ring and the latter catalyzes the hydrolysis of an amide bond between the homoserine lactone moiety and a fatty-acyl group. The degraded AHL products are no longer active in quorum sensing, therefore the phenomenon is often called "quorum quenching." A lactonase was originally identified and purified from a Gram-positive *Bacillus* strain and the enzyme was designated "AiiA," meaning autoinducer inactivation. The protein sequence has no significant similarity to any known sequences, but contains a HXHXDH zinc-binding motif that is conserved in glyoxalase II, metallo β-lactamase and arylsulfatase (Dong et al., 2000). The purified AiiA protein cleaves the homoserine lactone ring in C_4 to C_{12}-HSLs, with or without substitution at carbon three position including 3-oxo-C_6-HSL produced by a plant pathogen, *Erwinia carotovora*. Heterologous expression of the *aiiA* gene in *Erwinia carotovora* resulted in a remarkable decrease in quorum sensing-activating gene expression and less virulence to plants. Also, *aiiA*-expressing transgenic tobacco and potato were tolerant to the bacterial infection (Dong et al., 2001). There are similar bioengineering studies utilizing AiiA lactonase. For example, AiiA overexpression in *Pseudomonas aeruginosa* and *B. thailandensis* impaired their quorum sensing activities through the degradation of signals (Reimmann et al., 2002; Ulrich, 2004). Following these AiiA studies, *aiiA* homologs genes have been identified from other species such as *Agrobacterium tumefaciens* (Zhang et al., 2002) and *Arthrobacter* sp., (Park et al., 2003) and their enzymatic activities have been demonstrated. In addition, a subclass of AHL lactonases has been recently discovered in a species of soil bacterium. Unlike AiiA, they have no conserved HXHXDH zinc-binding motif. For example, QsdA in *Rhodococcus erythropolis* is a phosphotriesterase (PTE)-like protein that has other zinc-binding domains instead of the HXHXDH motif and can degrade AHLs which have an acyl chain of C_6–C_{14} in length (Uroz et al., 2008). However, unlike typical PTE enzymes, the protein is unable to cleave the phosphotriester bond. AiiM in *Microbacterium testaceum* isolated from a potato leaf (Wang et al., 2010) and three BpiB isomer proteins from soil metagenomic clones (Schipper et al., 2009) do not have any putative zinc-binding domains. AiiM has been deduced to belong to alpha/beta hydrolase fold family. The protein prefers C_6 to C_{12}-HSLs with 3-oxo substitution to those without substitution as degradation substrates. The expression of AiiM in the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum,* which is a 3-oxo-C_6-HSL producer, reduced virulence against the potato tissue (Wang et al., 2010). The series of BpiB proteins were originally isolated from soil-derived metagenomic libraries with a *traI-lacZ Agrobacterium tumefaciens* reporter strain that can respond to 3-oxo-C_8-HSL, then protein expressing clones which attenuated *traI-lacZ* activity were isolated. Two out of the three BpiB proteins, designated BpiB01 and BpiB04, show no similarity to any known proteins while the other protein, BpiB07 shares some sequence similarity with the esterase-lipase superfamily proteins. In addition to the degradation of 3-oxo-C_8-HSL, all the clones inhibited *Pseudomonas aeruginosa* swarming motility and biofilm formation controlled by 3-oxo-C_{12}-HSL and C_4-HSL (Schipper et al., 2009). AHL lactonase was originally characterized as a zinc-binding protein and it has been shown that mutations in the HXHXDH zinc-binding domain in some AiiA family lactonases result in them losing their function. However, based on their amino acid sequences, it is still unclear whether AiiM and BpiBs bind zinc. Recently, a new type of AHL lactonase has been isolated from the marine bacterium *Pseudoalteromonas byunsanensis*. The identified ORF appears to encode a hybrid membrane protein that has a GDSL (consensus Gly-Asp-Ser-Leu motif) hydrolase domain at the N-terminal of a RND (resistance-nodulation-cell-division)-type multidrug efflux transporter (Huang et al., 2012). The truncated form, including the GDSL hydrolase function, is designated as QsdH and has a catalytic activity for the C_4 to C_{12}-HSLs (with or without 3-oxo substitution) lactonase reaction, and co-inoculation of the plant pathogen *Erwinia carotovora* with a recombinant QsdH-overexpressing *Escherichia coli* has resulted in

milder lesions on potato tissues compared to a co-inoculation without QsdH.

In addition to environmental microorganisms, mammalian enzymes also have AHL lactonase activities (Chun et al., 2004). Human has three paraoxygenases (PON1, PON2, and PON3) with a distinct substrate specificity and expression pattern. There are reports that they cleave lactone rings in a series of AHLs (Draganov et al., 2005; Ozer et al., 2005)

The other family of AHL degradation enzymes is AHL acylase. This was first described in *Variovorax paradoxus*, although the gene which is responsible for the reaction has not been yet identified (Leadbetter and Greenberg, 2000; Leadbetter, 2001). The organism was isolated from soil based upon its ability to utilize 3-oxo-C_6-HSL as both an energy and nitrogen source. Hypothetically, AHLs is initially cleaved into a fatty acid and homoserine lactone moiety by an uncharacterized acylase in first reaction step, and subsequently the fatty acid is subjected to beta-oxidation as an energy material, while the homoserine lactone is degraded into ammonium chloride and carbon dioxide. The first AHL acylase to be characterized is AiiD from *Ralstonia eutropha* (Lin et al., 2003). The polypeptide is most similar to the aculeacin A acylase (AAC) from *Actinoplanes utahensis* and it also shares significant similarities with the cephalosporin and penicillin acylases, which are members of the N-terminal (Ntn) hydrolase superfamily. AiiD has been purified as a glutathione *S*-transferase (GST) fusion protein and its AHLs cleavage spectrum has been investigated. The GST-AiiD protein effectively hydrolyzes an amide bond on AHLs with longer fatty acyl side chains, such as 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL and 3-oxo-C_{12}-HSL whereas it is less active against shorter side chain substrates as 3-oxo-C_6-HSL. Heterologous AiiD expression in *Pseudomonas aeruginosa* has also been shown to abolish the accumulation of both 3-oxo-C_{12}-HSL and C_4-HSL and the killing of *Caenorhabditis elegans*. Based on the AiiD sequence, homologues have been identified in other organisms. Three acylases in *Pseudomonas aeruginosa* and closely related species, designated PvdQ, QuiP, and PA0305 (alternatively named HacB), respectively, are well-characterized (Huang et al., 2003, 2006; Wahjudi et al., 2011). They participate in the degradation of 3-oxo-C_{12}-HSL, but not C_4-HSL. However, their expression is considered to be highly regulated and turned off in standard experimental conditions (usually aerobically growth at 37 degree in rich medium) because wild-type *Pseudomonas aeruginosa* accumulates a large amount of 3-oxo-C_{12}-HSL during early stationary phase. When these acylases are constitutively produced from exogenous plasmids, a significant reduction of 3-oxo-C_{12}-HSL accumulation in the medium is observed. Some other homologous AHL acylases have also been identified in *Streptomyces* sp. from soil samples, the fish-associated bacterium *Shewanella* sp. and the nitrogen-fixing cyanobacterium *Anabaena* sp. (Park et al., 2005; Morohoshi et al., 2008; Romero et al., 2008). These studies provide us with not only extensive ideas for quorum sensing inhibitor applications, but also stimulate our general biological interest as to why AHLs-degrading organisms are widespread in nature. Thus far, the physiological benefits of degradation enzymes are presumed to be specific to AHL utilization as a nutrient resource, the detoxification of lactone ring compounds, the jamming of quorum sensing in pathogens as an innate bio-defense mechanism, and the modulation of the quorum sensing activity.

SIGNAL TRAPPING

An alternative technique for the attenuation of quorum sensing based on the trapping of AHLs has been created. This method arose from the observation that quorum sensing does not occur when the AHL concentration is maintained below a threshold level, thus an AHL interceptor would act as a quorum sensing inhibitor. Cyclodextrins are well known to form stable aqueous complexes with many organic compounds. In an initial study, C_4-HSL was reported to be an entry substrate for a cyclodextrin donor, and a bacterial culture containing the cyclodextrin suppressed RhlR-activated *rhlA* gene expression in *Pseudomonas aeruginosa* (Ikeda et al., 2002). The C_6-HSL, C_7-HSL, C_8-HSL, and 3-oxo-C_6-HSL from *Serratia marcescens* and C_4-HSL from *Pseudomonas aeruginosa* can be trapped and this results in the decrease in the production of the quorum sensing-induced red-pigment (prodigiosin; Kato et al., 2006). Currently, the use of cyclodextrin as a method of quorum sensing interference is still immature, although it is well studied as a cholesterol remover and as a carrier for medical applications such as in Niemann–Pick disease (See review Vance and Peake, 2011). For applications to antibacterial and bio-fouling materials, further technical studies are required, for example, a chemical engineering approach to increase the solubility and the stability of the cyclodextrin-AHL inclusion complex may be successful in the future.

UNKNOWN MECHANISM: MACROLIDE ANTIBIOTICS AS A CASE OF STUDY

Macrolide antibiotics have been shown to inhibit *Pseudomonas aeruginosa* quorum sensing. Since macrolides are relatively hydrophobic and are large sized-molecules, these antibiotics are generally believed to be ineffective against Gram-negative bacteria due to their low permeability and exclusion from the bacterial cytoplasm (Nikaido and Vaara, 1985; Nikaido, 1996). However, surprisingly, there have been reports from clinical trials showing that long term treatment with macrolide antibiotics at sub MIC eases the chronic lung infectious diseases caused by *Pseudomonas aeruginosa* in patients with CF and diffuse panbronchiolitis (DPB; Keicho and Kudoh, 2002; Southern et al., 2011). A number of mechanisms for the macrolide action on the bacterium and host have been proposed (See reviews, Tateda et al., 2007; Kanoh and Rubin, 2010). One proposal is that the drug influences quorum sensing. Azithromycin, the 15-membered ring macrolide has been shown to repress the activity of *Pseudomonas aeruginosa* quorum sensing based on both the levels of 3-oxo-C_{12}-HSL and C_4-HSL syntheses and the expression of *las* and *rhl*-activated gene/protein such as elastase, rhamnolipid, and pyocyanin (Tateda et al., 2001; Wagner et al., 2005; Nalca et al., 2006; Skindersoe et al., 2008). The azithromycin efficacy at sub-MIC is presumably attributed to the reduction of 3-oxo-C_{12}-HSL and C_4-HSL levels because a subset of genes involving SAM biosynthesis is partly repressed by azithromycin (Kai et al., 2009). Elucidation of its exact molecular action and target is the next question to be answered.

SUPPRESSORS OF QUORUM SENSING

It is known that some quorum sensing bacteria have regulatory elements which impede their quorum sensing. It is speculated that the physiological implications of an intrinsic modulation mechanism in quorum sensing is a tightly controlled repression of quorum sensing-controlled genes under a threshold population, a delay in quorum sensing initiation, and a slowing of its regulatory circuit or fine-tuning of its activity at a specific level. Apart from their actual roles, it might be possible to apply these suppressors to a quorum sensing inhibitory method, because if we are able to artificially manipulate the function and cellular level of these elements, quorum sensing will be controlled.

Anti-LuxR activators inhibiting quorum sensing activation have been reported in *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa.* TrlR from *Agrobacterium tumefaciens* is a homologue of TraR, an AHL receptor protein, but lacks a DNA binding domain (Chai et al., 2001). The protein forms an inactive heterodimer with TraR. The other anti-TraR proteins, TraM and its homologue TraM2, also interact with TraR to prevent its DNA binding (Fuqua et al., 1995; Hwang et al., 1995; Swiderska et al., 2001; Wang et al., 2006). These mutants confer constitutive AHL signal accumulation even in the absence of octopine, which is a quorum sensing initiator, and also confer hyper-plasmid conjugative transfer efficiency with excessive activation of the quorum sensing. Like anti-TraR in *Agrobacterium tumefaciens*, QslA is an anti-LasR protein in *Pseudomonas aeruginosa* (Seet and Zhang, 2011). The *qslA* null mutant is able to respond to much lower levels of the quorum sensing signal than the parent, resulting in higher quorum sensing activity, such as elevated exo-protease and elastase production. QscR, an orphan LuxR-family protein in *Pseudomonas aeruginosa,* inhibits a number of LasR- RhlR-activated genes by protein–protein interaction with LasR and RhlR, respectively, and suppresses virulence in a *Drosophila* infection model (Chugani et al., 2001; Ledgham et al., 2003). The unique small protein QteE controls the stability of LasR protein in *Pseudomonas aeruginosa,* but affects neither its transcription nor translation (Siehnel et al., 2011). In the absence of the *qteE* gene, LasR is more stable at a low cell density culture and overproduction of QteE reduces the LasR stability. In addition to these anti-activators, RsaL is a repressor and simultaneously binds to the *lasI* promoter with LasR in *Pseudomonas aeruginosa* (Rampioni et al., 2006, 2007). The *rsaL* mutant results in unlimited 3-oxo-C_{12}-HSL production, however, overproduction of the protein produces a lower level of virulence proteins, thereby RsaL manages homeostasis of the quorum sensing.

Non-coding regulatory RNAs are also involved in quorum sensing suppression. YenS from *Y. enterocolitica* is a non-translated *trans*-RNA (Tsai and Winans, 2011). At low cell densities, the signal-free receptor protein apo-YenR activates the *yenS* transcription binding to a particular sequence on the *yenS* promoter. The YenS base-pairs with 5′ region of the signal generator YenI mRNA and then inhibits YenI translation. At high densities, a signal-bound YenR (holo-YenR) cannot do so, resulting in the induction of the quorum sensing. Thus, YenS is a suppressor for the quorum sensing in *Y. enterocolitica.* The photosynthetic soil bacterium *Rhodopseudomonas palustris* produces a non-coding *cis*-RNA that affects the quorum sensing signal receptor expression (Hirakawa et al., 2012). The *cis*-RNA (named asrpaR) is an anti-sense transcript of *rpaR*, a *luxR*-family signal receptor gene. The transcript is induced by the quorum sensing signal *p*-coumaroyl-HSL and the RpaR protein. asrpaR inhibits RpaR translation, presumably by base-pairing with sense transcripts, thus suppressing the quorum sensing activity. Off-targeting technology to disrupt specific target functions utilizing RNA interference with siRNA (small interfering RNA) is undergoing extensive development in the mammalian area, but the major challenge of developing therapeutic applications is currently ongoing. Studies on RNA interference will also be carried out on bacteria.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Blocking bacterial cell-to-cell communication activity is a novel strategy in antibacterial therapy. In the last 20 years, several approaches to disrupting quorum sensing have been attempted, and these include antagonizing signal sensing, inhibition of signal generation, inactivation of signals, and a variety of agents have been discovered from natural and synthetic libraries. In addition, there are inhibitors like the macrolides where the mode of action has not yet been addressed. The aim of inhibiting quorum sensing is to suppress bacterial virulence and reduce drug resistance/tolerance accompanied with quorum sensing-activated biofilm formation and other innate bio-defense mechanisms by means other than killing bacteria. This strategy is the opposite of bacteriocidal therapies using antibiotics. The benefits might be the suppression of the development of antibiotic resistance, and the ability to expand chemotherapeutic strategies to combat multidrug resistant (MDR) pathogens. Some of the inhibitors have been evaluated in animal and plant infection models. However, there are many hurdles to overcome for this approach to be used in clinical applications. For example, do these agents only target quorum sensing without any critical and unexpected side effects in addition to their pharmacokinetics (ADME: absorption, distribution, metabolism and excretion)? If they do, can they be administrated with authentic antibiotics to promote healing against multidrug-resistant infections? Recently, a pilot study in clinical trial has been made (Smyth et al., 2010). They used garlic as a quorum sensing inhibitor for 13 CF patients, however, no significant effects were observed compared to placebo group. As they suggested in the preliminary study, reorganization of the study with some modifications (for example, to test in a larger trial) should be necessary. We also need to investigate the potential for selective pressure by quorum sensing inhibition (As we mentioned above, there is a report describing a *mexR* mutation that increases resistance to a furanone inhibitor). Quorum sensing cheater with mutations will be also a critical issue to keep in mind. We will need to answer these questions in the near future to enable us to use these agents as novel antibacterial agents.

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Design of a large-scale femtoliter droplet array for single-cell analysis of drug-tolerant and drug-resistant bacteria

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Single-cell analysis is a powerful method to assess the heterogeneity among individual cells, enabling the identification of very rare cells with properties that differ from those of the majority. In this Methods Article, we describe the use of a large-scale femtoliter droplet array to enclose, isolate, and analyze individual bacterial cells. As a first example, we describe the single-cell detection of drug-tolerant persisters of *Pseudomonas aeruginosa* treated with the antibiotic carbenicillin. As a second example, this method was applied to the single-cell evaluation of drug efflux activity, which causes acquired antibiotic resistance of bacteria. The activity of the MexAB-OprM multidrug efflux pump system from *Pseudomonas aeruginosa* was expressed in *Escherichia coli* and the effect of an inhibitor D13-9001 were assessed at the single cell level.

Keywords: single-cell analysis, microdevice, drug tolerance, persister, drug resistance, drug efflux, transporter

INTRODUCTION

Opportunistic infection with bacteria resistant to multiple antibiotics is a continuing clinical challenge (Taubes, 2008). The antibiotic resistance of bacteria can be classified into two categories, natural resistance (tolerance) and acquired resistance. In natural resistance, a very small proportion of the bacterial population is resistant to multiple antibiotics despite having the same genotype as the sensitive majority. These bacteria are often referred to as "persisters" (Lewis, 2010). However, the nature of these persisters is not fully understood because they occur at a very low frequency in a bacterial population (typically less than 1%), which makes systematic studies difficult (Allison et al., 2011; Balaban, 2011, Gerdes and Maisonneuve, 2012; Kint et al., 2012). In the first section of this review article, we describe a microdroplet-based method to identify and culture individual bacterial cells for efficient detection of persisters. In contrast, acquired antibiotic resistance is caused by a change in the genotype of the sensitive strain. The four main mechanisms of acquired resistance are suppression of drug influx into the cell due to decreased expression of membrane channel proteins, inactivation of drugs by intracellular and extracellular enzymes, mutations in the target proteins of drugs, and active efflux of the drugs from the cell due to increased expression of efflux pumps (Fischbach and Walsh, 2009; Nikaido, 2009). Here we focused on the active efflux of drugs from the cell. In the second section, we introduce a microdroplet-based method for assessing the drug efflux activity of single bacterial cells.

ADVANTAGES OF SINGLE-CELL ANALYSIS USING A MICRODEVICE

Single-cell analysis is a powerful approach for detecting variations among the cells in a population, such as differences in the expression of proteins, the copy number of genes, and the concentration of metabolites (Li and Xie, 2011; Trouillon et al., 2013). Single-cell analysis can overcome the limitations associated with ensemble-averaged data from multiple cells, and enable the identification of very rare cells with properties that differ from those of the majority. Microfabricated devices have contributed greatly to the development of massively parallel and high-throughput single-cell analyses.

However, in most microdevices, the target cells are eukaryotic, such as mammalian cells and yeasts (Sims and Allbritton, 2007; Gupta et al., 2010, Lindstrom and Andersson-Svahn, 2010), because their size, a few millimeters to tens of micrometers, allows for easy handling compared to bacteria, which are much smaller in size. Thus far, only a few studies have used microdevices for single bacterial cell analysis (Balaban et al., 2004; Cai et al., 2006, Ottesen et al., 2006; Weibel et al., 2007, Boedicker et al., 2009; Teng et al., 2013, Wakamoto et al., 2013). In addition, in many microdevices that are based on microfluidic channels and valves or droplets generated in a microfluidic channel, the closed nature of the system makes the collection of cells from the device and their subsequent use difficult. Therefore, the development of microdevices from which individual bacterial cells can be recovered has been highly anticipated.

LARGE-SCALE FEMTOLITER DROPLET ARRAY FOR SINGLE BACTERIAL CELL ANALYSIS

We recently developed a micron-sized femtoliter droplet array fixed on a hydrophilic-in-hydrophobic micropatterned surface (Sakakihara et al., 2010). In our new microdevice, a large number of dome-shaped femtoliter droplets can be prepared that enclose individual bacterial cells (Iino et al., 2012a). One prominent feature of this array is that the individual droplets containing the enclosed cells can be accessed and collected with a micropipette. The array can also be used for mass culture and gene and protein analyses.

We prepared the hydrophilic-in-hydrophobic micropatterned surface through conventional microfabrication (**Figure 1A**). A hydrophobic polymer of carbon-fluorine (CYTOP; Asahi Glass, Japan) was deposited on a SiO_2 cover glass, and photolithography was performed using a high-viscosity photoresist. The resist-patterned substrate surface was dry-etched with O_2 plasma by using a reactive ion-etching system to produce circular micropatterns on a hydrophilic SiO_2 glass surface. The diameter of the exposed hydrophilic SiO_2 surfaces was 10–30 μm, and they were surrounded by a hydrophobic polymer layer with a height of 1 μm. A fabricated micropatterned cover glass was attached to the bottom of a perforated petri dish (**Figure 1B**). The circular micropatterns were grouped into islands and were numbered to identify the individual droplets and the cells enclosed in each droplet.

To form a droplet array containing bacteria, we covered the micropatterned cover glass with medium containing a bacterial suspension (**Figure 1C**, left). Then, fluorinated oil (Fluorinert FC-40; Sigma Aldrich, USA), which has a higher density than water, was flowed over the medium near the surface. The hydrophilic SiO_2 glass surfaces retained the medium and the bacteria, whereas the hydrophobic surface was replaced with oil. As a result, many droplets containing one or more bacteria were formed (**Figure 1C**, right). More than 3×10^5 droplets could be simultaneously prepared in a 1-cm^2 area in a single device. Enclosure of the cells in the droplets was stochastic and was dependent on the cell density of the bacterial suspension. At an optical density (turbidity) at 600 nm (OD_{600}) of 0.6, approximately 20–30% of the droplets contained single cells. Increasing the diameter of the hydrophilic surfaces to 20 or 30 μm increased the fraction of droplets containing multiple cells; however, the fraction of droplets containing single cells did not increase significantly (**Figure 1D**). In contrast, the number of droplets formed increased significantly when we used a device with hydrophilic surfaces of a smaller diameter. Therefore, to increase the total number of droplets containing single cells, we used a microdevice containing hydrophilic surfaces with a diameter of 10 μm.

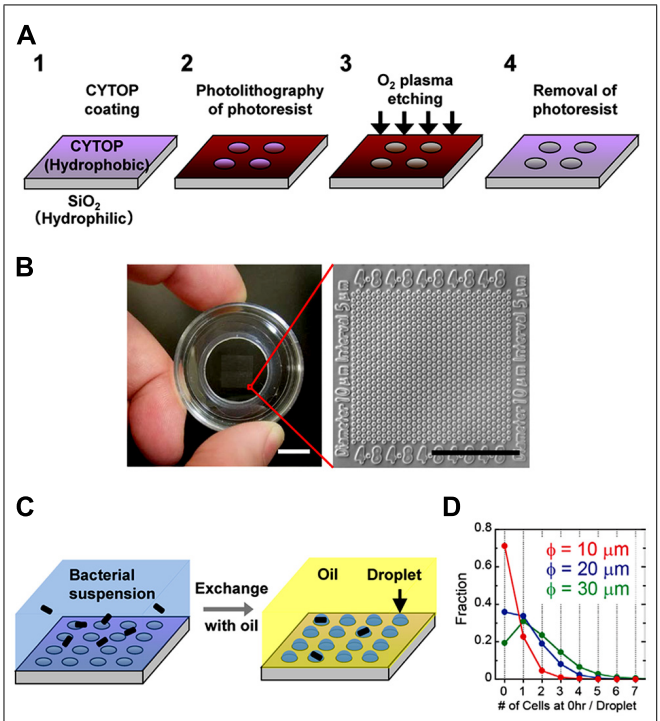

FIGURE 1 | Formation of a femtoliter droplet array containing bacteria. **(A)** Schematic of the fabrication procedure for preparation of a hydrophilic-in-hydrophobic micropatterned surface. **(B)** Image of the assembled device (left). Scale bar, 10 mm. Microscopic image of the hydrophilic-in-hydrophobic micropatterned surface (right). Scale bar, 200 μm. **(C)** Procedure for bacterial enclosure into the femtoliter droplet array. **(D)** The number of bacterial cells in each droplet in microdevices with different hydrophilic surface diameters just after enclosure.

DETECTION OF PERSISTER BACTERIA IN A FEMTOLITER DROPLET ARRAY

We generated a femtoliter droplet array of *Pseudomonas aeruginosa* PAO1 using our microdevice to detect persisters under an optical microscope. In the control experiment without antibiotic treatment, most cells underwent multiple cell divisions after incubation overnight at 37°C (**Figures 2A,B**). The divided cells showed active flagellar motion, indicative of high metabolic activity. To detect persisters, an antibiotic, carbenicillin (at final concentration of 5 mg/mL, which is ∼100 times higher than the minimal inhibitory concentration), was added to the bacterial suspension that was grown to late exponential phase (OD_{600} ∼1.0) in trypticase soy broth. The suspension was further incubated at 37°C for 3 h, and then the cells were collected, washed, resuspended in fresh medium (OD_{600} ∼0.2). This suspension was enclosed in a droplet array. After enclosure, the whole device was placed in an incubator at 37°C, and the cells were cultured overnight. The persisters were easily identified under an optical microscope the overnight culture (**Figure 3A**). The divided cells were not cells that acquired resistance, but were actually persisters. This was confirmed by collecting the cells with a micropipette with an aperture diameter of 10–15 μm (**Figures 3B,C**), inoculating a culture in test tubes, and antibiotic susceptibility testing.

Bacterial cells that divided multiple times were counted, and the frequency of persisters was calculated. The frequency of persisters in the femtoliter droplet array (1.5 ± 0.72%, $N = 4$; **Figure 3D**) was quite unexpectedly much higher than that estimated by conventional agar plate assays (0.10 ± 0.03%, $N = 4$). In the plate assays, the carbenicillin-treated preculture sample was prepared as described above along with an untreated culture sample, and then the samples were serially diluted and cultured overnight at 37°C on agar plates. The number of colonies on the plates

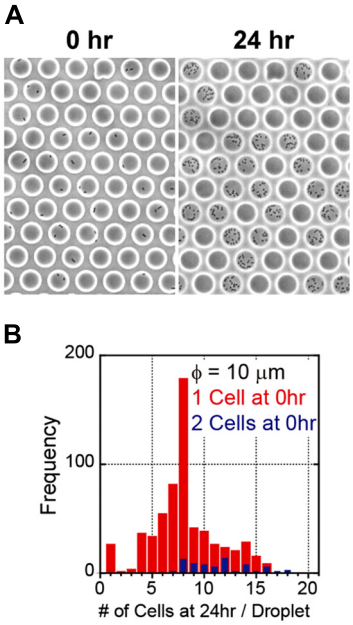

FIGURE 2 | Enclosure and culture of bacteria in a femtoliter droplet array. (A) Images of *P. aeruginosa* PAO1 after 0 h (left) and 24 h (right) of culture in the femtoliter droplet array. **(B)** Distribution of the number of cells after 24 h of culture.

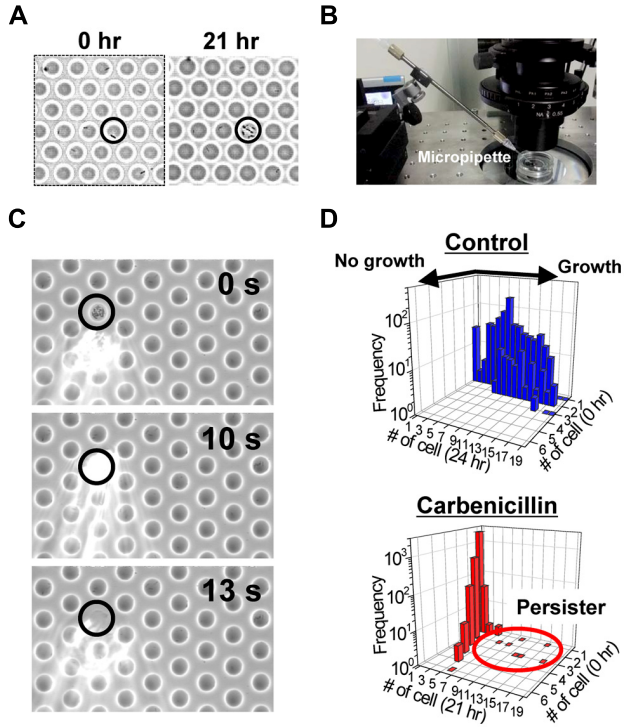

FIGURE 3 | Detection of the persisters in the femtoliter droplet array. (A) Images of *P. aeruginosa* PAO1 persisters (indicated by circle) after 0 h (left) and 21 h (right) of culture in the femtoliter droplet array. **(B)** Image of the micropipette used for droplet collection. **(C)** Sequential images of droplet collection. **(D)** Distribution of the number of cells in each droplet before and after overnight culture. Top, control cells without carbenicillin treatment. Bottom, carbenicillin-treated cells.

from carbenicillin-treated and untreated preculture samples were counted and compared. It has been recently reported that quorum sensing autoinducer increased the frequency of persister appearance (Moker et al., 2010; Leung and Levesque, 2012, Vega et al., 2012), and that inhibiting the quorum signal restored antibiotic susceptibility (Pan et al., 2012, 2013). Furthermore, the quorum-sensing signal could be transduced even in single isolated cells when PAO1 was enclosed in picoliter-volume droplets (Boedicker et al., 2009). Therefore, enclosure of a single cell in a femtoliter droplet may enhance the quorum sensing signal and increase persister frequency. The effect of the quorum sensing signal on the frequency of persister appearance in the femtoliter droplet array can be more clearly confirmed by treating the cells with antibiotic after enclosure in the droplets by adding the antibiotic with a micropipette (Sakakihara et al., 2010).

A SINGLE-CELL DRUG EFFLUX ASSAY IN A FEMTOLITER DROPLET ARRAY

The AcrAB-TolC multicomponent efflux pump system recognizes and expels a wide variety of compounds, including antibiotics, dyes, and detergents. In this system, AcrA is the membrane fusion protein that stabilizes the complex (Zgurskaya and Nikaido, 1999), AcrB is the inner membrane transporter protein that belongs to the resistance-nodulation-division (RND) family (Murakami et al., 2006; Nakashima et al., 2011, 2013), and TolC is the outer membrane channel protein (Koronakis et al., 2000). The AcrAB-TolC efflux system is responsible for both intrinsic and acquired drug resistance of Gram-negative bacteria such as *Escherichia coli* and *Salmonella enterica* (Nishino and Yamaguchi, 2008; Nikaido and Takatsuka, 2009). Two systems *P. aeruginosa* that are homologous to the AcrAB-TolC system, MexAB-OprM and MexXY-OprM, lead to multidrug resistance in clinical isolates (Morita et al., 2001; Livermore, 2002; Hocquet et al., 2006, 2007; Henrichfreise et al., 2007).

We have recently developed a single-cell drug efflux assay using the femtoliter droplet array (**Figure 4**; Iino et al., 2012a). In this assay, *E. coli* cultured in test tubes was mixed with a fluorogenic substrate, fluorescein-di-β-D-galactopyranoside (FDG), enclosed in a droplet array, and then cultured for 15–20 min at room temperature. Upon entering the cytoplasm of *E. coli*, FDG is hydrolyzed into the fluorescent dye fluorescein by β-galactosidase. Both FDG and fluorescein are substrates for the AcrAB-TolC system (Matsumoto et al., 2011; Iino et al., 2012b). In wild-type cells, FDG was effectively pumped out before hydrolysis, and no fluorescence was detected (**Figure 4A**, left, and **Figure 4B**, top). In contrast, when FDG was imported into Δ*acrB* (ΔB) and Δ*tolC* (ΔC) strains it was hydrolyzed to fluorescein. In ΔB cells, not only the cells, but also the droplets themselves fluoresced (**Figure 4A**, center, and **Figure 4B**, middle) because the remaining minor RND efflux pumps slowly pumped out the fluorescein. Although only a small amount of the dye was pumped out, it could be easily detected because it was confined to the femtoliter droplet (Rondelez et al., 2005; Sakakihara et al., 2010, Kim et al., 2012). In ΔC cells, fluorescein accumulated in the cell (**Figure 4A**, right, and

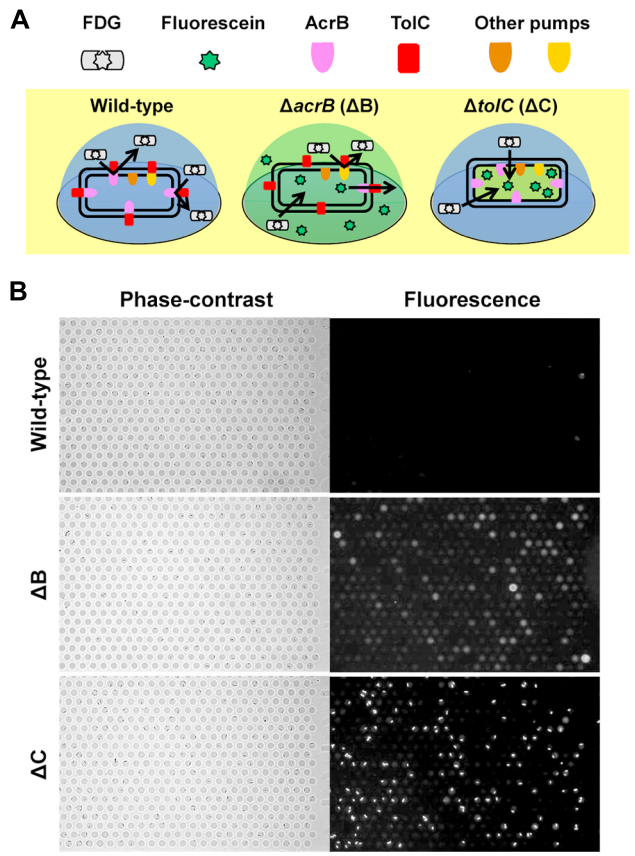

FIGURE 4 | Single-cell drug efflux assay. (A) Schematic of the principle of the single-cell drug efflux assay. **(B)** A representative assay. Phase-contrast (left) and fluorescence (right) images of the same field are shown.

Figure 4B, bottom) because TolC is a channel protein common to both the major and minor RND efflux pumps in *E. coli*.

With this method, the inhibitory effect of chemical compounds against the efflux pump can be easily assessed. The effect of an efflux pump inhibitor, D13-9001 (Yoshida et al., 2007), is shown in **Figure 5**. D13-9001 has been reported to enhance the antibacterial activities of several antibiotics by binding tightly to the drug binding pockets of AcrB and MexB (Nakashima et al., 2013). A ΔBΔC double-knockout *E. coli* strain that stably expresses MexAB-OprM from *P. aeruginosa* was used for the experiment. This strain did not fluoresce in our assay, indicating that the exogenously expressed MexAB-OprM worked well in *E. coli*, and that the cells recovered drug efflux activity (**Figure 5**, top). Addition of D13-9001 increased the number of fluorescent cells (**Figure 5**, bottom). The fluorescence intensity of the cells increased as the concentration of D13-9001 increased, indicating a concentration-dependent inhibitory effect. D13-9001 is a specific inhibitor of MexB, a major efflux pump in *P. aeruginosa*. However, it does not inhibit MexY, which is another major efflux pump in *P. aeruginosa* (Yoshida et al., 2007). Our simple and rapid approach would be useful to screen for new inhibitors that are also effective against MexY and other efflux pumps.

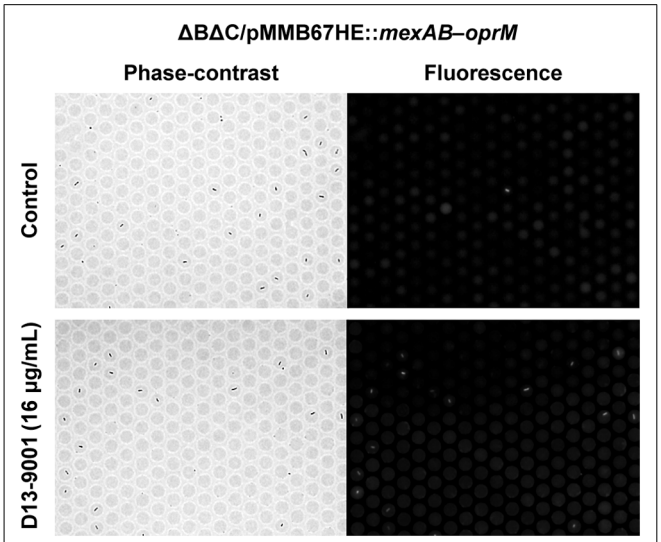

FIGURE 5 | Effect of the pump inhibitor D13-9001 on the efflux activity of MexAB-OprM expressed in the ΔBΔC *E. coli* strain. Phase-contrast (left) and fluorescence (right) images of untreated control cells (top) or cells treated with 16 μg/mL D13-9001 (bottom).

PHENOTYPIC CHANGE AFTER GENETIC TRANSFORMATION

As a demonstration of the rapid phenotypic change after genetic transformation, we introduced the *S. enterica tolC* gene into *E. coli* ΔC cells. After electroporation with the expression vector, the cells were incubated for different time durations in the presence of the selection marker kanamycin and drug efflux activity was assessed. The efflux-active phenotype was observed after 3 h (**Figure 6**, top), whereas no phenotypic change was observed in the control experiment (**Figure 6**, bottom).

A prominent feature of the femtoliter droplet array is the ability to access individual droplets. Using a micropipette, not only droplets but also the cells within the droplets can be collected (**Figures 3B,C**). Collected single cells divide multiple times after

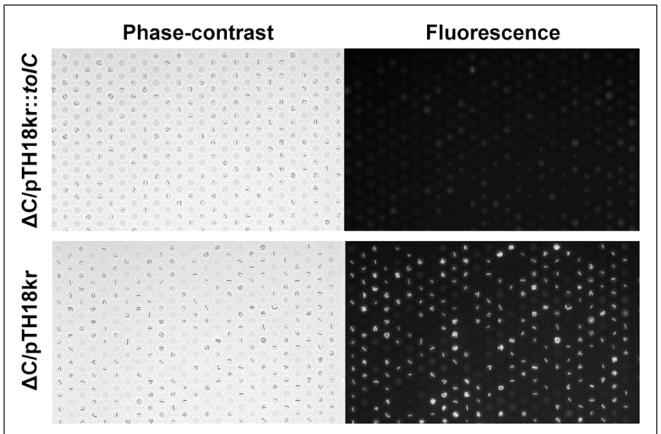

FIGURE 6 | Phenotypic change after genetic transformation. Phase-contrast (left) and fluorescence (right) images of *E. coli* ΔC cells transformed with a vector expressing *tolC* from *Salmonella enterica* (pTH18kr::tolC; top) or a control vector pTH18kr (bottom).

transfer to growth medium in a test tube. The plasmid in the divided cells can be extracted and used for subsequent gene analysis (Iino et al., 2012a). Culturing after cell collection can be omitted by amplifying the DNA by single-cell PCR (Ottesen et al., 2006). Considering the rapid detection (3 h) of the phenotypic change after genetic transformation, single-cell gene analysis would enable high-throughput screening.

PERSPECTIVE

As described above, the femtoliter droplet array is useful for single-bacterial cell analysis. Single-cell analysis of persister bacteria could help elucidate the mechanism of persister appearance and the reversible switching dynamics between persister and sensitive cells. This single-cell drug efflux assay can be used to screen for pump inhibitors, which requires the testing of numerous compounds. Our method is a direct evaluation of efflux activity, it takes only 20-30 min, and its advantage over the conventional method, based on the shift in the minimal inhibitory concentration is evident. Furthermore, with the advantage of individual droplet accessibility, single persister cells and cells exhibiting the efflux-active phenotype can be easily collected and used for subsequent analysis. It should be possible to screen for genes encoding functional efflux pump systems with a plasmid library of cloned genomic fragments. We believe that our approach will aid in addressing the challenge of infectious diseases caused by bacteria that are multidrug tolerant and resistant.

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Mutation in ribosomal protein S5 leads to spectinomycin resistance in *Neisseria gonorrhoeae*

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Spectinomycin remains a useful reserve option for therapy of gonorrhea. The emergence of multidrug-resistant *Neisseria gonorrhoeae* strains with decreased susceptibility to cefixime and to ceftriaxone makes it the only medicine still effective for treatment of gonorrhea infection in analogous cases. However, adoption of spectinomycin as a routinely used drug of choice was soon followed by reports of spectinomycin resistance. The main molecular mechanism of spectinomycin resistance in *N. gonorrhoeae* was C1192T substitution in 16S rRNA genes. Here we reported a Thr-24→Pro mutation in ribosomal protein S5 (RPS5) found in spectinomycin resistant clinical *N. gonorrhoeae* strain, which carried no changes in 16S rRNA. In a series of experiments, the transfer of *rpsE* gene allele encoding the mutant RPS5 to the recipient *N. gonorrhoeae* strains was analyzed. The relatively high rate of transformation [ca. 10^{-5} colony-forming units (CFUs)] indicates the possibility of spread of spectinonycin resistance within gonococcal population due to the horizontal gene transfer (HGT).

Keywords: spectinomycin resistance, spot-transformation, horizontal gene transfer, ribosomal proteins, mutations

INTRODUCTION

Neisseria gonorrhoeae is an obligate pathogen causing gonorrhea, one of the most abundant sexually transmitted diseases. The expansion of drug resistant *N. gonorrhoeae* strains is one of the global contemporary problems. The emergence and spreading of multidrug resistant (MDR) strains, which are resistant to penicillin, tetracycline, and ciprofloxacin, are reported throughout the world. In some countries, including Russia, about 50% of clinical *N. gonorrhoeae* strains identified as MDR (Kubanova et al., 2010; Allen et al., 2011). Until recently, all these strains remain susceptible to spectinomycin and to extended-spectrum cephalosporins (ceftriaxone and cefixime). Recently, *N. gonorrhoeae* strains displayed reduced susceptibility to the extended-spectrum cephalosporins. Moreover, several cases of clinical failures during the cefixime treatment have been reported (Wang et al., 2003; Heymans et al., 2012; Unemo et al., 2012). The spectinomycin remains the only antibiotic still effective in analogous cases. However, adoption of spectinomycin as the routinely used drug of choice was soon followed by reports of spectinomycin resistance (Boslego et al., 1987). The treatment of gonorrhea infection caused by extremely drug resistant (XDR) *N. gonorrhoeae* strains is very difficult, and the extended knowledge of molecular mechanisms of drug resistance is required for development of new tests needed to for routine diagnose in clinical practice.

In general, these are several mechanisms occurring in the bacteria which confer them antibiotic resistance. The most common are known to be drug inactivation, efflux-pumping of the drug out of the cell, and target modification due to single nucleotide change polymorphisms (SNPs) mainly (Davies and Davies, 2010). A bacterial strain can acquire resistance either by mutation of its own genes or by the uptake of exogenous genes by horizontal transfer from other microbes. Within bacterial population horizontal gene transfer (HGT) occurs via conjugation, transformation and transduction. With regards to neisseria genus these bacteria are naturally transformable and are capable to exchange their genetic material with high frequency (Koomey, 1998). This property leads to the rapid dissemination of antibiotic resistance markers and to the panmictic structure of the gonococcal and meningococcal populations.

Spectinomycin belongs to an aminocyclitol antibiotic class which blocks biosynthesis of bacterial proteins. After entering the bacterial cells, spectinomycin binds a ribosome beneath the 34 helix of 16S rRNA and interrupts elongation of the polypeptide during protein synthesis apparently preventing the translocation of the peptidyl tRNA from the A-site to the P-site (Carter et al., 2000; Borovinskaya et al., 2007).

Various bacteria demonstrate spectinomycin resistance, which results from three different mechanisms. The most frequent mechanism is the drug inactivation by adenylylation. Until now a diverse number of adenyltransferases, which exhibit the spectinomycin resistant (Spt-R) phenotype, was described (Shaw et al., 1993). One group of enzymes referred as AAD(3″)(9) [or ANT(3″)(9)] confers combined resistance to spectinomycin and streptomycin. These enzymes were found in a variety of gram-negative bacteria (Yamada et al., 1968; Hollingshead and Vapnek, 1985; Kehrenberg et al., 2005) and also in gram-positive bacteria (Clark et al., 1999). The other group of adenyltransferase referred as AAD(9) [or ANT(9)] confers the Spt-R phenotype only (LeBlanc et al., 1991).

On the other hand, the spectinomycin resistance can result from alteration of 30S subunit of bacterial ribosome due to

mutations in chromosomal genes encoding ribosomal RNAs or proteins. Thus, mutations in the spectinomycin binding region of helix 34 of 16S rRNA encompassing the cross-linked positions from 1063 to 1066 and from 1190 to 1193 (in *Escherichia coli* numbering) lead to high level resistance to spectinomycin (Sigmund et al., 1984; Brink et al., 1994). These mutations were discovered for various bacteria such as *Chlamydia psittaci, Borrelia burgdorferi, E. coli, Pasteurella multocida* (Johanson and Hughes, 1995; Binet and Maurelli, 2005; Criswell et al., 2006; Kehrenberg and Schwarz, 2007) including *N. gonorrhoeae* and *N. meningitidis* (Maness et al., 1974; Galimand et al., 2000). Although ribosomal protein S5 (RPS5) is not involved in spectinomycin binding, it is located very close to the antibiotic binding site (within 5 A°) (Wirmer and Westhof, 2006). Accordingly, it has been found that mutations in RPS5 can lead to spectinomycin resistance in *E. coli* (Funatsu et al., 1972; Bilgin et al., 1990) and in *P. multocida* (Kehrenberg and Schwarz, 2007).

Until recently, the only 16S rRNA substitutions were found in Spt-R bacteria from *Neisseria* genus (Maness et al., 1974; Galimand et al., 2000). In this study, we reported the discovery of isolated mutation in RPS5 in Spt-R clinical *N. gonorrhoeae* strain, confirmed by Unemo et al. (2013), and estimate the potential of Spt-R horizontal spread. We established that the 10^{-5} colony-forming units (CFU) from transformation experiments represents a high risk of spectinomycin resistance spread within gonococcal population.

MATERIALS AND METHODS

BACTERIAL STRAINS

N. gonorrhoeae clinical isolates were collected as a part of previous study (Ilina et al., 2008) and stored at a temperature of −80°C. The studied *N. gonorrhoeae* clinical isolates and strains are shown in **Table 1**.

For any manipulations, gonococci were cultivated on the BBL™ GC agar base (Becton Dickinson & Co, USA) supplemented with 1% BBL™ IsoVitaleX™ Enrichment (Becton Dickinson & Co, USA) at 37°C in a humid atmosphere with 5% CO_2 during 20–24 h.

PHENOTYPIC CHARACTERIZATION

Susceptibility testing to penicillin G, tetracycline, ciprofloxacin, spectinomycin and ceftriaxone was performed by agar dilution method in accordance with CLSI recommendations (CLSI document M100-S17, 2007). All antibiotics were manufactured by Sigma, USA. *N. gonorrhoeae* strain ATCC 49226 was used as a control.

GONOCOCCAL TRANSFORMATION

Spot-transformation of gonococci was performed as described (Dillard, 2011). Briefly, 200 ng of amplified DNA fragments in a 10 μl volume were placed into spots (diameter approximately 1.5 cm) on a pre-warmed (37°C) GC base agar plate. The spot was allowed to soak into the plate. Then piliated colonies of *N. gonorrhoeae* recipient strains were streaked across the plate through the DNA spots. After overnight incubation at 37°C in a humid atmosphere with 5% CO_2, colonies were swabbed from the spot with a Dacron swab and re-suspended in 100 μl of storage medium (50% BBL™ Brain Heart Infusion, Becton Dickinson & Co, USA, 30% Fetal Bovine Serum, Gibco, USA, 20% glycerin, Sigma, USA). This suspension of cells was then diluted and plated on both GC base agar alone, and GC base agar supplemented with 64 mg/L of spectinomycin. These plates were incubated at 37°C in a humid atmosphere with 5% CO_2 for one to two days, and individual transformants were picked up from spectinomycin supplemented plate. Selected colonies were cultivated once again on spectinomycin supplemented plates; the obtained

Table 1 | Characteristics of gonococci involved into current investigation.

Name	Description	MICs (mg/L) for					Serotype	NG-MAST sequence type
		PEN	TET	CIP	SPT	CRO		
e03.04	Clinical isolate	0.015	< 0.03	0.001	32–64	< 0.002	P1B3	5187
NG3	Clinical strain derived from e03.04 isolate	0.015–0.03	0.03–0.06	0.001–0.002	4–8	0.0005–0.001	P1B3	5187
NG3.1	Clinical strain derived from e03.04 isolate	< 0.015	0.03–0.06	0.0005–0.001	64–128	0.0005–0.001	P1B3	5187
NG3.2	Clinical strain derived from e03.04 isolate	0.03–0.06	0.03–0.06	0.0005–0.001	64–128	0.001–0.002	P1B3	5187
NG7	Clinical strain, using as a recipient for spot-transformation	0.12–0.25	0.015	0.001	16–32	0.002	P1B3	8633
NG94	Clinical strain, using as a recipient for spot-transformation	0.015–0.03	< 0.03	0.001	16–32	< 0.002	P1B3	8401

PEN, penicillin; TET, tetracycline; CIP, ciprofloxacin; SPT, spectinomycin; CRO, ceftriaxone.

N. gonorrhoeae strains were stored at −80°C. Transformation frequency was calculated as the number of spectinomycin-resistant colony-forming units (SPT^r CFU/ml) per total CFU (CFU/ml).

GENOTYPIC CHARACTERIZATION USES FEW METHODS: PCR AND SEQUENCING AND MALDI-TOF MS

Total genomic DNA from tested *N. gonorrhoeae* strains was isolated by "DNA express" kit (Lytech Ltd, Russia). When necessary, the prepared DNA samples were stored at a temperature of −20°C.

Conventional PCR was done using the genomic DNA as a template and oligonucleotide primers described in **Table 2**. All primers were newly designed using the Oligo_6.31 software (Molecular Biology Insights Inc., USA) and sequences of *N. gonorrhoeae* strain FA1090 (GenBank accession number is NC_002946). Amplification was carried out in 10 μl of 66 mM Tris-HCl pH 9.0; 16.6 mM $(NH_4)_2SO_4$; 2.5 mM $MgCl_2$; 0.2 mM of each dNTP; 5 pmol of each primer (**Table 2**) and 1 unit of TaqPolymerase (Fermentas, Lithuania) under the following conditions: 94°C for 20 s, 60°C for 20 s, and 72°C for 15 s, in 35 cycles. A programmed thermocycler TETRAD DNA ENGINE (MJ Research Inc.) was used. The amplification products were analyzed by electrophoresis in 2% agarose gel.

For further sequencing analysis dephosphorylation of the 5′-end phosphate groups of dNTPs and cleavage of primers in the post-amplification reaction mixture was done by incubation with 0.5 U of shrimp alkaline phosphatase and 0.1 U of exonuclease I (both enzymes from Fermentas, Lithuania) for 20 min at 37°C, followed with inactivation by heating at 85°C for 10 min. Sequence analysis of 16S rRNA genes as well as genes cording the ribosomal proteins S5, S4, and S8 was performed with dedicated primers (**Table 2**) by the modified Sanger method using the ABI Prism™ BigDye® Terminator v. 3.1 Cycle Sequencing Kit and 3730xl DNA Analyzer (Applied Biosystems, USA) according to the manufacturer's instruction. Analysis of the nucleotide sequences as well as deduced amino acid sequences was done using the Vector NTI Advance v. 9.0 software (Infomarks Incorporation, USA).

N. gonorrhoeae multi-antigen typing (NG–MAST) was performed as originally described (Martin et al., 2004). Sequence types were identified via NG-MAST (http://www.ng-mast.net) websites.

The rapid identification of A70C nucleotide substitution in *rpsE* gene, leading to Thr-24→Pro amino acid mutation in RPS5, was done by primer extension reaction followed by MALDI ToF mass spectrometry. For this, PCR was performed as mentioned above with primers form **Table 2**. The dephosphorylation of the 5′-end phosphate groups of dNTPs in the post-amplification reaction mixture was carried out by incubation with 0.5 U of shrimp alkaline phosphatase (Fermentas, Lithuania) for 20 min at 37°C, followed by inactivation of the enzyme by heating for 10 min at 85°C. After that amplicon was used as template in thermocyclic primer extension reaction, carried out in 20 μL of the mixture of 66 mM Tris-HCl pH 9.0; 16.6 mM $(NH_4)_2SO_4$; 2.5 mM $MgCl_2$;

Table 2 | List of primers selected for PCR and sequencing of *N. gonorrhoeae* genes encoding 16S rRNAs, and ribosomal proteins S4, S5, and S8.

Genetic characterization of selected *N. gonorrhoeae* strains		
Target	**Primers for PCR, 5′-3′ sequence(amplicon length)**	**Primers for sequencing, 5′-3′ sequence**
1st copy of 16S rRNA	aaccgatgacccccgcttg; gaaaaatgcatggcaggccg (2194 bp)	aaccgatgacccccgcttg tgacgtgtgaagccctggtcataa acgctaccaagcaatcaagttgcc tcatcggccgccgatattggcaac
2nd copy of 16S rRNA	aaccgatgacccccgcttg; accgtaaccgaatgcccctg (2409 bp)	
3rd copy of 16S rRNA	aaccgatgacccccgcttg; gcaaacccatgacatttacc (2435 bp)	
4th copy of 16S rRNA	aaccgatgacccccgcttg; acgggatcgggaacgaaaac (2432 bp)	
RPS4 (rpsD gene)	tattaaaggtccaggtccaggtcg; catggatgacagtaagatacggcg (954 bp)	as for PCR
RPS5 (rpsE gene)	cgtgcacatgcacgatgagattca; tgagaaggctaaagcagcaggtgt (727 bp)	as for PCR
RPS8 (rpsH gene)	tcaatccattcctcgtaatgcggc; tttacttctacaccagcgggaacc (637 bp)	as for PCR
Genomic DNA fragment for spot transformation	tcaaagcaactgtaggtcgg; tgcgttctggtgctaaaggc (12931 bp)	

Identification of RPS5 Thr-24→Pro mutant strains					
Target	Primers for PCR, 5′-3′ sequence (amplicon length)	Primer for primer extension (minisequencing) reaction, 5′-3′ sequence, molecular weight	Composition of dNTP and ddNTP mix	Predicted molecular weight of primer extension reaction products for wild type	Predicted molecular weight of primer extension reaction products for mutant type
A70C mutation in *rpsE* gene	cgtgcacatgcacgatgagattca; tgagaaggc-taaagcagcaggtgt (727 bp)	gacgacctttaactactttgg (6397 Da)	dG, ddT	6685 Da (primer + ddT)	7014 Da (primer + dG +ddT)

The scheme of identification of A70C mutation in rpsE gene by primer extension reaction followed by mass spectrometry measuring is also presented.

0.2 mM of each dGTP and ddTTP, 10 pmol of internal primer (see **Table 2**) and 2 units of TermiPol DNA Polymerase (Solis Biodyne, Estonia) according to the followed profiling: 94°C for 20 s, 58°C for 20 s, and 72°C for 15 s, in 70 cycles.

The products from primer extension reaction were purified using the SpectroCLEAN Kit (Sequenom, USA) according to the manufacturer's instruction.

An aliquote (0.2–1 μL) of purified products from primer extension reaction was spotted onto a matrix, which was preliminarily dried on the MALDI target AnchorChip™ with 400 μm diameter of anchor sports (Bruker Daltonics, Germany). The matrix employed was a saturated solution of 3-hydroxypicolinic acid (Fluka, Germany) in a 1:1 acetonitrile/water mixture (Merck, Germany) mixed with 0.4 M dibasic ammonium citrate (Fluka, Germany) in 9:1 vol. ratio. All solvents were of quality suitable for mass spectrometry. Mass spectra were collected on a Microflex LT MALDI ToF mass spectrometer (Bruker Daltonics, Germany) that was operated in the positive linear mode. A 337 nm nitrogen laser with the 9 Hz pulse frequency was used. Mass spectrometer parameters were optimized for the range of m/z values from 1000 to 10,000, using a peptide standards set for calibration. Each mass spectrum was collected at 30 laser pulses at constant laser power and constant threshold value, in order to enhance the resolution.

ACCESSION NUMBERS

DNA sequences of *rspE* gene alleles of *N. gonorrhoeae* strains NG3, NG3.1, and NG3.2 were deposited into GenBank [accession numbers KF021591, KF021592, and KF021593, respectively].

RESULTS AND DISCUSSION

In this study we identified a molecular mechanism responsible for spectinomycin resistance in gonococci. As a part of unrelated experiment, *N. gonorrhoeae* strains with Spt-R phenotype were occasionally isolated. They were derived from clinical isolate e03.04 belonged to Por1B3 serotype. Collected in 2004 it was identified as susceptible to penicillin, tetracycline, ciprofloxacin, and ceftriaxon. Spectinomycin MIC was defined as 32-64 mg/L, which corresponded to intermediate resistant (Spt-I) phenotype. When the susceptibility profile of this isolate was retested in 2008 two colonies emerged on the plate supplemented with 64 mg/L of spectinomycin. Each colony was seeded once again on plates with the same antibiotic concentration, and then on the plates with 128 mg/L of spectinomycin. It was a poor growth on plates with 128 mg/L of spectinomycin, so two clones grown well under the 64 mg/L of spectinomycin were stored.

It should be noted that neither one of other *N. gonorrhoeae* isolates tested in this experiment nor the same e03.04 clinical isolate examined in repeats revealed any additional colonies survived under 64 mg/L of spectinomycin. Thus we have considered two occasionally isolated Spt-R clones as a small fraction of heterogeneous e03.04 clinical isolate. The collected strains were designated as NG3.1 and NG3.2. Gonococci derived from e03.04 isolate susceptible to spectinomycin treatment were also collected and stored as NG3 *N. gonorrhoeae* clinical strain.

Susceptibility testing data for clinical e03.04 isolate as well as for selected *N. gonorrhoeae* strains is presented in **Table 1**. In accordance with NG-MAST scheme all of them belonged to the same 5187 sequence type that supported the assumption of a common origin of the strains studied. Since, the only previously reported mechanism for Spt-R gonococci was C1192T mutation in 16S rRNA gene (Maness et al., 1974; Galimand et al., 2000), we have sequenced all four copies of 16S rRNA genes located in *N. gonorrhoeae* genomic DNA (for the number of rRNA genes copies see rrnDB database, http://ribosome.mmg.msu.edu/rrndb/index.php). For each tested strain, all four copies of 16S rRNA genes were found identical. Indeed, the 1192 position as well as a locus encoding for the 34 helix of 16S rRNA, which forms the spectinomycin-binding site of ribosome (Carter et al., 2000; Wirmer and Westhof, 2006) possessed the wild-type sequence in NG3 strain as well as in NG3.1 and NG3.2 ones. So, we have proposed that other molecules located close by to the ribosomal spectinomycin-binding site could be involved in formation of Spt-R phenotype.

The crystal structure of the 30S subunit of bacterial ribosome from *E. coli* in complex with spectinomycin (Carter et al., 2000) was taken into account and the ribosomal proteins S4, S5, and S8 were picked up for further examination due to their vicinity to a spectinomycin-binding site. Accordingly, *rpsD*, *rpsE*, and *rpsH* genes were amplified and sequenced. No differences were found between *rpsD*, and *rpsH* genes. However, both Spt-R *N. gonorrhoeae* strains (NG3.1 and NG3.2) carried the same A70C mutation in *rpsE* genes, leading to Thr-24→Pro amino acid substitution at RPS5, which mapped to Ser-22 residue of RPS5 from *E. coli* (**Figure 1**).

The 19–33 loop of RPS5 is known to be RNA binding site, and is the most frequent zone for location of mutations related

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              1                                                  50
ATCC49226  MAKHEIEERG DGLIEKMVAV NRVTKVVKGG RIMAFSALTV VGDGDGRIGM
  FA1090   MAKHEIEERG DGLIEKMVAV NRVTKVVKGG RIMAFSALTV VGDGDGRIGM
     NG3   MAKHEIEERG DGLIEKMVAV NRVTKVVKGG RIMAFSALTV VGDGDGRIGM
   NG3.1   MAKHEIEERG DGLIEKMVAV NRVPKVVKGG RIMAFSALTV VGDGDGRIGM
   NG3.2   MAKHEIEERG DGLIEKMVAV NRVPKVVKGG RIMAFSALTV VGDGDGRIGM
  NM_MC58  MAKHEIEERG DGLIEKMVAV NRVTKVVKGG RIMAFSALTV VGDGDGRIGM
   EC_K12  MA-H-IEKQA GELQEKLIAV NRVSKTVKGG RIFSFTALTV VGDGNGRVGF
  PM_1398  MA-N-IEKQA GELQEKLIAV NRVSKTVKGG RIMSFTALTV VGDGNGRVGF
```

FIGURE 1 | Multiple alignment of RPS5 sequences of NG3, NG3.1, and NG3.2 strains. The Thr-24→Pro mutation is bolded. Deduced amino acid sequences of RPS5 of wild-type *N. gonorrhoeae* (ATCC49226, FA1090), *N. meningitidis* (NM_MC58), *E. coli* (EC_K12), and *P. multocida* (PM_1398) are also presented. Regions involved in formation of resistance to spectinomycin are underlined [according to Davies et al. (1998); Kehrenberg and Schwarz (2007)].

to spectinomycin resistance (Funatsu et al., 1972; Wirmer and Westhof, 2006; Kehrenberg and Schwarz, 2007). Mostly, RPS5 mutations associated with Spt-R phenotype occur simultaneously with 16S rRNA changes. However, the isolated Ser-22→Pro RPS5 mutation in resistant to spectinomycine *E. coli* strain was also described (Wilcox et al., 2001). Basing on this knowledge, we have suggested that the Thr-24→Pro substitution found in RPS5 from NG3.1 and NG3.2 *N. gonorrhoeae* strains confer the Spt-R phenotype.

As an argument, that the single Thr-24→Pro mutation in RPS5 can support a Spt-R phenotype alone, we performed a transformation of spectinomycin sensitive *N. gonorrhoeae* strains by the exogenic DNA fragment contained mutant *rpsE* gene. Furthermore, the experiments described below demonstrated a potential role of HGT in spread of spectinomycin resistance.

The 12931 bp fragments of *N. gonorrhoeae* chromosomal DNA, which include whole *rpsE* gene and the nearest DNA uptake sequences (DUS12, 5′ atgccgtctgaa 3′) on their ends were amplified with primers indicated in **Table 2**. It was done using the NG3 (wild type) and NG3.1 (mutant) genomic DNAs as templates. The presence of DUS12 is known to be necessary for DNA recognition and uptake and therefore, mediates an increase in transformation efficiency (Duffin and Seifert, 2010).

Two different *N. gonorrhoeae* strains—NG7, and NG94, susceptible to spectinomycin were chosen as recipients. For each recipient, the spot transformation by purified amplified DNA fragment contained mutant (or wild) variant of *rpsE* gene was carried out. The principal scheme of an experiment is represented in **Figure 2**. The transformation of recipients by a DNA fragment carried a wild variant of *rpsE* gene was used as a negative control. Each experiment was repeated in triplicate.

As a result, 74.7 ± 5.7 and 15.6 ± 2.5 colonies emerged on GC agar plates supplemented with 64 mg/L of spectinomycin and derived from NG7 and NG94 bacterial cells transformed by mutant *rpsE* gene, respectively (**Table 3**). Bacterial cells transformed by DNA fragment carried wild type of *rpsE* gene formed no colonies on spectinomycin supplemented GC agar plates. Transformation frequency calculated as the number of SPT^r CFU per total CFU was found $2.2 \pm 0.4 \times 10^{-5}$ and $1.7 \pm 0.2 \times 10^{-5}$ for NG7 and NG94 *N. gonorrhoeae* strains, respectively.

Each transformant survived after a re-cultivation on spectinomycin supplemented (64 mg/L) agar plates was tested by sequencing. All tested strains carried A70C mutation in *rpsE* gene identical to NG3.1 and NG3.2 *N. gonorrhoeae* strains and leading to Thr-24→Pro amino acid substitution in RPS5. Otherwise, in accordance with NG MAST, they belonged to the same sequence types as the recipient strains from which they originated.

The spectinomycin MICs were measured for the transformants derived both from NG7 and NG94 *N. gonorrhoeae* recipient strains. In all cases, spectinomycin MICs increased fourfold (from 16–32 mg/L to 64–128 mg/L) and became identical to the ones of NG3.1 and NG3.2 *N. gonorrhoeae* strains.

Described experiments verified that single Thr-24→Pro amino acid substitution in RPS5 confers the Spc-R phenotype of *N. gonorrhoeae* strains.

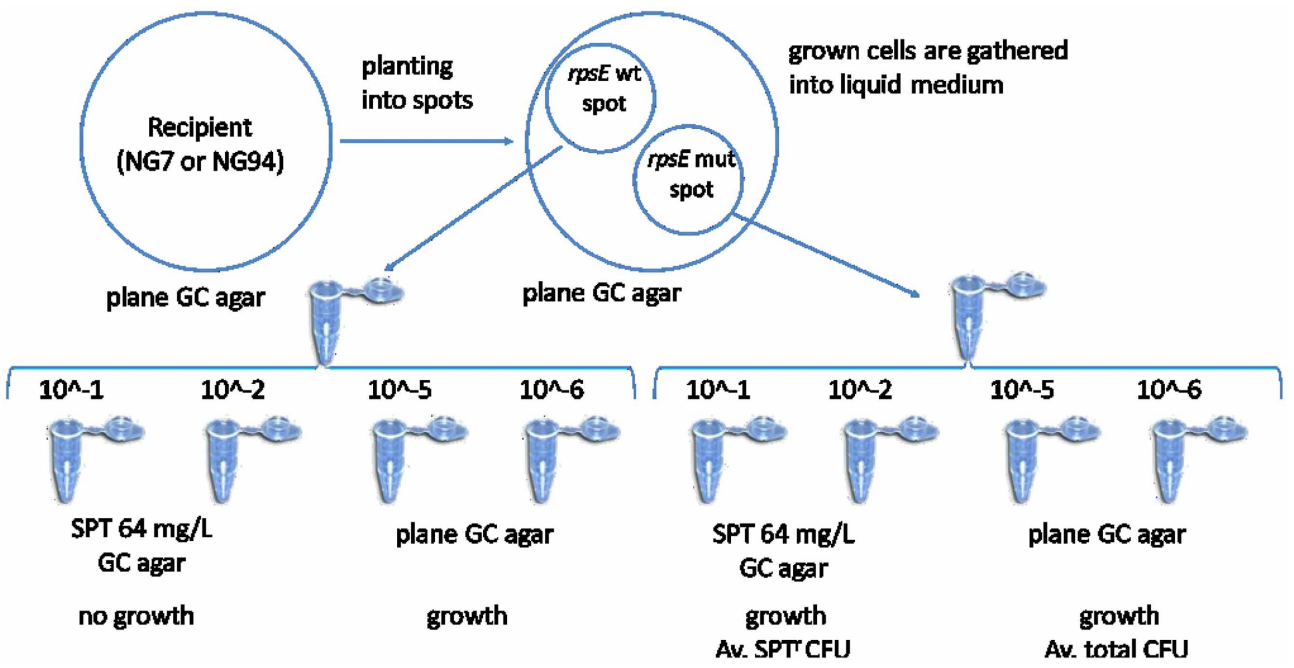

FIGURE 2 | The principal scheme of sport transformation. Amplified DNA fragments carried mutant (mut) and wild type (wt) of *rpsE* gene cording the ribosomal protein S5 (RPS5) were placed in spots on a GC base agar plate. Then piliated colonies of recipient strains (NG7 and NG94) were streaked across the plate through the DNA spots. After overnight incubation, bacterial cells were swabbed from the spots into a liquid medium, diluted and plated on both GC base agar alone, and GC base agar supplemented with 64 mg/L of spectinomycin (SPT). The individual transformants emerged on SPT-supplemented plates were picked up for further examination. Colony-forming units (CFUs) were counted on each plate.

Table 3 | The calculated results of transformation experiments.

Recipient	Av. SPT^r CFUs	Av. SPT^r CFU/ml	Av total CFU/ml	Transformation efficiency
NG7	74.7 ± 5.7	$3.5 \pm 0.4 \times 10^4$	$1.6 \pm 0.5 \times 10^9$	$2.2 \pm 0.4 \times 10^{-5}$
NG94	15.6 ± 2.5	$0.8 \pm 0.2 \times 10^4$	$4.7 \pm 0.2 \times 10^8$	$1.7 \pm 0.2 \times 10^{-5}$

An average data from three replicas is presented.

Furthermore, the transformation efficiency was found relatively high (ca. 10^{-5}) and was not statistically different between both recipient strains. This finding supports the statement that the HGT through the natural transformation permits movement of mutant allele of *rpsE* gene and increases the opportunity for the spread of spectinonycin resistance within gonococcal population.

The RPS5 mutation we verified here appeared to be the same that was described for *N. gonorrhoeae* WHO A strain (Unemo et al., 2013). This mutation results in low-level spectinomycin resistance in gonococci that agrees with our conclusion.

The main issue is that in this study, conducted independently the mutant allele of *rpsE* gene was found in clinical *N. gonorrhoeae* strain isolated in Russia. This emphasizes that the described molecular mechanism of Spt-R phenotype formation is a universal one and spreads worldwide.

To extend the knowledge of frequency of RPS5 Thr-24→Pro mutants the entire laboratory collection of *N. gonorrhoeae* genomic DNA ($n = 1131$) acquired since 2002–2008 years was analyzed. The majority of them ($n = 1113$) were susceptible to spectinomycin, and the 18 strains revealed the Spt-I phenotype (MICs = 32–64 mg/L). Keeping in mind that NG3.1 and NG3.2 strains possessed Thr-24→Pro mutation in RPS5 were found occasionally as a small fraction of a wild population, we did not exclude susceptible to spectinomycin gonococci from the examination.

No more mutant *N. gonorrhoeae* strains have been found. It seems the tested Thr-24→Pro substitution in RPS5 is very rare among gonococci. This may reflect the uncommon use of spectinomycin in therapy of gonorrhea. On the other hand, we cannot deny that gonococci with mutation in ribosomal protein could be somehow defective in their survival and population spreading. Undoubtedly, fitness cost due to such mutation requires further careful investigation.

Nevertheless, our study is an additional evidence of the existing of clinical *N. gonorrhoeae* strains with moderate spectinomycin resistance due to isolated mutations in RPS5. It is very important in terms of the surveillance and the therapy of gonococcal infection. The extended knowledge of the potential mutations responsible for resistant phenotypes is essential in the development of new tests suitable in clinical setting, which aim to establish the correct antimicrobial therapy.

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Characterization of fluoroquinolone resistance and *qnr* diversity in *Enterobacteriaceae* from municipal biosolids

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Municipal biosolids produced during activated sludge treatment applied in wastewater treatment plants, are significant reservoirs of antibiotic resistance, since they assemble both natural and fecal microbiota, as well as residual concentrations of antibiotic compounds. This raises major concerns regarding the environmental and epidemiological consequences of using them as fertilizers for crops. The second generation fluoroquinolone ciprofloxacin is probably the most abundant antibiotic compound detected in municipal biosolids due to its widespread use and sorption properties. Although fluoroquinolone resistance was originally thought to result from mutations in bacterial gyrase and topoisomerase IV genes, it is becoming apparent that it is also attributed to plasmid-associated resistance factors, which may propagate environmental antibiotic resistance. The objective of this study was to assess the impact of the activated sludge process on fluoroquinolone resistance. The scope of resistances and mobile genetic mechanisms associated with fluoroquinolone resistance were evaluated by screening large collections of ciprofloxacin-resistant *Enterobacteriaceae* strains from sludge ($n = 112$) and from raw sewage ($n = 89$). Plasmid-mediated quinolone resistance determinants (*qnrA*, *B*, and *S*) were readily detected in isolates from both environments, the most dominant being *qnr*S. Interestingly, all *qnr* variants were significantly more abundant in sludge isolates than in the isolates from raw sewage. Almost all ciprofloxacin-resistant isolates were resistant to multiple antibiotic compounds. The sludge isolates were on the whole resistant to a broader range of antibiotic compounds than the raw sewage isolates; however, this difference was not statistically significant. Collectively, this study indicates that the activated sludge harbors multi-resistant bacterial strains, and that mobile quinolone-resistance elements may have a selective advantage in the activated sludge.

Keywords: activated sludge, biosolids, fluoroquinolone, ciprofloxacin, antibiotic resistance, *qnr*, integron

INTRODUCTION

Municipal biosolids produced during the activated sludge process in secondary wastewater treatment are often utilized as fertilizers for a wide array of crops, due to their availability and high nutrient content (Dröge et al., 2000; Golet et al., 2003). However, despite the obvious advantages of this practice, sludge application may have highly negative ecological and epidemiological ramifications. Wastewater treatment plants (WWTPs) represent an endless pool of commensal human and farm animal bacteria (Dröge et al., 2000; Zhang et al., 2009), which are constantly exposed to a wide range of anthropogenic compounds, such as antibiotics. These compounds can select for resistant strains of both pathogenic and non-pathogenic bacteria, by means of both vertical and horizontal gene transfer (Pellegrini et al., 2011).

The application of municipal biosolids to soil may not only result in transfer of resistant bacterial strains, but also in dissemination of antibiotic resistance genes (ARGs) that can enhance environmental antibiotic resistance reservoirs. Indeed, there is growing perception that ARGs are emerging contaminants with the ability to spread from anthropogenic sources, such as WWTPs, into natural environments (LaPara et al., 2011; Li et al., 2012; Pruden et al., 2012). Although municipal biosolids are generally stabilized before application to soils, a recent study detected significant levels of ARGs in biosolids subsequent to different stabilization methods (Ma et al., 2011; Munir and Xagoraraki, 2011), and elevated levels of these genes were even observed in soil samples after land application of biosolids (Munir and Xagoraraki, 2011).

Fluoroquinolones are fully synthetic broad-spectrum antibacterial agents that are becoming increasingly popular in the treatment of clinical infections. The mechanism by which fluoroquinolones inhibit cell proliferation was well elaborated by Jacoby (2005) and by Strahilevitz et al. (2009).

Ciprofloxacin is a second generation fluoroquinolone and is the fifth most commonly prescribed antibacterial with over 20 million outpatient prescriptions written in the US alone in 2010.

The metabolism of ciprofloxacin in the human body is only partial and so it is excreted in both urine (45–62%) and feces (15–25%), and transported to WWTPs through municipal sewage systems (Golet et al., 2003). During wastewater treatment, a large fraction of ciprofloxacin is removed from the aqueous phase and absorbed into the sludge, thereby accumulating in dewatered sludge, where concentrations of up to 50 mg/kg dry weight have been detected (Golet et al., 2002; McClellan and Halden, 2010).Field experiments have demonstrated that sorption of fluoroquinolones to municipal sludge results in long-term persistence, which continues even after application to agricultural soils (Golet et al., 2003).

When fluoroquinolones were first introduced for clinical use in the mid 1980s the likelihood for the emergence of resistance was considered to be negligible, because bacteria would have to spontaneously acquire two or more non-fatal mutations in the catalytic sites of the gyrase/topoisomerase IV enzymes to evade the antibiotic effect (Robicsek et al., 2006). Nonetheless, shortly after fluoroquinolones became one of the top antibiotic compounds in nosocomial use, resistance became a common global phenomenon (Robicsek et al., 2006; Strahilevitz et al., 2007). Originally, fluoroquinolone resistance was attributed to mutations in specific areas of DNA gyrase genes (*gyrA*, *parC*, and *parE*), known as quinolone resistance-determining regions. However, in the late 1990s emerging evidence indicated acquisition of fluoroquinolone resistance in a non-clonal manner (Robicsek et al., 2006), with the detection of a plasmid-mediated-quinolone-resistance mechanism by Martinez-Martinez et al. (1998). This plasmid encoded a pentapeptide-repeat protein termed QnrA (for quinolone resistance) that increased quinolone resistance levels between 8- and 64-fold in *Escherichia coli*. Although the resistance of these isolates was approximately 10-fold lower than traditional mutation-associated minimal inhibitory concentration (MIC), they were found to have increased probabilities for acquisition of additional resistance mechanisms (Tran and Jacoby, 2002). Experiments conducted with a purified, His6 QnrA determined that it eliminated the quinolone action by directly binding to gyrase and preventing the antibiotic from binding to the DNA, thus being trapped in the lethal gyrase–DNA–quinolone cleavage complex (Tran and Jacoby, 2002; Tran et al., 2005; Strahilevitz et al., 2009).

The worldwide expansion of *qnr* was rapid. In a study conducted in Alabama in 1994 by Martinez-Martinez et al. (1998), the newly defined *qnr*A variant was identified in only one host-associated *Klebsiella pneumoniae* isolate, and was not detected among any other of 350 Gram-negative isolates that were screened over 6 months. Ten years later, the prevalence of *qnr*A among ciprofloxacin-resistant *K. pneumoniae* isolates rose to 11% (in a 3-year survey done in six US states; Wang et al., 2004). During this period of time, *qnr*A-like determinants were also found in 7.7% of ciprofloxacin-resistant *E. coli* isolates in Shanghai, China (Wang et al., 2003). In light of these, and many other studies, it may be suggested that the increase in fluoroquinolone resistance was driven by the increase in *qnr* prevalence, which is horizontally transferred and confers a selective advantage to plasmid bearing bacteria (Martinez-Martinez et al., 1998; Nordmann and Poirel, 2005; Strahilevitz et al., 2007).

Several other variants of the Qnr protein have been discovered to date, including QnrB, QnrS, and the rarer QnrC and QnrD. All Qnr proteins involve a similar mechanism of protecting the bacterial gyrase/topoisomerase IV from the quinolone, and vary in size from 214 to 221 amino acids (Strahilevitz et al., 2009). Other studies show that although originating in different bacterial hosts, *qnr* genes are mostly found adjacent to sulfonamide resistance genes – *sul-I* and *sul-II*, and are often carried on class-1 integrons, in proximity to the integrase (*intl-1*) gene, which are prevalent in a wide range of ecological habitats (Tran and Jacoby, 2002; Strahilevitz et al., 2009; Pellegrini et al., 2011).

The objective of this study was to assess the phenotypic and genotypic scope of quinolone resistance in raw sewage and in WWTP sludge and to determine whether quinolone resistant bacteria, and specifically if plasmid-associated quinolone-resistance genes are enriched in WWTPs during sewage treatment. We isolated a large collection of ciprofloxacin-resistant *Enterobacteriaceae* from both the raw sewage entering the WWTP, and from dewatered sludge, and these isolates were screened for *qnrA*, *B*, and *S* variants, and for class-1 integron-associated integrase (*intl-1*) genes. Ciprofloxacin-resistant isolates were also screened against 1 μg/ml ciprofloxacin and six other antibiotic compounds to assess levels of multi-resistance in these environments. The initial isolation of the analyzed *Enterobacteriaceae* was conducted on 0.4 μg/ml ciprofloxacin, which corresponds to intermediate ciprofloxacin (IC) resistance that is a characteristic of *qnr*-harboring isolates. This is approximately 2.5-fold below MIC values associated with gyrase/topoisomerase IV mutant strains (Hopkins et al., 2005; Nordmann and Poirel, 2005; Gosling et al., 2012).

MATERIALS AND METHODS

SITE DESCRIPTION AND SAMPLING PROTOCOL

The Dan Region Wastewater Treatment and Reclamation Project (termed Shafdan), is the largest and most complex wastewater treatment facility in Israel. It treats approximately 340,000 m^3 of sewage daily from the entire metropolitan Tel Aviv area including five major hospitals. Secondary treatment is conducted in 12 activated sludge reservoirs with retention times ranging from 12–14 h. Approximately 15,000 m^3 of sludge are produced daily (1% dry weight).

Triplicate dewatered sludge and raw sewage samples were taken from the Shafdan WWTP in three individual sampling profiles (April 2012, May 2012, and October 2012). Dewatered sludge (18% water content) was sampled in sterile 100 ml test tubes, and the raw sewage was collected from WWTP influent in 1 l bottles. Samples were transferred to the lab on ice within 1 h, and raw sewage samples were concentrated by centrifuging for 10 min at 1000 *g*.

ENTEROBACTERIACEAE ISOLATION AND CHARACTERIZATION

Five grams of dewatered activated sludge or raw sewage samples were suspended in 40 ml of sterile 0.1 M ammonium phosphate buffer [$(NH_4)_2HPO_4$], shaken laterally at 350 rpm for 45 min, at room temperature, and centrifuged twice for 10 min at 500 rpm; each time supernatant was transferred into new 50 ml sterile tubes and pellet was discarded. Supernatant was

then centrifuged for 10 min at 8,000 rpm, and the pellet was resuspended in 20 ml sterile saline solution. Samples were serially diluted and filtered onto 25 mm, 0.45 μm pore size polycarbonate membranes (GE Healthcare, Chalfont St. Giles, UK). Filters were aseptically placed on 45 mm Petri dishes containing modified membrane thermotolerant *Escherichia coli* (mTEC) agar, which selects for *Enterobacteriaceae*. The modified mTEC method www.epa.gov/nerlcwww/documents/1603sp02.pdf uses the chromogen 5-bromo-6-chloro-3-indolyl-β-D-glucuronide (Magenta Gluc) as the differential agent (Francy and Darner, 2000).

Serial dilutions were platted on media with or without IC concentrations (0.4 μg/ml). This sub-MIC value was selected, because, as mentioned above, it is believed to be a ciprofloxacin concentration which will allow us to screen for *Enterobacteriaceae* that harbor plasmid-mediated fluoroquinolone resistance (Hopkins et al., 2005; Gosling et al., 2012). Plates were incubated for 2 h at 37°C and then for 20–22 h at 45°C as previously described (Francy and Darner, 2000).

The taxonomic affiliation of the isolates was determined by plating on CHROMagar Orientation medium agar plates (CHROMagar Microbiology, Paris, France). CHROMagar typing indicated that all of the *Enterobacteriaceae* isolates from both the raw sewage and the sludge biosolids were affiliated with either the *E. coli* (16.1% of sludge isolates and 11.2% of raw sewage isolates) or *Klebsiella* (78.6% of sludge isolates and 70.8% of raw sewage isolates) genera.

There was no statistical difference in the ration of *E. coli* to *Klebsiella* genera in the sludge relative to the raw sewage.

All of the non-*Enterobacteriaceae* isolates were not used for further analysis. The taxonomic affiliation of 20 of the isolates was further assessed by phylogenetic analysis of partial 16S rRNA gene fragments. Standard Sanger sequencing was conducted at HyLabs (Rehovot, Israel) and the taxonomic affiliation of the sequences was determined by basic local alignment search tool (BLAST). In total, 201 sub-MIC *Enterobacteriaceae* isolates were obtained (112 from the activated sludge, and 89 from the raw sewage), and further analyzed as described below.

RESISTANCE PHENOTYPING

Ninety activated sludge and 87 raw sewage, sub-MIC ciprofloxacin-resistant isolates were screened for resistance to other antibiotic compounds as follows: isolates were transferred aseptically to Müller-Hinton (MH)-agar containing one of the eight different antibiotics at European Committee on Antibiotic Susceptibility Testing (EUCAST) clinical MIC breakpoint concentrations according to standard procedures (http://www.eucast.org/clinical_breakpoints): ciprofloxacin (1 μg/ml), ampicillin (8 μg/ml), ceftriaxone (2 μg/ml), chloramphenicol (8 μg/ml), and gentamicin (4 μg/ml). In addition, isolates were also screened against nalidixic acid (32 μg/ml) and tetracycline (30 μg/ml). Plates were incubated at 37°C overnight, and colony formation was assessed.

DNA EXTRACTION FROM ISOLATES

DNA was extracted from all samples using a modified bead-beating method. Bacteria cells were grown overnight in 5 ml Luria-Bertani (LB) broth containing ciprofloxacin (0.4 μg/μl). After harvest, cells were lysed in extraction buffer [100 mM Tris–HCl, pH 8.0; 100 mM potassium phosphate buffer pH 8.0; 1% cetyltrimethylammonium bromide (CTAB); and 2% sodium dodecyl sulfate (SDS)] followed by bead-beating (in the Fast Prep FP 120, Bio101, Savant Instruments Inc., Holbrook, NY, USA). The crude extracts were mixed with KCl to a final concentration of 0.5 M, incubated for 5 min, and centrifuged. DNA present in the supernatant was bound to glassmilk (0.5–10 μm silica particles, Sigma Chemical Co., St. Louis, MO, USA) with 6 M NaI. The silica was then resuspended in an ethanol-based wash buffer solution (Boyle and Lew, 1995) and transferred to a centrifuge tube filter (0.22 μm cut-off nylon filter, Costar, Corning Inc., Corning, NY, USA) that bound the DNA to the silica and retained it on the filter until eluted with 60°C Tris–EDTA (TE) into a sterile tube. Extracted DNA samples were stored at −20°C until use.

PCR DETECTION OF *qnr* VARIANTS AND *intI-1* GENES

Detection of the *qnr* variants was accomplished by means of a modified multiplex, touchdown PCR protocol based on a previously described method and primers (Cattoir et al., 2007). Briefly, the protocol involved 20 initial cycles: 5 min 94°C, 30 s 94°C, 30 s 60–50°C (each cycles reducing 0.5°C), 20 s 72°C, repeating the latter three steps; 15 additional cycles: 30 s 94°C, 30 s 50°C, 20 s 72°C; and a final cool down step for 10 min, at 72°C followed by a slow descent to 10°C.

For detection of the *intI-1* gene, the following PCR protocol was conducted, using previously reported primers (Gaze et al., 2011): 25 cycles: 3 min 95°C, 1 min 95°C, 30 s 58°C, 10 s 72°C repeating the latter three steps, 10 min 72°C and a slow descent to 10°C. Both PCR programs were executed in a final volume of 12.5 μl containing: 8 ng of purified DNA, 5 U Dream-Taq polymerase (MBI Fermentas, Lithuania), 1× Dream-Taq Buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM primers.

STATISTICAL ANALYSES

Statistical analyses were conducted using the Statistica software application (StatSoft, Inc., Tulsa, OK, USA). Multiple statistical comparisons were conducted using the Tukey's HSD (honestly significant difference) test, in which mean values that do not share any letters are considered significantly different. Pairwise comparisons were conducted using the Fisher's exact test. Compared values were considered statistically significant when p values were less than 0.05.

RESULTS

ESTIMATION OF CIPROFLOXACIN-RESISTANT *ENTEROBACTERIACEAE*

Enterobacteriaceae levels in both raw sewage and dewatered, activated sludge ranged between 10^7 and 5×10^8 CFU/mg. Resistance to IC concentrations (0.4 μg/ml) reduced the number of colony forming units in both sample types by approximately two orders of magnitude (**Figure 1**), with no significant differences in the relative abundances of IC-resistant isolates in the sludge in comparison to the sewage isolates.

SCREENING FOR MULTI-RESISTANCE

The raw sewage and activated sludge *Enterobacteriaceae* isolates were highly resistant to EUCAST clinical MIC breakpoint

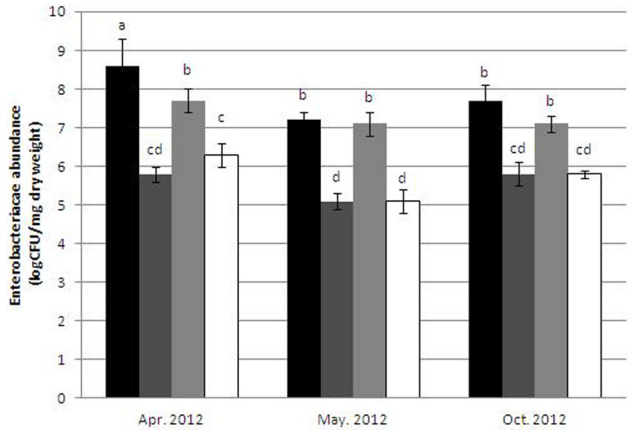

FIGURE 1 | Abundance of *Enterobacteriaceae* in dewatered sludge: non-selective media (black bars) and 0.4 μg/ml ciprofloxacin-amended media (dark gray bars); and in raw sewage: non-selective media (light gray bars), and 0.4 μg/ml ciprofloxacin-amended media (white bars). Error bars indicate standard deviation. Mean values that do not share any letters are considered significantly different (Tukey's HSD $p < 0.05$).

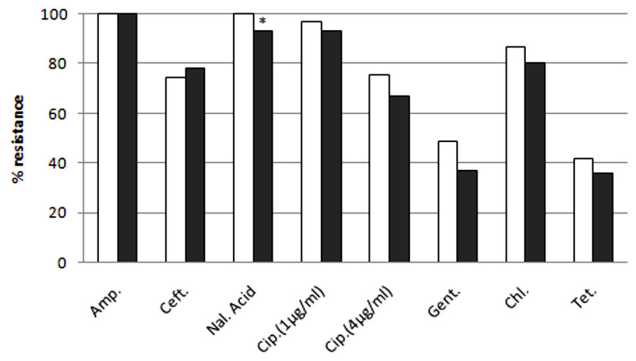

FIGURE 2 | Resistance of sludge (white bars) and raw sewage (gray bars) intermediate ciprofloxacin-resistant isolates to additional antibiotic compounds at MIC concentrations. Asterisk indicates statistically significant (Fisher's exact test $p < 0.05$) differences between sampling locations.

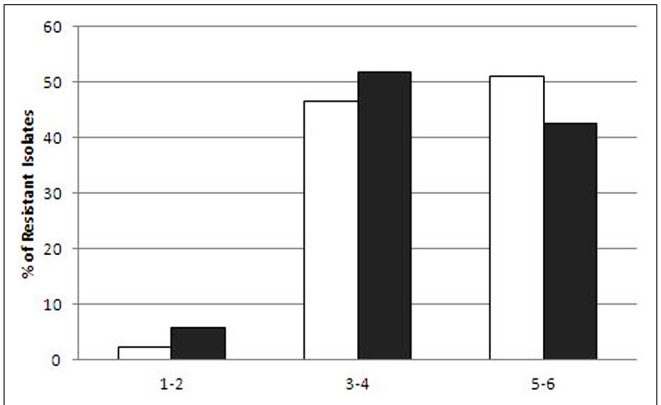

FIGURE 3 | Level of multi-resistance in intermediate ciprofloxacin-resistant isolates from sludge (white bars) and raw sewage (gray bars). The *x*-axis shows the abundance of the strains resistant to 1–2, 3–4, and 5–6 additional antibiotic compounds.

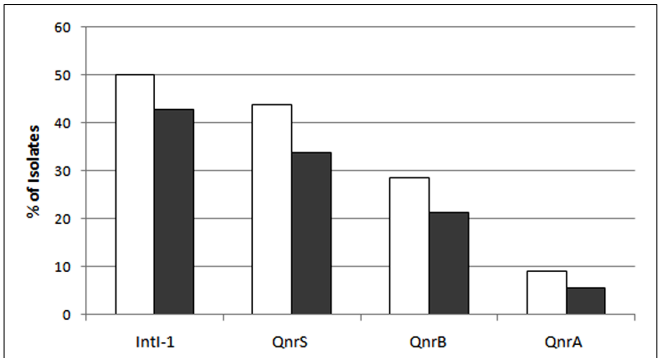

FIGURE 4 | The abundance of the different variants of *qnr* and of the *intI-1* genes in sludge (white bars) and raw sewage (gray bars) intermediate ciprofloxacin-resistant isolates.

concentrations (**Figure 2**). With the exception of ceftriaxone, the ratio of IC isolates resistant to additional antibiotic compounds (at MIC concentrations) was generally higher in the dewatered sludge than in the raw sewage; however, this was only statistically significant for nalidixic acid. The efficacy of different antibiotics against the sewage and sludge isolates was highly similar for all of the antibiotics tested, as shown in **Figure 2**. Additional discrepancies were observed in the resistance profiles of the two environments when examining the scope of multi-resistance of the IC isolates. The sludge IC isolates were generally resistant to a larger range of antibiotic compounds; however, this was not statistically significant (**Figure 3**).

DETECTION OF *qnr* VARIANTS AND PRESENCE OF CLASS-1 INTEGRONS

In total, 75% of the sludge IC isolates and 59.6% of the raw sewage IC isolates were found to harbor at least one *qnr* variant. This suggests a possible enrichment of plasmid-mediated quinolone resistance in the activated sludge ($p < 0.05$). The *qnrS* variant was the most abundant, detected in 43.8 and 33.7% of the sludge and raw sewage IC isolates, respectively; the *qnrB* variant accounted for 28.5 and 21.3%, respectively; and *qnrA* was detected in 9 and 5.6%, respectively (**Figure 4**).

The *intI-1* gene was found in 50 and 42.7% of sludge and raw sewage IC isolates, respectively (**Figure 4**). Although *qnr*S was the most prevalent variant detected in both sludge and raw sewage, *qnr*B was found to have the strongest association with *intI-1* (**Figure 5**).

Contingency tests were conducted on the question whether specific populations of bacteria (*E. coli* and *Klebsiella*) are more or less prone to carry the different *qnr* variants or the *intI-1* gene. No such correlation was found.

Several studies have found that *qnr* genes are associated with class-1 integrons. Therefore, the linkage between *qnr* and *intI-1* genes was more comprehensively explored. Approximately 81.5% of the raw sewage *intI-1*-positive IC isolates were *qnr* positive (I^+Q^+), whereas only 29.4% of the *intI-1*-negative IC isolates harbored *qnr* (I^-Q^+). This stands in complete contrast to the

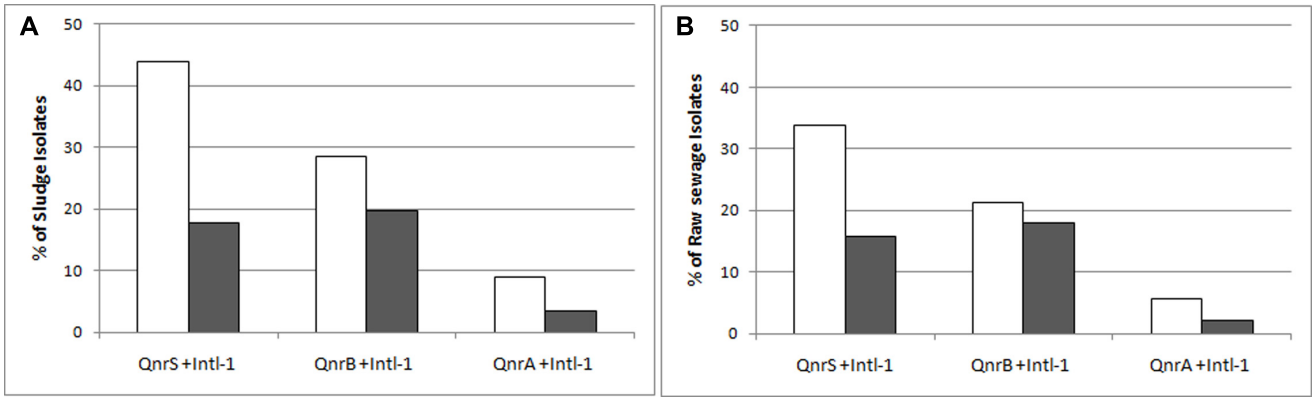

FIGURE 5 | *White bars*: the relative abundance of intermediate ciprofloxacin-resistant isolates carrying *qnr* variants (A, B, S) from the sludge (A), and raw sewage (B); *Gray bars*: the abundance of *intI-1* positive isolates among those *qnr*-positive isolates.

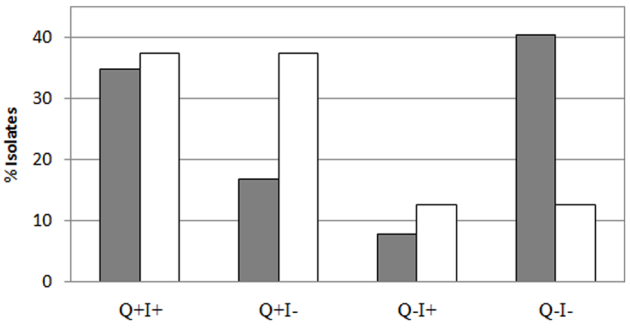

FIGURE 6 | Correlation between *qnr* and *intI-1* presence: isolates exhibited a negative correlation between *qnr* (Q) and their *intI-1* (I) content in sludge (white bars) and raw sewage (gray bars).

activated sludge, where 75% of both the I^+ and I^- IC isolates contained *qnr* genes (**Figure 6**).

DISCUSSION

Wastewater treatment plants are man-made ecological niches that assemble high concentrations of fecal and environmental bacteria. These giant "chemostats" have the potential to serve as hotspots for horizontal gene transfer. Horizontal transfer of ARGs in activated sludge is an actual concern, due to the potential evolution of multi-resistance strains, which are later discharged into agricultural environments when municipal biosolids are used as fertilizer. These ARGs can readily enter water supplies and the food chain, and henceforth may have significant epidemiological ramifications. Bacterial pathogens from municipal biosolids are generally not considered as high risk factors for disease propagation, since the sludge is generally stabilized by anaerobic digestion or compostation prior to application as fertilizer. However, a fraction of sludge bacteria, and more notably, horizontally transferable genetic entities harboring ARGs can survive sludge stabilization processes and this gene pool may be disseminated into the soil microbiome following municipal sludge application.

In this study we focused on plasmid-mediated quinolone resistance (Qnr) determinants. Although these determinants have been found to occur on chromosomal DNA in certain environmental bacteria (Poirel et al., 2005), they are considered to be almost exclusively plasmid-borne in *Enterobacteriaceae* (Rodriguez-Martinez et al., 2011). This characteristic is especially concerning due to the clinical importance of fluoroquinolones and the rapid spread of plasmid-associated resistance. We isolated a large group of *Enterobacteriaceae* resistant to IC concentrations from both raw sewage ($n = 89$) and activated sludge ($n = 112$, in order to assess the potential influence of the activated sludge process on (a) the relative abundance of IC resistance; (b) the scope of resistance to other antibiotics; and (c) the abundance of selected mobile genetic elements.

Interestingly, although no significant increase in the level of IC-resistant isolates was observed when comparing the sludge to the raw sewage, the sludge IC isolates were characterized by a significantly higher ratio of *qnr*-positive variants, indicating that these plasmid-associated elements enhance the fitness of the *Enterobacteriaceae* isolates in the activated sludge. This may be due to the *qnr* genes themselves, or alternatively to additional genes that are carried on mobile genetic elements that also harbor the *qnr* genes. The acquisition of plasmids carrying *qnr* genes increases the MIC of ciprofloxacin for wild-type *E. coli* J53 from 0.016 to 0.25 μg/ml (Robicsek et al., 2006) and therefore, it may be assumed that *qnr* confers a selective advantage to bacteria residing in the sludge, where ciprofloxacin concentrations of up to 0.05 μg/mg have been detected (Golet et al., 2002; McClellan and Halden, 2010).

We discovered that although the activated sludge process did not appear to select for a specific resistance, there appeared to be an increase in the degree of multi-resistance of the sludge IC isolates relative to the raw sewage isolates. However, with the exception of nalidixic acid, the increase of IC sludge vs. raw sewage isolates resistant to additional antibiotics was not statistically significant, and therefore a larger pool of isolates will need to be screened before this hypothesis can be established.

Typically, plasmid-mediated quinolone resistance was thought to be associated with class-1 integrons (Robicsek et al., 2006; Lapierre et al., 2008). However, the increased abundance of *qnr* elements observed in the sludge isolates relative to the raw sewage appears to stem from *qnr* elements that were not *intl-1* positive (**Figure 6**), suggesting that the *qnr* genes that proliferate in the sludge IC isolates are not associated with class-1 integrons. In total, raw sewage IC isolates that harbored the *intl-1* gene had a probability of 81.5% to carry a *qnr* gene, whereas only 75% of the *intl-1* positive sludge IC isolates carried a *qnr* gene (I^+Q^+). If the integron was indeed the genetic platform on which *qnr* resides, one would expect to see a correlated decrease between *qnr* and *intl-1* in class-1 integron negative isolates in both raw sewage and sludge. In reality, while only 29% of *intl-1* negative raw sewage isolates harbored a *qnr* gene, 75% of *intl-1* negative sludge isolates were. In contrast, 67% of the *qnr*-positive raw sewage isolates were also positive for *intl-1*, whereas only 50% of the sludge isolates did. Furthermore, only 16% of the *qnr*-negative raw sewage IC isolates carried the *intl-1* gene, whereas 50% of the *qnr*-negative sludge isolates did. This strongly suggests that *qnr* genes are not essentially associated with type-1 integrons, and that the dynamics of *qnr* in sludge may differ from that in raw sewage. A few studies have shown that the association between qnr and class-1 integrons is not mandatory. For example, Richter et al. (2010) identified an association between a *qnr* gene and a Tn2012 transposon. This novel transposon was comprised of a transposase gene (ISEcp1C) and a *qnr*B19 gene that were inserted into a Tn1721 transposon, a non-conjugative transposon from the Tn3 family. This genetic structure allows the *in vitro* dissemination of the *qnr*B19 gene into conjugative strains (Cattoir et al., 2008).

In conclusion, as depicted from our data, the presence of *qnr* (both with and without corresponding *intl-1* genes) is favored in the WWTP process irrespective of class-1 integron presence, thereby eliminating the suggested linkage between class-1 integrons and plasmid-associated quinolone resistance. This is of particular interest since it was previously described that qnr tend to be found in correlation to the class-1 integrons, and could suggest a different, negative correlation between those two genetic entities. Future research will need to screen the flanking regions of these *qnr*s in order to determine which mobile genetic elements the *qnr*s are located on, and if there are ubiquitous flanking genes that potentially bestow a selective advantage in the activated sludge environment.

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Mechanisms of antimicrobial resistance in finfish aquaculture environments

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Consumer demand for affordable fish drives the ever-growing global aquaculture industry. The intensification and expansion of culture conditions in the production of several finfish species has been coupled with an increase in bacterial fish disease and the need for treatment with antimicrobials. Understanding the molecular mechanisms of antimicrobial resistance prevalent in aquaculture environments is important to design effective disease treatment strategies, to prioritize the use and registration of antimicrobials for aquaculture use, and to assess and minimize potential risks to public health. In this brief article we provide an overview of the molecular mechanisms of antimicrobial resistance in genes found in finfish aquaculture environments and highlight specific research that should provide the basis of sound, science-based policies for the use of antimicrobials in aquaculture.

Keywords: antimicrobials, antimicrobial resistance, fish, aquaculture

INTRODUCTION

Through a continuous process of expansion, intensification, and diversification, aquaculture has become the fastest-growing food industry in the world (Bostock et al., 2010). With production of new species and the expansion of the production of current species to new geographical locations, the risk of disease and need for treatment continues to increase. This risk is compounded by the uncertainties introduced by global climate change, which may affect the emergence and dynamics of new and existing pathogens (Tirado et al., 2010), and consequently the use of antimicrobials and the prevalence of antimicrobial resistance.

The major concern surrounding the use of antimicrobials in aquaculture is considered to be the potential to favor the development of a reservoir of antimicrobial resistance genes (ARGs) that may be eventually transferred to clinically relevant bacteria (FAO/OIE/WHO, 2006). So far, lack of data, methodological constraints, and the complexity of characterizing exposure pathways have prevented the derivation of quantitative estimates of the risk that this may pose to public health. Nevertheless, there is consensus in the scientific community and international organizations concerned with human health, animal health, and food security that steps should be taken to minimize it (FAO/OIE/WHO, 2006; Smith, 2008; Heuer et al., 2009; WHO, 2011). On a global scale, several of the major classes of antimicrobials are being used or have been used in aquaculture. Among these are sulphonamides, penicillins, macrolides, quinolones, phenicols, and tetracyclines (Sapkota et al., 2008), all of which are listed as critically or highly important antimicrobials in human medicine (WHO, 2011). However, the judicious use of antimicrobials in global aquaculture is important to effectively treat bacterial fish diseases and maintain fish health and welfare.

The molecular mechanism, genomic context, and prevalence of genes conferring resistance to antimicrobials determine their clinical relevance (i.e., whether they confer low- vs. high-level clinical resistance, their ability to be mobilized by lateral gene transfer, and their frequency of occurrence). Thus, knowledge of ARGs from aquaculture environments is important to design and prioritize monitoring programs that may generate data that eventually becomes relevant for performing quantitative risk assessments and develop sound treatment strategies to control fish disease. Within this general scope, we provide this article as an overview of the molecular mechanisms, genomic context, and prevalence of quinolone, tetracycline, and phenicol resistance genes that have been reported to occur in aquaculture environments.

ANTIMICROBIAL RESISTANCE GENES IN AQUACULTURE ENVIRONMENTS

Quinolones (i.e., oxolinic acid, flumequine, and enrofloxacin), tetracyclines [i.e., oxytetracycline (OTC)], and phenicols (i.e., florfenicol) are among the most widely used antimicrobial compounds in aquaculture, and they have been used extensively to control bacterial fish disease in salmon farming [SERNAPESCA (Chile), 2009; Burridge et al., 2010; Rico et al., 2012]. Although the use of quinolones in salmon farming currently accounts for less than 1% of the overall use of antimicrobials, they continue to be used in aquaculture production in several Asian countries [SERNAPESCA (Chile), 2009; Burridge et al., 2010; Rico et al., 2012]. Quinolones, tetracyclines, and phenicols have been reported to be selective for a variety of ARGs and tend to occur in mobile genetic elements that favor their dissemination (i.e., transposons, plasmids, and integrons; Cloeckaert et al., 2000; Chopra and Roberts, 2001; Kümmerer, 2004; Schwarz et al., 2004; Roberts, 2005).

QUINOLONE RESISTANCE

The protein targets of quinolones are the bacterial enzymes, DNA gyrase and topoisomerase IV. DNA gyrase is a tetrameric enzyme

encoded by the *gyrA* and *gyrB* genes, and its main activity is to catalyze the negative supercoiling of bacterial DNA. Topoisomerase IV is also a tetrameric enzyme and it is encoded by the *parC* and *parE* genes; its function is to decatenate and relax the activity of daughter replicons following DNA replication (Anderson et al., 1998; Hawkey, 2003).

The acquisition of quinolone resistance is primarily due to chromosomal mutations in topoisomerases genes (i.e., *gyrA*, *gyrB*, *parC*, and *parE*) and mutations that reduce drug accumulation by decreasing uptake or increasing efflux (Drlica and Zhao, 1997; Ruiz, 2003). Additionally, at least three mechanisms of quinolone resistance are known to be plasmid encoded: (1) Qnr proteins; (2) AAC(6)-Ib-cr aminoglycoside acetyltransferases; and (3) QepA and OqxAB efflux pumps. Qnr proteins protect DNA gyrase and type IV topoisomerase from quinolone inhibition (Tran and Jacoby, 2002; Jacoby, 2005) and the AAC(6)-Ib-cr determinant acetylates several fluoroquinolones. Plasmid-encoded QepA and OqxAB are active efflux pumps that may extrude hydrophilic fluoroquinolones such as enrofloxacin (Li, 2005; Poirel et al., 2008, 2012; Cattoir and Nordmann, 2009; Rodríguez-Martínez et al., 2011).

Various point mutations in the quinolone resistance-determining regions of the *gyrA* and/or *parC* genes have been detected in quinolone-resistant strains of the fish pathogens *Aeromonas hydrophila*, *Vibrio anguillarum*, and *V. parahaemolyticus* (Okuda et al., 1999; Rodkhum et al., 2008; Lukkana et al., 2012). Levels of quinolone resistance in Gram-negative bacteria are suggested to be high when associated with point mutations in both the *gyrA* and *parC* genes, whereas only an intermediate level of resistance is associated with point mutations in the *gyrA* gene only. However, high-level resistance to oxolinic acid associated with a single mutation in the *gyrA* gene has been reported for strains of the fish pathogens *Aeromonas salmonicida*, *Edwardsiella tarda*, and *Photobacterium damselae* (Oppegaard and Sørum, 1994; Goñi-Urriza et al., 2002; Kim et al., 2005, 2011; Ozanne et al., 2005). The extensive administration of quinolones in fish farming has been linked to increased mutations in DNA gyrase and topoisomerase IV in quinolone-resistant fish pathogens such as *Yersinia ruckeri*, *Flavobacterium psychrophilum*, and *V. anguillarum* (Gibello et al., 2004; Izumi and Aranishi, 2004; Colquhoun et al., 2007; Izumi et al., 2007; Shah et al., 2012).

Plasmid-mediated quinolone resistance in bacteria associated with fish farms has been detected in several countries. Ishida et al. (2010) detected the *qnr* and *aac(6′)-Ib-cr* resistance determinants in bacterial strains isolated from fish farm water samples in Egypt. Recently, Buschmann et al. (2012) reported the occurrence of topoisomerase protection genes *qnrA*, *qnrB*, and *qnrS*, the putative enzymatic inactivation gene *aac(69)-Ib-cr* and the efflux pump gene *oqxA* among strains isolated from un-polluted and fish farm-impacted marine sediments in Chile. Jiang et al. (2012) found a high prevalence of *qnrB* and *qnrS* genes in *Escherichia coli* strains recovered from Chinese farmed fish while *qnrD* and *aac(6′)-Ib-cr* genes occurred less frequently. Han et al. (2012) found 17 strains encoding chromosomal mutations in *gyrA*, 11 strains encoding mutations in *parC*, and a few strains carrying the *qnrS1*-like and *qnrS2* genes among 33 *Aeromonas* spp. isolated from diseased fish and from water samples. The identification of QnrS determinants in *Aeromonas* spp. suggests that they may act as an environmental reservoir of *qnrS* genes, as already described for *tet* genes (Rhodes et al., 2000; Schmidt et al., 2001a). Some *qnr* genes have also been described in the Vibrionaceae family and it is suggested that water-borne Vibrionaceae may constitute a natural reservoir for Qnr-like quinolone-resistance determinants (Poirel et al., 2005; Cattoir et al., 2007; Cattoir and Nordmann, 2009). Evidence suggests that *qnr*-plasmids are most commonly integron associated and carry multiple resistance determinants, providing resistance to several classes of antimicrobials, including beta-lactams and aminoglycosides (Li, 2005).

TETRACYCLINE RESISTANCE

Oxytetracycline is a broad-spectrum bacteriostatic antimicrobial, active against a wide variety of Gram-positive and Gram-negative bacteria, which is extensively used in fish farming. Tetracyclines bind reversibly to the 70S ribosome of prokaryotes and block protein synthesis (Chopra, 1985; Roberts, 1996).

Mechanisms of tetracycline resistance include active efflux, ribosomal protection, ribosomal RNA mutations, and tetracycline inactivation (Speer and Salyers, 1989; Salyers et al., 1990; Burdett, 1991; Levy, 1992; Speer et al., 1992; Taylor and Chau, 1996). Tetracycline resistance in fish farm-associated bacteria has been found to be mainly mediated by one or more of the Tet family of proton-dependent efflux pumps or via ribosomal protection by cytoplasmic proteins found widely in Gram-negative bacteria (Roberts, 2005; Roberts et al., 2012).

Several *tet* determinants have been identified in fish farm bacteria from a number of geographical locations and fish species (DePaola et al., 1988; Adams et al., 1998; Rhodes et al., 2000; Schmidt et al., 2001a; Furushita et al., 2003; Miranda et al., 2003; Akinbowale et al., 2007; Seyfried et al., 2010; Gao et al., 2012). The genes *tet*(A), *tet*(B), *tet*(E), *tet*(H), *tet*(L), *tet*(34), and *tet*(35) were found in tetracycline-resistant bacteria isolated from Chilean salmon farms (Miranda et al., 2003). Recently, Seyfried et al. (2010) detected the presence of *tet*(A), *tet*(B), *tet*(D), *tet*(E), *tet*(G), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), and *tet*(W) genes in medicated and non-medicated feed samples and water samples from non-commercial fish farms in the United States. Similarly, Jun et al. (2004) found a high prevalence of *tet*(A) and *tet*(D) genes associated with mobile plasmids and *tet*(B) and *tet*(G) genes associated with non-mobile elements in *Edwardsiella tarda* strains isolated from fish farms in Korea. Nonaka et al. (2007) found a high incidence of *tet*(M)-carrying *Vibrio* strains in fish farms and Agersø et al. (2007) detected *tet*(E) in *Aeromonas* strains from Danish fish farms associated with large plasmids capable of horizontal transfer to *Escherichia coli*. Nawaz et al. (2009) found a high prevalence of *tet*(B) and, to a lesser extent, *tet*(A), *tet*(C) and the co-occurrence of *tet*(A) and *tet*(B) in *Escherichia coli* isolated from farm-raised catfish. Kim et al. (2004) reported the occurrence of *tet*(M) and *tet*(S) in tetracycline-resistant bacteria from fish farms in Korea and Nonaka and Suzuki (2002) found the novel OTC-resistance determinant *tet*(34) in a *Vibrio* strain isolated from cultured yellowtail (*Seriola quinqueradiata*).

The spread of *tet* genes is often facilitated by their location on mobile genetic elements, such as plasmids and transposons (Roberts, 1994; DePaola and Roberts, 1995; Chopra and Roberts,

2001). Tn1721 and Tn1721-like elements, for example, are known to play a significant role in the global dissemination of the *tet*(A) gene (Rhodes et al., 2000; Sørum et al., 2003; Chenia and Vietze, 2012). Miranda et al. (2003) found the *tet*(H) gene as part of the transposon Tn5706 in *Moraxella* and *Acinetobacter* strains isolated from salmon farms. In addition, they were able to transfer *tet*(B) and *tet*(34) genes from strains of *Serratia liquefaciens*, *Pseudomonas pseudoalcaligenes*, and *Brevundimonas vesicularis*. Similarly, Furushita et al. (2003) transferred *tet*(B), *tet*(D), and *tet*(Y) genes from bacterial isolates from different Japanese fish farms by conjugation and Adams et al. (1998) reported that various OTC-resistant isolates of *Aeromonas salmonicida* transferred R-plasmids carrying the *tet*(A) gene to environmental and clinical isolates of *Aeromonas* spp. Class 1 integrons harboring different combinations of the resistance gene cassettes *ant(3″)Ia*, *aac(6′′)Ia*, *dhfr1*, *oxa2a*, and/or *pse1* and *tet* genes were also detected in a large number of plasmid bearing *Aeromonas* spp. strains isolated from tilapia, trout, and koi cultures in South Africa (Jacobs and Chenia, 2007). In *Edwardsiella ictaluri* strains isolated from diseased freshwater catfish in Vietnam, Dung et al. (2009) found the *tet*(A) gene associated with a high-molecular weight plasmid belonging to the Inc*K* group. In addition, all strains were able to transfer their *tet*(A)-carrying plasmids to *Escherichia coli* recipients.

Several studies have also reported the co-occurrence of tetracycline and sulphonamide resistances genes. Agersø and Petersen (2007) showed that *tet*(39) and *sul2* genes located on plasmids of different sizes to be common among clonally distinct *Acinetobacter* spp. from fish farms in Thailand. Su et al. (2011) detected the genes *tet*(A), *tet*(C), and the sulphonamide-resistance gene, *sul2*, in more than 50% of the strains of Enterobacteriaceae they isolated from fish farms in China. Gao et al. (2012) recently reported the co-occurrence of tetracycline- and sulphonamide-resistance genes in *Bacillus* species isolated from aquaculture farms in China.

PHENICOL RESISTANCE

Florfenicol is a synthetic fluorinated analog of chloramphenicol whose bacteriostatic activity is based on a reversible binding to the 50S subunit of 70S bacterial ribosomes that prevents peptide elongation (Schwarz et al., 2004). The replacement of a hydroxyl group with a fluorine atom protects florfenicol from inactivation by chloramphenicol acetyltransferases (CATs), a common mechanism of bacterial resistance to chloramphenicol (Shaw and Leslie, 1991; Schwarz et al., 2004). The effectiveness of florfenicol against a number of relevant fish pathogens makes it a very valuable drug for the fish farming industry (Fukui et al., 1987; Nordmo et al., 1994; Samuelsen et al., 1998, 2003; Bruun et al., 2000; Gaunt et al., 2003; McGinnis et al., 2003; Michel et al., 2003; Samuelsen and Bergh, 2004).

Mechanisms of resistance to florfenicol include specific and non-specific drug transporters, RNA methyltransferases, and specific hydrolases (Paulsen et al., 1996; Schwarz et al., 2004; Poole, 2005; Long et al., 2006; Tao et al., 2012). Genes *floR* and *fexA* belong to the major facilitator superfamily and code for efflux proteins that export florfenicol out of the cell (Schwarz et al., 2004). The gene *cfr*, which has been shown to be an RNA methyltransferase that belongs to the recently discovered radical *S*-adenosylmethionine (SAM) superfamily of proteins (Sofia et al., 2001), inhibits ribose methylation and thereby causes resistance to florfenicol, chloramphenicol, and clindamycin (Long et al., 2006).

Most studies of florfenicol resistance in fish farming have reported the occurrence of the *floR* gene. Dang et al. (2007) detected the *floR* gene in tetracycline-resistant bacteria isolated from aquaculture sites in China, and Ishida et al. (2010) detected the *floR* gene in four strains of Gram-negative bacteria isolated from fish farms in Africa. In North America, McIntosh et al. (2008) reported the occurrence of *Aeromonas salmonicida* strains carrying a conjugative IncA/C plasmid harboring *floR*, *sul2*, and *tetA* genes that were transferable to *Aeromonas hydrophila* and *Edwardsiella tarda*. Welch et al. (2009) detected IncA/C plasmid-mediated florfenicol resistance in the catfish pathogen *Edwardsiella ictaluri*, and Gordon et al. (2008) reported a multiresistant *Aeromonas bestiarum* strain carrying a plasmid harboring the *floR*, *tetY*, *sul2*, and *strA–strB* resistance genes. A relatively recent study showed that many florfenicol-resistant bacterial strains isolated from Chilean salmon farms carried the *floR* gene, whereas others possessed non-specific efflux pumps that conferred florfenicol resistance (Fernández-Alarcón et al., 2010). In fish farm impacted marine sediments, Buschmann et al. (2012) recently reported the occurrence of several strains containing plasmid-borne *floR*, *tet*, and *qnr* genes.

PRIORITIZING RESEARCH AND POLICY NEEDS

The studies discussed in the previous sections support the hypothesis that fish farms represent a reservoir of diverse ARGs, many of which may be readily mobilized by lateral gene transfer. Given the extent of global aquaculture and its fast-paced growth, it is imperative that research needs with regards to the use of antimicrobials and the emergence and potential spread of antimicrobial resistance are prioritized. This research should form the basis of sound, science-based policies that contribute to the sustainability of the aquaculture industry and minimize risks to public health. Further studies are needed to explore the prevalence of antimicrobial resistance in zoonotic fish pathogens such as *Aeromonas hydrophila*, *Edwardsiella tarda*, *Mycobacterium fortuitum*, *Mycobacterium marinum*, *Photobacterium damselae*, *Pseudomonas fluorescens*, and *Streptococcus iniae* (Austin and Austin, 2012). Moreover, there are important knowledge gaps regarding the co-occurrence of antimicrobial-resistant bacteria from aquaculture environments with human pathogens throughout production cycles and across a range of aquaculture environments, the transfer rate of resistance genes between aquaculture and clinically relevant bacteria under field or semi-field conditions, and the epidemiology of antimicrobial resistance in areas of intense aquaculture activity.

Good management strategies can make a significant contribution to minimize the use of antimicrobials in fish and the emergence and spread of antimicrobial resistance in aquaculture environments. Among these precautionary practices are the use of "good quality" fish stocks, reducing stocking densities, maintaining overall good environmental conditions (e.g., dissolved O_2 levels), the implementation of proper biosecurity measures, the development of effective vaccines and vaccination programs, and

the rotation of antimicrobial compounds in the treatment of fish disease. Although the use of antimicrobials in human medicine places constraints on the type of antimicrobials that may be used in veterinary medicine, the rotation of antimicrobials in aquaculture may play an important role in reducing the chances of selection, co-selection, and dissemination of antimicrobial resistance. In this context, it is also important that efforts are directed toward creating incentives for the development and registration of antimicrobials for aquaculture use in addition to implementation of antimicrobial stewardship practices.

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MexXY multidrug efflux system of *Pseudomonas aeruginosa*

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Anti-pseudomonas aminoglycosides, such as amikacin and tobramycin, are used in the treatment of *Pseudomonas aeruginosa* infections. However, their use is linked to the development of resistance. During the last decade, the MexXY multidrug efflux system has been comprehensively studied, and numerous reports of laboratory and clinical isolates have been published. This system has been increasingly recognized as one of the primary determinants of aminoglycoside resistance in *P. aeruginosa*. In *P. aeruginosa* cystic fibrosis isolates, upregulation of the pump is considered the most common mechanism of aminoglycoside resistance. Non-fermentative Gram-negative pathogens possessing very close MexXY orthologs such as *Achromobacter xylosoxidans* and various *Burkholderia* species (e.g., *Burkholderia pseudomallei* and *B. cepacia* complexes), but not *B. gladioli*, are intrinsically resistant to aminoglycosides. Here, we summarize the properties (e.g., discovery, mechanism, gene expression, clinical significance) of the *P. aeruginosa* MexXY pump and other aminoglycoside efflux pumps such as AcrD of *Escherichia coli*, AmrAB-OprA of *B. pseudomallei*, and AdeABC of *Acinetobacter baumannii*. MexXY inducibility of the PA5471 gene product, which is dependent on ribosome inhibition or oxidative stress, is noteworthy. Moreover, the discovery of the cognate outer membrane component (OprA) of MexXY in the multidrug-resistant clinical isolate PA7, serotype O12 deserves special attention.

Keywords: aminoglycoside resistance, *Pseudomonas aeruginosa*, efflux, MexXY, PA5471, OprA

INTRODUCTION

Pseudomonas aeruginosa has been recognized as an increasingly important and worrisome species in health care-associated infections (Poole, 2011). This bacterium possesses intrinsic resistance to many antimicrobials because of the low permeability of its outer membrane barrier and the presence of multidrug efflux transporters (Nikaido, 1994; Hancock, 1998). Although fluoroquinolones (e.g., ciprofloxacin), broad-spectrum β-lactams (e.g., imipenem), and anti-pseudomonas aminoglycosides (e.g., amikacin) are often available for treatment, *P. aeruginosa* readily acquires resistance to these anti-pseudomonas agents via chromosomal mutations and lateral gene transfer (Poole, 2011). The emergence and spread of multidrug-, extensive drug-, and pandrug-resistant *P. aeruginosa* infections is of great concern as very few agents are effective against these strains (Fischbach and Walsh, 2009; Poole, 2011). The problem of increasing antimicrobial resistance is even more threatening when considering the very limited number of new antimicrobial agents in development (Fischbach and Walsh, 2009). In particular, the prospects of finding new antibiotics for Gram-negative pathogens are poor because of the low permeability of their outer membrane barriers and the presence of multidrug efflux transporters (Fischbach and Walsh, 2009). To combat these bacteria efficiently, it is necessary to understand the molecular basis of the efflux mechanisms involved in limiting the intracellular (or periplasmic) concentration of many antibiotics (Nikaido and Pages, 2012).

The most clinically relevant multidrug efflux systems in Gram-negative bacteria are those of the resistance-nodulation-cell division (RND) family (Poole, 2004, 2012; Piddock, 2006; Lister et al., 2009). *P. aeruginosa* expresses several RND-type multidrug efflux systems, of which MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY are significant determinants of multidrug resistance in laboratory and clinical isolates (Poole, 2004, 2012; Piddock, 2006; Lister et al., 2009). These pumps are three-component systems comprising antiporters of the RND family driven by proton motive force (MexB, MexD, MexF, and MexY), outer membrane factors (OMF; OprM, OprJ, and OprN), and periplasmic membrane fusion proteins (MFP; MexA, MexC, MexF, and MexX; Piddock, 2006; Lister et al., 2009). These pumps probably function in a similar manner with AcrAB-TolC, which is the best-studied RND-type multidrug pump of *Escherichia coli* (Nakashima et al., 2011; Nikaido, 2011).

Among them, the MexXY system is intriguing in that it is a significant determinant of aminoglycoside resistance only in *P. aeruginosa*, with numerous reports of clinical isolates during the last decade (Nikaido and Pages, 2012; Poole, 2012). Relatively few bacterial drug efflux systems are known to accommodate aminoglycosides (Poole, 2012). In addition to MexXY, AmrAB-OprA of *B. pseudomallei* is noteworthy for its contribution to this organism's intrinsic aminoglycoside resistance, while AdeABC of *Acinetobacter baumannii* is implicated in acquired aminoglycoside resistance (Poole, 2012). The upregulation of the MexXY pump is considered the most common mechanism of resistance (Armstrong and Miller, 2010) and appears to be the major determinant of aminoglycoside resistance in cystic fibrosis (CF) lung isolates of *P. aeruginosa* (Poole, 2011). Here, we summarize the properties

of these pumps and discuss how to combat efflux-mediated aminoglycoside resistance.

PRE-MexXY DISCOVERY ERA: RND MULTIDRUG EFFLUX PUMPS AS DETERMINANTS OF RESISTANT TO A WIDE RANGE OF ANTIMICROBIALS, BUT NOT AMINOGLYCOSIDES

In 1993, the first RND-type multidrug efflux system of *P. aeruginosa*, MexAB-OprM (OprM was called OprK at that time), was discovered at approximately the same time as the AcrAB (AcrB was called AcrE at that time) system of *E. coli* (Poole et al., 1993b). It was the first genetic evidence that an efflux operon was involved in multiple antibiotic resistance in *P. aeruginosa* (Poole et al., 1993b). The following year, the efflux activity of tetracycline, chloramphenicol, norfloxacin, and benzylpenicillin was shown using antibiotic accumulation assays in intact cells, which was the first biochemical evidence of the role of efflux in intrinsic multidrug resistance in *P. aeruginosa* (Li et al., 1994a,b; Li et al., 1995). Taken together, the MexAB-OprM system was shown to contribute to the intrinsic resistance of *P. aeruginosa* to a wide range of antimicrobial compounds including fluoroquinolones, tetracycline, chloramphenicol, and β-lactams such as carbenicillin (Poole et al., 1993b; Li et al., 1995). Its homologs (MexCD-OprJ and MexEF-OprN) were then discovered as determinants of *nfxB* and *nfxC* fluoroquinolone-resistant (e.g., norfloxacin) mutants from *P. aeruginosa* (Poole et al., 1996; Kohler et al., 1997). Incidentally, these two pumps are not expressed in normal laboratory growth conditions, but are induced under some conditions in wild-type *P. aeruginosa* (e.g., Morita et al., 2003; Fetar et al., 2011). An unidentified efflux system that requires OprM was shown to contribute to resistance to quinolones and cephalosporins, such as cefpirome, erythromycin, and tetracycline, but not β-lactams, such as cefoperazone and carbenicillin, in the *P. aeruginosa* PAO1 background (Zhao et al., 1998). More details on these three pumps can be found in recent reviews (e.g., Li and Nikaido, 2004, 2009; Lister et al., 2009).

In those days, RND multidrug efflux systems such as MexAB-OprM and AcrAB-TolC, which can handle a wide variety of drugs that appear to contain hydrophobic domains of significant sizes (Nikaido, 1996), were considered to be similar to the P-glycoprotein multidrug efflux pump of mammalian cells, which extrudes not only basic compounds but also neutral and weakly acidic compounds (Nikaido, 1994). However, there was no evidence for the efflux of aminoglycosides, which are very hydrophilic compounds, among the antibiotics used to treat *P. aeruginosa* infections (Li et al., 1994a; Nikaido, 1996).

MexXY SYSTEM OF *P. aeruginosa* WAS IDENTIFIED BY THREE DIFFERENT GROUPS

The MexXY system was discovered in 1999 in Japan as the fourth RND-type multidrug efflux system of *P. aeruginosa* PAO1 (Mine et al., 1999). This system was functionally expressed and conferred resistance to fluoroquinolones, tetracycline, erythromycin, etc. in the *E. coli* KAM3 mutant (Morita et al., 1998) lacking the *acrB* gene, which is an RND transporter component of the major multidrug efflux pump (AcrAB-TolC) in *E. coli*. Interestingly, unlike the other three already known systems, no open-reading frame encoding the outer membrane component, such as OprM, was found in the region downstream from the *mexY* gene (Mine et al., 1999). However, this system was found to function cooperatively with OprM of *P. aeruginosa* and TolC of *E. coli* (Mine et al., 1999).

Nine months after the discovery described above, a French group showed that MexXY was involved in the natural resistance of *P. aeruginosa* PAO1 to aminoglycosides as well as tetracycline and erythromycin (Aires et al., 1999). Although the overexpression of MexXY increased the level of resistance to fluoroquinolones in *P. aeruginosa* PAO1 cells, disruption of *mexXY* from PAO1 had no detectable effect on susceptibility to these agents (Aires et al., 1999). *mexZ*, which is located upstream of but transcribed separately from *mexXY*, was identified (Aires et al., 1999). Its product, MexZ, contains a helix-turn-helix motif, which is characteristic of DNA-binding proteins, at its N-terminus, similar to the repressors of RND-type multidrug efflux genes (e.g., AcrR, a repressor of *acrAB* in *E. coli*), supporting the notion that *mexZ* negatively controls the expression of the operon (Aires et al., 1999).

The following month (10 months after the first discovery), a group in the USA showed that MexXY, which they called AmrAB, was an aminoglycoside impermeability factor in spontaneous aminoglycoside-resistant mutants of the impermeability phenotype from *P. aeruginosa* PAO1 (Westbrock-Wadman et al., 1999). Interestingly, a dramatic decrease in the amount of OprM was observed in the mutants compared to wild-type PAO1, indicating that OprM is unlikely to be the outer membrane component associated with this efflux system in the mutants (Westbrock-Wadman et al., 1999). In addition, MexXY was shown to be upregulated in clinical *P. aeruginosa* isolates displaying aminoglycoside impermeability, suggesting that the pump is a clinically relevant mechanism of aminoglycoside resistance in *P. aeruginosa* (Westbrock-Wadman et al., 1999).

The following year, the complete genomic sequence of *P. aeruginosa* strain PAO1 (PAO1-UW) was published in *Nature* (Stover et al., 2000). Although the locus IDs PA2019-18 of the PAO1-UW genome sequence correspond to the *mexXY* genes, the nucleotide sequences of PA2019-18 were not identical with those of previously published *mexXY* (Aires et al., 1999; Mine et al., 1999) findings. This is probably because the DNA sequencing technology at that time was unable to analyze GC-rich bacteria such as *P. aeruginosa* (66–67% GC content; Winsor et al., 2011). Therefore, we analyzed MexXY using the nucleotide sequences from the PAO1-UW complete genome (Winsor et al., 2011) because we live in the post-genome era.

STRUCTURE AND FUNCTION OF MexY

The RND components of RND-type tripartite multidrug efflux pumps determine substrate specificity (e.g., Srikumar et al., 1997; Eda et al., 2003); therefore, we focused on the structure and function of MexY rather than MexX or OprM. Very recently, the crystal structure of the RND-type multidrug efflux pump AcrB of *E. coli* revealed the presence of two discrete, high-volume multisite binding pockets that contribute to the remarkably broad substrate recognition of AcrB and its homologs (Nakashima et al., 2011). Although we basically assume that MexY pumps out antimicrobials in a similar manner as AcrB, it will be intriguing to uncover the molecular basis of how MexY accommodates aminoglycosides

because they are strongly hydrophilic molecules that are completely different from the relatively hydrophobic compounds (e.g., minocycline, doxorubicin, rifampicin, and erythromycin) used as substrates of AcrB (Nakashima et al., 2011).

Generally speaking, the function of a transporter (e.g., substrate specificity and energy coupling) should be determined by its time course efflux assay and evaluated using kinetic constants (e.g., Yerushalmi et al., 1995; Edgar and Bibi, 1997; Mine et al., 1998; Morita et al., 2000). However, it is difficult to conduct such an assessment in a small bacteriology laboratory. The reconstitution of proteoliposomes revealed that AcrB, AcrD, and MdtBC of *E. coli* were H^+/drug antiporters (Zgurskaya and Nikaido, 1999; Aires and Nikaido, 2005; Kim et al., 2010), and we assume that MexY pumps out antimicrobials coupled with the same energy. In addition, five charged and polar amino acid residues that are involved in the proton translocation pathway are conserved between MexY and AcrB of *E. coli* (Takatsuka and Nikaido, 2006). Unfortunately, the purification, reconstitution, and characterization of the MexXY pump remain to be established, and the energy coupling and substrate specificity of MexXY has not been examined through its efflux activity. On the other hand, the MexXY-mediated energy-dependent efflux activity of ethidium (Mine et al., 1999), aminoglycosides (Aires et al., 1999; Vogne et al., 2004), tetracycline (Aires et al., 1999), Ala-Nap (MC-005,556) (Mao et al., 2001), and fluorescein-di-β-D-galactopyranoside (Matsumoto et al., 2011) has been measured in whole cells.

It is conventional to use differences in minimum inhibitory concentrations (MICs) between bacterial cells with and without a multidrug efflux transporter to estimate substrate specificity (e.g., Nishino and Yamaguchi, 2001; Nishino et al., 2003). Although the comparison of MICs can sometimes be a misleading indicator of pump function (e.g., Nagano and Nikaido, 2009), it can still indicate the possible clinical relevance of a pump (e.g., Poole, 2004, 2012; Piddock, 2006). Mutant strains lacking major multidrug efflux pump(s) have been used to determine substrate specificity (e.g., Nishino and Yamaguchi, 2001; Morita et al., 2001b). The substrate specificity of MexXY-OprM was determined using a mutant from PAO1 that overproduced MexXY-OprM, but not MexAB (and AmpC in the case of β-lactams), and were compared with a mutant lacking MexXY/MexAB-OprM (and AmpC in the case of β-lactams; Masuda et al., 2000b). MexXY-OprM-mediated resistance was then observed for quinolones, macrolides, tetracyclines, aminoglycosides, chloramphenicol, lincomycin, and most β-lactams, but not for novobiocin, polymyxin B, and some β-lactams (carbenicillin, sulbenicillin, cefsulodin, ceftazidime, oxacephem, imipenem, and aztreonam) among a wide variety of antimicrobial agents (Masuda et al., 2000b). In conclusion, MexXY-OprM is a multidrug efflux transporter whose specificity is extraordinary broad, but different compared with MexAB-OprM, MexCD-OprJ, MexEF-OprN, and other RND efflux transporters in *P. aeruginosa*. In addition, MexXY-OprM was the only pump to mediate aminoglycoside resistance and was thus considered to recognize aminoglycosides as substrates (Masuda et al., 2000b).

Basic local alignment search tool (BLAST) analysis showed that MexY was highly conserved in *P. aeruginosa* strains: more than 99% (99%) identity (positive) for most strains and 97% (98%) identity (positive) for PA7 (Morita et al., 2012). There was no functional difference between the MexYs of PAO1 and PA7 when they were expressed in either *E. coli* or *P. aeruginosa* (Morita et al., 2012). MexY was more similar [70–73% (83–86%) identity (positive)] to orthologs of *B. pseudomallei* and various *B. cepacia* complexes than other RND pumps of *P. aeruginosa* and other *Pseudomonas* species (Morita et al., 2012). These *Burkholderia* species, except for *B. mallei*, are intrinsically resistant to aminoglycosides (e.g., Kenny et al., 1999; Thibault et al., 2004; Vermis et al., 2003; Jassem et al., 2011). *B. gladioli* is also known to be involved in human infections (Segonds et al., 2009); however, no MexY (AmrB) ortholog exists in *B. gladioli* BSR3 (Seo et al., 2011), consistent with the fact that all isolates tested were susceptible to aminoglycosides (Segonds et al., 2009). Interestingly, the most similar functional ortholog to MexY exists in *Achromobacter xylosoxidans* and has a 74% identity (86% positive); this pump was named AxyY in strain AXX-A (Bador et al., 2012). *A. xylosoxidans* is also an opportunistic human pathogen capable of causing a wide range of infections (Glupczynski et al., 1988; Bador et al., 2011). Most *A. xylosoxidans* clinical isolates were resistant to the tested aminoglycosides, including amikacin (Ngeow and Puthucheary, 1985; Glupczynski et al., 1988). The AxyY pump contributes to aminoglycoside resistance in a similar manner to MexY and AmrBs (Bador et al., 2012).

COBALT analysis is a multiple sequence alignment tool for finding a collection of pairwise constraints. Such constraints are derived from data of the conserved domain database, protein motif database, and sequence similarity of RND pumps (Papadopoulos and Agarwala, 2007), including all pumps from *P. aeruginosa* PAO1-UW and *E. coli* K12 (MG1655). The exception is heavy metal efflux pumps, which are characterized by their relationships. Therefore, we focused on the four branches containing the four Mex pumps in *P. aeruginosa* (**Figure 1**). The MexY branch is located next to the MexD branch and includes the AmrBs of *Burkholderia* species (e.g., Mima and Schweizer, 2010) and AxyY of *A. xylosoxidans* (Bador et al., 2012). The MexD branch includes the AdeB pump of *A. baumannii* (Magnet et al., 2001), MtrD of *Neisseria gonorrhoeae* (Hagman et al., 1997), and BdeB of *Bradyrhizobium japonicum* (Lindemann et al., 2010). The SmeZ pump of *S. maltophilia*, which can mediate aminoglycoside resistance (Crossman et al., 2008), also belongs to the MexD branch. Many pumps in the MexY/MexD branches can mediate aminoglycoside resistance (e.g., Magnet et al., 2001; Crossman et al., 2008; Lindemann et al., 2010; Mima and Schweizer, 2010), which hints at the structure-function relationship of pumps involved in aminoglycoside resistance. MexB is located in the branch that contains the AcrB/D/F and MdtF pumps of *E. coli* (Nishino and Yamaguchi, 2001; Nishino et al., 2003), AdeJ of *A. baumannii* (Damier-Piolle et al., 2008), BpeB of *B. pseudomallei* (Mima and Schweizer, 2010), AxyB of *A. xylosoxidans* (Bador et al., 2011), and VmeB of *Vibrio parahaemolyticus* (Matsuo et al., 2007). Among them, some pumps (e.g., AcrD and MexB) were reported to be involved in aminoglycoside resistance under some conditions (Li et al., 2003; Aires and Nikaido, 2005). The MexF branch includes AdeG of *A. baumannii* (Coyne et al., 2010) and MdsB of *Salmonella enterica* (Nishino et al., 2006).

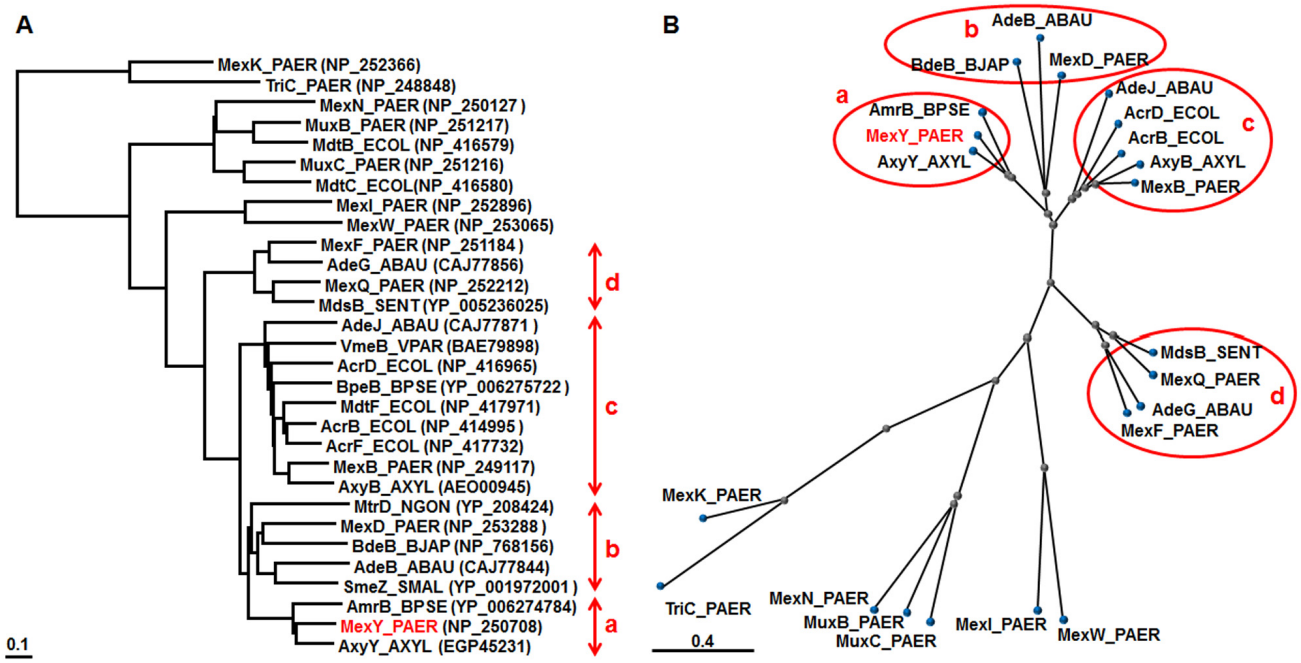

FIGURE 1 | Phylogenetic trees for representative RND transporters. According to the COBALT program, the trees were constructed using the Fast evolution method and rendered with **(A)** Rectangle and **(B)** Radical. Protein names are abbreviated; for example, "MexY_PAER" stands for "MexY of *P. aeruginosa*." Accession numbers are shown in parentheses. The four branches shown in red are: (a) MexY, (b) MexD, (c) MexB, and (d) MexF.

Substrate specificity is determined almost entirely by the periplasmic domain (i.e., two large extramembrane loops that largely protrude toward the periplasmic space) of MexY (and MexB; Eda et al., 2003). $MexY_{F1018L}$ (the F1018L mutation is located in TMS-12 of MexY base on the structure of AcrB; Murakami et al., 2002), enhanced the function of MexY, presumably by increasing the efflux of aminoglycosides, cefepime, and fluoroquinolones, which was the first example of an improved efflux pump *in vivo* (Vettoretti et al., 2009b).

GENE EXPRESSION OF THE MexXY SYSTEM

MexXY was shown to be induced by sub-inhibitory concentrations of tetracycline, erythromycin, aminoglycosides, tigecycline, and LMB415 (a peptide deformylase inhibitor), but not ofloxacin in *P. aeruginosa* PAO1 (Masuda et al., 2000a; Dean et al., 2003; Caughlan et al., 2009). Moreover, ofloxacin and cefpirome were also shown to be inducers, but only in a PAO1 mutant lacking MexAB (and AmpC in the case of cefpirome; Masuda et al., 2000a,b). MexZ was shown to bind an inverted repeat region located in the *mexZ-mexX* intergenic region directly as a homodimer, which encompasses the putative *mexXY* promoter, but the inducers failed to alter the MexZ-operator interactions (Matsuo et al., 2004). The crystal structure of MexZ has since been solved (Alguel et al., 2010). Induction of the MexXY efflux pump in *P. aeruginosa* PAO1 was shown to be dependent on drug–ribosome interactions (Jeannot et al., 2005), and the pump remained inducible, but to a lesser degree, by ribosomal inhibitors, even in the *mexZ* mutant (Jeannot et al., 2005). These data demonstrate the physiological interactions between MexXY and the ribosome and are suggestive of an alternative function for MexXY beyond the efflux of antibiotics (Jeannot et al., 2005). Microarray analysis showed that *mexXY* were the most highly upregulated genes in *P. aeruginosa* PAO1 after 4 h of interaction with primary normal human airway epithelial cells (Frisk et al., 2004) and in response to sub-inhibitory concentrations of tobramycin under normal aerobic conditions, but not under lethal aerobic conditions or anaerobic conditions (Kindrachuk et al., 2011).

The antibiotic inducibility of the MexXY multidrug efflux system of *P. aeruginosa* was shown to be involved in the modulation of MexZ activity by the antibiotic-inducible PA5471 gene product (Morita et al., 2006; **Table 1**). PA5471 encodes a predicted product of 43.5 kDa, which was identified as a hypothetical protein conserved between bacteria and archaea, and is a representative of the uncharacterized protein family UPF0027 in the Pfam protein families database (Morita et al., 2006) or the PRK09588 cluster in ProtClustDB (NCBI Protein Clusters Database; Klimke et al., 2009). Recently, it was demonstrated that RctB of *E. coli*, which is related to members of this family, is a novel RNA ligase and functions as a *bona fide* RNA repair protein *in vivo* (Tanaka and Shuman, 2011). PA5471 is found upstream of and in a possible operon with an open-reading frame dubbed PA5470; RT-PCR confirmed both the drug inducibility of PA5470 and its expression from a polycistronic message that also contains PA5471 (Morita et al., 2006). PA5470 is predicted to encode a peptide chain release factor of 22.3 kDa (Morita et al., 2006). A homolog of PA5471 from *E. coli* K12, *ykfJ* (b0235), which was, however, C-terminally truncated (approximately 1 kb; Baranov et al., 2006), was also shown to be inducible by 4-azaleucine, which is known

to interfere with translation, and it too is linked to a putative peptide release factor gene (Morita et al., 2006). *P. aeruginosa* senses antibiotic-mediated ribosomal disruption and links it to PA5471 gene expression by monitoring the translation of a 13-amino-acid-leader peptide region (PA5471.1) found ∼250 bp upstream of the PA5471 coding sequence on PA5471 mRNA (Morita et al., 2009). The antimicrobial-inducible PA5471 gene product has been shown to interact with the repressor MexZ and interfere with its DNA binding activity *in vitro* (Yamamoto et al., 2009), and this finding contributed to elucidating the molecular mechanisms of the MexXY induction. However, PA5471 is not sufficient for MexXY recruitment in response to antibiotic exposure, and additional antibiotic-dependent effects are needed in *P. aeruginosa* (Morita et al., 2009). Exposure to reactive oxygen species (ROS; e.g., peroxide) induces the expression of the PA5471 gene, leading to MexXY-dependent aminoglycoside resistance (Fraud and Poole, 2011). Moreover, long-term (8-day) exposure of *P. aeruginosa* to peroxide (mimicking chronic *in vivo* ROS exposure) increased the frequency of PA5471- and *mexXY*-dependent aminoglycoside resistance (Fraud and Poole, 2011). Recently, reduced (approximately twofold) expression of the *rplU-rpmA* operon (encoding the 50S ribosomal proteins L21 and L27) was shown to promote *mexXY* expression via the PA5471 gene in pan-aminoglycoside resistant mutants from PAO1 and a CF clinical isolate (Lau et al., 2012). Such expression was in the form of ribosomal protein mutations that influence *mexXY* expression, including *rplY* (encoding ribosomal protein L25; El'Garch et al., 2007) and *rplA* (encoding ribosomal protein L1; Westbrock-Wadman et al., 1999). Transcriptome profiling revealed that significantly increased expression was observed for the *mexXY* and PA5471 genes in both the PA2572 and PA2573 mutants compared with the wild-type PAO1 strain during exponential growth in Luria–Bertani media (McLaughlin et al., 2012). PA2572 encodes a putative response regulator of a two-component system required for full virulence to *Galleria mellonella* (Wax moth) and PA2573 also encodes an ophan chemotaxis sensor which seems to function in part through signal transduction involving PA2572 (McLaughlin et al., 2012).

A recent study identified a gene, *parR*, encoding the response regulator of a two-component system, ParRS, which promotes either induced or constitutive *mexXY* upregulation, thereby activating the MexXY efflux system as well as OprD porin loss and lipopolysaccharide modification in a MexZ-independent manner (Muller et al., 2011). Overexpression of PaeIII, a small non-coding RNA between PA3505 and PA3536 in the genome of *P. aeruginosa* PAO1, in the stationary phase increased the expression of the *mexXY* and *mexZ* genes as well as type III secretion genes, while reducing the expression of genes for arginine metabolism (Goldberg et al., 2008).

MexXY SYSTEM AS AN ANTIMICROBIAL RESISTANCE DETERMINANT IN *P. aeruginosa*

Pseudomonas aeruginosa shows intrinsic resistance against many antimicrobials because of the low permeability of its outer membrane and the presence of efflux systems (Nikaido, 1994; Hancock, 1998). MexXY was shown to be involved in natural resistance to aminoglycosides, tetracycline, tigecycline, erythromycin, and LBM415 in *P. aeruginosa* PAO1 (Aires et al., 1999; Masuda et al., 2000a; Morita et al., 2001a; Dean et al., 2003; Caughlan et al., 2009). MexXY was also shown to be necessary for the adaptive resistance of *P. aeruginosa* PAO1 to aminoglycosides (Hocquet et al., 2003). It is of note that MexXY is the only pump of the 12 identified RND systems that mediates aminoglycoside resistance in *P. aeruginosa* PAO1 (Poole, 2011). The antagonism of aminoglycosides by the divalent cations Mg^{2+} and Ca^{2+} is well documented (Medeiros et al., 1971), and culture in cation-adjusted Mueller–Hinton broth is recommended as a susceptibility test to ensure acceptable results when *P. aeruginosa* isolates are tested (Barry et al., 1992). MexXY was shown to be required for the antagonism of aminoglycosides by divalent cations in *P. aeruginosa* PAO1 (Mao et al., 2001). Although Phe-Arg-β-naphthylamide (PAβN, MC-207,110) is known as a non-specific inhibitor against RND-type multidrug efflux pumps (Lomovskaya et al., 2001), this inhibitor, as observed for divalent cations, antagonized the activity of aminoglycosides (amikacin and netilmicin) in a MexXY-dependent manner, even though it also inhibited MexXY-dependent fluoroquinolone (levofloxacin) resistance (Mao et al., 2001). Conversely, PAβN inhibited MexXY-mediated aminoglycoside (gentamicin) resistance (Mesaros et al., 2007). The reason for the discrepancy between these two results remains unknown. Increased susceptibility to aminoglycosides in *nfxB* mutants, which upregulate *mexCD-oprJ* expression, was correlated with increased resistance to fluoroquinolones and some β-lactams, such as cefepime, concomitant with a higher susceptibility to aminoglycosides and some β-lactams, such as ticarcillin, aztreonam, and imipenem. This was shown to be partly due to the impaired activity of MexXY-OprM because of major changes in cell physiology, but not the expression/production of *mexY*/MexY and *oprM*/OprM (Jeannot et al., 2008; Mulet et al., 2011). The increased susceptibility to aminoglycosides in MexEF-OprN-overproducing *nfxC* mutants was also observed, apparently owing to impairment of the MexXY system (Sobel et al., 2005). *mexXY* expression (and so MexXY-mediated resistance) was independent of the AmgRS two-component system in which mutations enhanced aminoglycoside action to control an adaptive response to membrane stress (Lee et al., 2009).

Multidrug resistant *P. aeruginosa* clinical isolates have often been reported to be MexXY overproducers (e.g., Llanes et al., 2004, 2006; Wolter et al., 2004; Deplano et al., 2005; Henrichfreise et al., 2007; Hocquet et al., 2007; Maniati et al., 2007; Vettoretti et al., 2009a; Beaudoin et al., 2010; Xavier et al., 2010; Fehlberg et al., 2012; Pasca et al., 2012). Time series analysis (January 1999 to January 2005) revealed a significant relationship between antibiotic use (aminoglycosides, fluoroquinolones, and cefepime, but not carbapenems) and the incidence of MexXY-overproducing *P. aeruginosa* in a French hospital (Hocquet et al., 2008). MexXY ($n = 39$) and MexAB ($n = 31$) were the most frequently overproduced pumps in 85 non-CF *P. aeruginosa* strains with low-level ciprofloxacin resistance (MICs ranging from 0.25 to 2 μg/mL, which are still susceptible or intermediate according to the CLSI breakpoints; Llanes et al., 2011). A large proportion of the strains were MexXY overproducers in genotypically distinct *P. aeruginosa* clinical isolates that were less susceptible to cefepime than to ceftazidime, and these were identified in Europe (Hocquet et al., 2006; Pena et al., 2009; Campo Esquisabel et al., 2011) and the USA (Laohavaleeson et al., 2008). In contrast, both cefepime and ceftazidime

are potent β-lactam antibiotics with similar MICs (1–2 μg/mL) for wild-type *P. aeruginosa* strains. Moreover, ceftobiprole, similar to cefepime, selected MexXY overproducers in clinical studies (Baum et al., 2009). Actually, a single step MexXY overproducer was selected *in vitro* by cefepime and ceftobiprole, but not ceftazidime (Queenan et al., 2010). MexXY contributed very significantly to the development of high-level (100–1000 μg/mL MIC) aminoglycoside resistance via a combination of aminoglycoside-modifying enzymes (AMEs) in multidrug resistant *P. aeruginosa* non-CF clinical isolates (Morita et al., 2012). However, AMEs are common determinants of aminoglycoside resistance in *P. aeruginosa*, except for CF isolates (Poole, 2011). In clinical CF isolates, MexXY has been primarily implicated in pan-aminoglycoside resistance (e.g., Sobel et al., 2003; Vogne et al., 2004; Islam et al., 2004, 2009). MexXY was also shown to be necessary in subpopulations of *P. aeruginosa* CF isolates that are hypersensitive to ticarcillin (called Tic^{hs}; Vettoretti et al., 2009b). *mexZ* was shown to be one of the most frequently mutated genes during chronic infection by *P. aeruginosa* in CF patients (Smith et al., 2006; Feliziani et al., 2010). However, a number of studies highlighted the absence of mutations in *mexZ* or the *mexXY* promoter region in MexXY-overproducing *P. aeruginosa* CF isolates (Sobel et al., 2003; Vogne et al., 2004; Islam et al., 2009). To date, three kinds of mutants (*agrZ*, *agrW1*, and *agrW2*) have been recognized as MexXY overproducers as a result of genetic mechanisms: mutants with impaired binding or unbinding of MexZ due to alterations in the *mexZ* or *mexZ-mexX* intergenic region (type *agrZ*); mutants with impaired protein synthesis (type *agrW1*); and mutants with alterations in *parRS* (type *agrW2*; de Bentzmann and Plesiat, 2011). Oxidative stress, a component of the host's immune system in the CF lung, induced *mexXY* expression via PA5471 and promoted aminoglycoside resistance (Fraud and Poole, 2011). Under conditions of oxidative stress, *P. aeruginosa* can develop aminoglycoside resistance, even in the absence of aminoglycosides (Poole, 2012). It is also very plausible that the routine use of aminoglycosides (e.g., tobramycin) might simply select for MexXY-overproducing *P. aeruginosa* in the CF lung (Smith et al., 2006).

Although it is obvious that MexXY is one of the determinants of antimicrobial resistance in *P. aeruginosa* in the clinical setting (Poole, 2011), only a few reports have assessed the *in vivo* impact of the MexXY system on antibiotic therapy for *P. aeruginosa* infections (e.g., Martha et al., 2006).

COGNATE OUTER-MEMBRANE COMPONENT OprA OF THE MexXY PUMP IS FOUND IN SEROTYPE O12 BUT IS LOST IN OTHERS

The *mexXY* operon lacks a gene coding for the outer membrane protein in *P. aeruginosa* PAO1 (Mine et al., 1999). OprM is necessary for the function of MexXY and MexAB in *P. aeruginosa* PAO1 (Aires et al., 1999; Masuda et al., 2000a; Morita et al., 2001a), although overproduced OpmB (PA2525) can function as an outer membrane component of MexXY, MexAB, and MexCD (Murata et al., 2002). Intriguingly, the multidrug resistant taxonomic outlier *P. aeruginosa* PA7 possesses a unique gene (*oprA*) downstream of *mexXY* encoding an outer membrane channel that is absent in most *P. aeruginosa* strains (Roy et al., 2010). MexXY in this strain utilizes either the OprA or OprM outer membrane channel (Morita et al., 2012; **Table 1**). While OprM is functional with both MexXY and MexAB, OprA did not associate as strongly with MexAB as it did with MexXY (Morita et al., 2012). We compared the OprA of *P. aeruginosa* PA7 with the OprM family (Remans et al., 2010) from *P. aeruginosa* PAO1 as well as TolC of *E. coli* K12 (Mine et al., 1999), OprA of *B. pseudomallei* 1026b (Mima and Schweizer, 2010), OprZ of *A. xylosoxidans* AXX-A (Bador et al., 2012), and AdeC *of A. baumannii* AYE (Magnet et al., 2001; **Figure 2**). COBALT analysis showed that OprA of *P. aeruginosa* PA7 and its close orthologs (OprA of *B. pseudomallei* 1026b and OprZ of *A. xylosoxidans* AXX-A) is located close to OprJ and is followed by OprM of the OprM outer membrane family of *P. aeruginosa* PAO1 (**Figure 2**).

Interestingly, a small portion of the *oprA* gene immediately downstream of the *mexY* gene in PAO1 was identified, suggesting that non-PA7 *P. aeruginosa* strains might have possessed, but lost, the intact *mexXY-oprA* efflux pump locus (Morita et al., 2012; **Table 1**). Consistent with this, the majority of a panel of serotype strains possessed the truncated *oprA*, but the serotype O12 isolate had an intact *mexXY-oprA* locus, similar to PA7 and the related strain DSM 1128 (Morita et al., 2012). O12 is a predominant serotype associated with multidrug resistance to a number of antibiotic classes, including aminoglycosides and β-lactams, although it represents a minor serotype in the environment (Pirnay et al., 2009; Roy et al., 2010). O12 might be more dominant due, in part, to the presence of *oprA* in hospitals in which antimicrobials promoting MexXY-OprA-mediated multidrug resistance, such as aminoglycosides, were used. *P. aeruginosa* PA7 isolated before 1984 from a wound infection in Buenos Aires, Argentina (Pirnay et al., 2009; Roy et al., 2010), might also have acquired multidrug resistance via the heavy use of antibiotics, including gentamicin or tobramycin, to treat wounds at that time. Apparently, a slightly increased resistance (two- to fourfold) to amikacin, ciprofloxacin, and cefpirome was shown in the presence and absence of *oprA* (Morita et al., 2012). Such a small difference might be significant during antibiotic treatment or in the presence of sub-inhibitory concentrations of antibiotics.

AcrD IS AN AMINOGLYCOSIDE EFFLUX PUMP THAT IS THE MOST SIMILAR TO MexY AMONG THE RND PUMPS IN *E. coli* K12

AcrD has the highest similarity score at the amino acid level to MexY of the *E. coli* K12 RND pumps and was shown to be an aminoglycoside efflux pump as judged by MIC determination and the aminoglycoside efflux assay (Rosenberg et al., 2000). However, differences in aminoglycoside resistance (uptake) between the parent strain JC7623 and its *acrD*-deletion mutant JZM320 was possibly not limited to AcrD function because JZM320 was constructed by inserting the *tet* gene from pBR322 into *acrD* (Rosenberg et al., 2000). The increased aminoglycoside uptake might be due to not only AcrD deficiency but also to the production of an aberrant cytoplasmic membrane protein (the product of *acrD* with the inserted *tet*) and/or the tetracycline/H^+ antiporter itself (Merlin et al., 1989a,b; Wyka and St John, 1990). While disruption of *tolC* or *acrA* did not increase the susceptibility of K12 to aminoglycosides (Rosenberg et al., 2000), both of them were

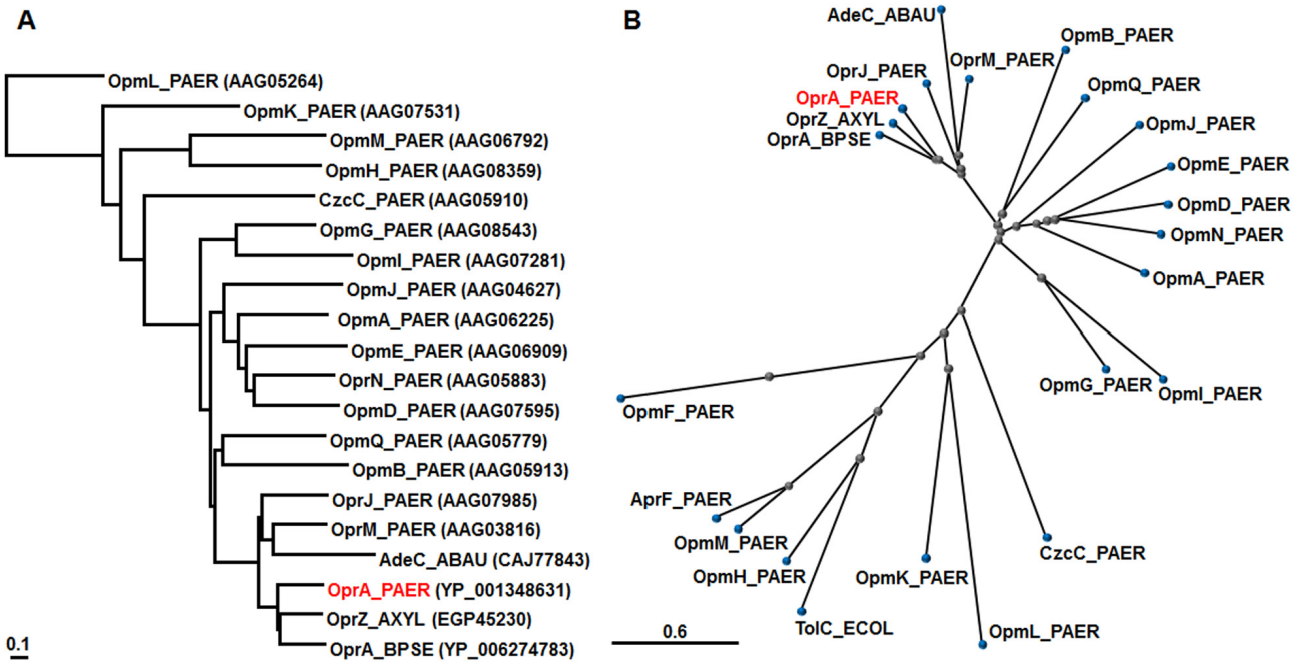

FIGURE 2 | Phylogenetic trees for representative OMPs. According to the COBALT program, the trees were constructed using the Fast evolution method and rendered with **(A)** Rectangle and **(B)** Radical. Protein names are abbreviated; for example, "OprA_PAER" stands for "OprA of *P. aeruginosa*." Accession numbers are shown in parentheses.

necessary for the function of *acrD* against various antimicrobials; however, no aminoglycosides were used in the study (Hirakawa et al., 2003). We do not rule out the hypothesis of Rosenberg et al. (2000) that AcrD protein can perhaps function without the participation of AcrA and TolC in the case of aminoglycoside efflux.

It is evident that purified AcrD can function as an H^+-driven aminoglycoside efflux pump (Aires and Nikaido, 2005). Especially, strong stimulation of proton efflux was observed when aminoglycosides (e.g., streptomycin) were added to the more acidic intra-vesicular space of reconstituted AcrD proteoliposomes containing AcrA and Mg^{2+} (Aires and Nikaido, 2005), indicating that AcrD captures aminoglycosides exclusively from the periplasm in *E. coli* (Nikaido, 2011). The difference in the MICs of amikacin and gentamicin between a parent strain and its in-frame *acrD*-deletion mutant or between an *acrBD*-deletion mutant and its *acrD*-overexpressing complementation mutant was approximately twofold (Elkins and Nikaido, 2002; Aires and Nikaido, 2005). There was no significant difference in kanamycin resistance in the case of an in-frame deletion (Hirakawa et al., 2003), and a twofold difference was observed in the case of overproduction (Nishino and Yamaguchi, 2001; Nishino et al., 2010). An *acrA* in-frame deletion mutant also showed an approximately twofold increased susceptibility to aminoglycosides (Aires and Nikaido, 2005). However, similar observations were not seen for AcrB (Nishino and Yamaguchi, 2001; Elkins and Nikaido, 2002; Aires and Nikaido, 2005). A comparison of the entrances of the vestibules, which are found in the central cavities (Murakami et al., 2002) of AcrD (which transports aminoglycosides) and AcrB (which does not) crystal structures, shows that this area in AcrD is in line with many more acidic residues that may attract polycationic substrates (Yu et al., 2003). Treatment with sub-inhibitory concentrations of kanamycin induced adaptive resistance to aminoglycosides, which was dependent on *acrD* (Sidhu et al., 2012). Aminoglycosides are very hydrophilic and polycationic and assumingly permeate through the porin channel in *E. coli*, unlike *P. aeruginosa* (Nikaido and Pages, 2012) in addition to so called "self-promoted" aminoglycoside uptake across the outer membrane of both of *E. coli* and *P. aeruginosa* (Hancock et al., 1991; Hancock, 1998). The MICs of aminoglycosides on *E. coli*, unlike *P. aeruginosa*, might be poor indicators of aminoglycoside efflux. There are numerous AcrD homologs in other Enterobacteriaceae (Poole, 2004). Although the AcrD of *S. enterica* serovar Typhimurium ATCC 14028s was studied comprehensively, no significant difference between the AcrDs of *E. coli* and *S. enterica* has been observed so far (Nishino et al., 2009; Horiyama et al., 2011; Yamasaki et al., 2011). Interestingly, AcrD pumps mediate resistance to the substrates of MexAB (e.g., carbenicillin, aztreonam, and novobiocin). However, AcrD pumps did not mediate resistance to the substrates of MexXY (e.g., cefpirome, erythromycin, and tetraphenylphosphonium) or shared substrates of both MexAB and MexXY (e.g., fluoroquinolone and tetracycline) when differences of the MICs were compared between a parent strain and its transformant overproducing the pump (Srikumar et al., 1997; Mine et al., 1999; Morita et al., 2001a; Nishino et al., 2003, 2009; Horiyama et al., 2011; Yamasaki et al., 2011). MexAB-OprM was also shown to contribute to aminoglycoside resistance, presumably via active efflux in the

low-ionic-strength medium used in this particular study (Li et al., 2003). AcrD and the MdtABC pump were iron-regulated, induced in low-iron conditions, and export the siderophore enterobactin (Bochner et al., 2008), which reminds us that MexAB-OprM was inducible under conditions of iron limitation and compensated for a growth defect in an iron-deficient medium in the presence of the non-metabolizable iron chelator 2,2′-dipyridyl (Poole et al., 1993a,b). AcrD seems to be a functional homolog of MexB rather than MexY, as determined from substrate specificity and physiological function, consistent with the fact that phylogenetic analysis showed that AcrD is closer to MexB than to MexY (**Figure 1**).

AmrAB-OprA IS A MULTIDRUG EFFLUX SYSTEM THAT MEDIATES AMINOGLYCOSIDE RESISTANCE IN *B. pseudomallei*

Burkholderia pseudomallei is the etiologic agent of melioidosis, a rare but serious disease endemic to South Asia, Northern Australia, and other parts of the tropics (Mima and Schweizer, 2010). Melioidosis is very difficult to treat because of the intrinsic resistance to many antimicrobial agents including aminoglycosides, macrolides, polymyxins, and some β-lactams (Mima and Schweizer, 2010). AmrAB-OprA was identified as an efflux determinant of resistance to aminoglycosides and macrolides in the *B. pseudomallei* 1026b clinical isolate (Moore et al., 1999). This pump was actually the first to be demonstrated responsible for the aminoglycoside resistance of RND pumps in Gram-negative bacteria. The gene product of *amrR*, which is located immediately upstream and divergently transcribed from *amrAB-oprA* in *B. pseudomallei* 1026b (Moore et al., 1999), showed strong homology [60% (73%) identity (positive)] to MexZ, which acts as a transcriptional repressor of the *mexXY* operon of *P. aeruginosa* PAO1 (Matsuo et al., 2004; Alguel et al., 2010; **Table 1**).

While the majority of *B. pseudomallei* clinical isolates exhibit high levels of aminoglycoside and macrolide resistance, rare isolates are susceptible to these antibiotics (Simpson et al., 1999; Trunck et al., 2009). While it is noted that the resistance profile of those isolates matches that of the *amrAB-oprA* mutants (Simpson et al., 1999), it was shown experimentally that *amrAB-oprA* was missing in *B. pseudomallei* 708a, an aminoglycoside- and macrolide-susceptible clinical isolate, and this loss was associated with the deletion of >130 kb of genetic material (Trunck et al., 2009). The expression of *amrAB-oprA* increased resistance to not only aminoglycosides and macrolides but also fluoroquinolones and tetracyclines in a BpeAB-OprA pump-deficient mutant of 1026b (Mima and Schweizer, 2010). Judging from the substrate specificity and sequence similarity (Mima and Schweizer, 2010), we have no doubt that AmrAB is a functional ortholog of MexXY in *B. pseudomallei*. BpeAB-OprB of *B. pseudomallei* also reportedly mediates aminoglycoside resistance in strain KHW (Chan et al., 2004), while this pump did not confer aminoglycoside resistance in 1026b (Mima and Schweizer, 2010). In addition, the BpeB RND transporter was also shown to be closely related to MexB of *P. aeruginosa*, both functionally and phylogenetically (Mima and Schweizer, 2010), consistent with our phylogenetic analysis (**Figure 1**).

Table 1 | Genetic organization of aminoglycoside efflux operons of clinical significance and their regulators in non-fermentative Gram-negative pathogens.

Organism	Efflux operon	Product	Function	Regulator	
				Cognate	Other
P. aeruginosa	*mexXY* (*-oprA*)[a]	MexX	MFP	MexZ	PA5471
		MexY	RND		ParRS
		(OprA)[a]	OMF		
		OprM			
A. xylosoxidans	*axyXY-oprZ*	AxyX	MFP	AxyR	
		AxyY	RND		
		OprZ	OMF		
B. pseudomallei (*B. cepacia* complex)	*amrAB-oprA*	AmrA	MFP	AmrR	
		AmrB	RND		
		OprA	OMF		
A. baumannii	*adeAB* (*-adeC*)[b]	AdeA	MFP	AdeRS	?[c]
		AdeB	RND		
		(AdeC)[b]	OMF		
		?[b]			

The genetic organization of genes involved in aminoglycoside efflux and their regulation of expression is summarized. No obvious significant differences on substrate specificities were observed among the pumps. TetR-type negative regulators are encoded by genes located upstream of the operons in P. aeruginosa, A. xylosoxydans, and B. pseudomallei, while the AdeRS two-component regulatory proteins are encoded by genes located upstream of the adeAB (-adeC) operon.

[a] *oprA gene found in the multidrug-resistant clinical isolate PA7 and relatives, all of which are serotype O12, is absent and often OprM encoded by the mexAB-oprM multidrug efflux operon is associated with the MexXY component in most P. aeruginosa strains (Morita et al., 2012). MexXY can utilize OprA or OprM as an outer membrane channel (Morita et al., 2012).*

[b] *AdeC is not essential for AdeAB-mediated resistance (Marchand et al., 2004), suggesting that AdeAB recruits another yet unknown outer membrane protein as indicated by the question mark.*

[c] *The question mark signifies other unknown regulatory mechanism(s) involved in adeABC overexpression (Sun et al., 2010).*

As described above, AmrB orthologs are conserved among various human pathogens belonging to *Burkholderia* species, but not *B. gladioli*. Actually, an AmrAB-OprA ortholog was shown to be a major aminoglycoside resistance contributor in *B. cenocepacia*, a member of the *B. cepacia* complex (Hamad et al., 2010).

AdeABC IS A MULTIDRUG EFFLUX SYSTEM THAT MEDIATES AMINOGLYCOSIDE RESISTANCE IN *A. baumannii*

Acinetobacter baumannii is the most frequently implicated species in nosocomial infections among *Acinetobacter* spp. (Coyne et al., 2011). AdeABC was identified as an RND-type efflux pump involved in resistance to multiple antimicrobials including aminoglycosides, fluoroquinolones, tetracycline, erythromycin, cefotaxime, trimethoprim, and chloramphenicol in *A. baumannii* BM4454, a low-level pan-aminoglycoside resistant clinical isolate

(Magnet et al., 2001). The polycistronic *adeABC* transcript was confirmed experimentally to encode AdeA (MFP), AdeB (RND transporter), and AdeC (OMF; Magnet et al., 2001; Marchand et al., 2004). Because the substrate specificity of AdeAB is very similar to that of MexXY, we have no doubt that AdeAB is a functional homolog of MexXY in *A. baumannii*. AdeC is not essential for AdeAB-mediated resistance (Marchand et al., 2004), indicating that AdeAB recruits another outer membrane protein to form a functional tripartite complex, as observed for the MexXY pump with OprM in *P. aeruginosa* (Aires et al., 1999; Mine et al., 1999). We do not rule out the possibility that AdeAB is functional with AdeC, as observed for the MexXY pump with its linked outer-membrane channel OprA, which was not essential in *P. aeruginosa* PA7 (Morita et al., 2012). The *adeAB* genes are usually present, but the *adeC* gene was not found in ∼40% of clinical isolates (Nemec et al., 2007). Our phylogenetic analysis showed that AdeC is more closely related to OprM and OprJ than to OprA in the OprM outer membrane family of *P. aeruginosa* (**Figure 2**).

The *adeABC* operon is expressed at low levels in natural isolates of *A. baumannii* due to stringent control by the AdeRS two-component system, which is encoded adjacent to *adeABC*, but transcribed in the opposite direction (Marchand et al., 2004; **Table 1**). Mutations (e.g., $AdeR_{Pro116Leu}$, $AdeS_{Thr153Met}$, or $AdeS_{Gly30Asp}$) in AdeRS have been shown to be responsible for the constitutive expression of AdeABC (Marchand et al., 2004), which reminds us that mutations (e.g., $ParR_{Met59Ile}$) in the ParRS two-component system are responsible for the constitutive expression of MexXY in *P. aeruginosa* (Muller et al., 2011). Overexpression of the AdeABC system in a tigecycline non-susceptible clinical isolate was due to the transposition of a copy of IS*Aba1* into *adeS* (Ruzin et al., 2007). Very recently, a truncated AdeS kinase protein generated by an IS*Aba1* insertion was shown to be correlated with enhanced *adeABC* expression in *A. baumannii* (Sun et al., 2012). Other regulatory mechanism(s) were shown to be involved in *adeABC* overexpression without any previously known mutation (Sun et al., 2010). Recently the AdeABC ortholog was shown to be a contributor to multiple antimicrobials, including aminoglycosides, in *Acinetobacter* genomospecies 13TU, a non-*A. baumannii* species (Roca et al., 2011).

FUTURE PERSPECTIVES

MexXY is one of the potential targets for novel anti-pseudomonas agents. Its inhibitor is able to not only potentiate previously used ineffective antimicrobial agents (e.g., aminoglycosides against aminoglycoside-resistant *P. aeruginosa* and *B. cepacia* complex), but also to speed up the development of novel anti-pseudomonas agents. Because there are a significant number of potential drug targets encoded by the genome of *P. aeruginosa* (e.g., products of essential genes; Morita et al., 2010), it is the most promising therapeutic strategy to conquer the impermeability barriers of these bacteria. The efflux inhibitor MP 601384, which has specificity toward aminoglycoside-accommodating RND efflux systems and is not toxic to bacteria, is the only MexXY inhibitor reported so far (Jassem et al., 2011). Uncultured bacteria and plants are predicted to be a significant reservoir of novel antimicrobial agents (Stavri et al., 2007; Piel, 2011). Screening novel antibacterial agents, including a MexXY inhibitor, is currently in progress in our laboratory (e.g., Shiota et al., 2004).

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Low-dose antibiotics: current status and outlook for the future

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Antimicrobial therapy is a key factor in our success against pathogens poised to ravage at risk or infected individuals. However, we are currently at a watershed point as we face a growing crisis of antibiotic resistance among diverse pathogens. One area of intense interest is the impact of the application of antibiotics for uses other than the treatment of patients and the association with such utilization with emerging drug resistance. This Research Topic "Low-dose antibiotics: current status and outlook for the future" in *Frontiers in Microbiology: Antimicrobials, Resistance, and Chemotherapy* details various aspects of the wide ranging effects of antimicrobial therapy from areas such as the regulation of host responses to modulation of bacterial virulence factors to acquisition of antibiotic resistance genes.

A remarkable and often overlooked fundamental of antibiotics is that they have biological activities beyond microbial killing. The host modulatory aspects of macrolides, tetracyclines, and beta-lactams are reviewed by Aminov (2013a) underscoring how, for example, macrolides such as azithromycin are routinely used for immunomodulation in patients with chronic pulmonary disease rather than for an antimicrobial effect. Azithromycin is also used as a tool by Imperi et al. to detail how non-conventional thinking about regulating virulence factors or modifying host inflammatory cascades are useful to combating major pathogens such as *Pseudomonas aeruginosa* (Imperi et al., 2014). Along this line, Morita and colleagues carefully detail the pleotropic responses of *P. aeruginosa* to sub-therapeutic levels of several antibacterials and propose avenues to pursue to combat this pathogen, such as developing efflux pump inhibitors (Morita et al., 2014). In their article, Charlebois et al. show *Clostridium perfringens* biofilm can be regulated by certain antibiotics at low concentrations (Charlebois et al., 2014). For example, low dose bacitracin significantly enhances biofilm formation whereas low dose penicillin reduces biofilm. This work underscores how there are untoward effects that are not predictable when antimicrobials are administered at low concentrations. Providing a view on specific host effector pathways with antimicrobials, Mihu et al. detail how antifungal medications effectively stimulate host responses via engagement with toll-like receptors (Mihu et al., 2014). In light of the expanding difficulties with drug resistance and a lack of therapeutics to combat them, Clark presents a cogent call for pursing Ca^{2+} modulating strategies where by host Ca^{2+} homeostasis is modulated to block pathogens from effectively utilizing this essential element (Clark, 2013).

An important focus in this Research Topic is the use of antibiotics as growth enhancers in animals. Sorensen and colleagues provide key insights into the effects of scientific evidence on the policy decisions on the use of low-dose antimicrobials in livestock for growth promotion and disease prevention particularly delineating how data have led to the European Union's ban of low-dose antimicrobials whereas their use in the United States of America remains in flux (Sorensen et al., 2014). The bottom line is that there is an urgent need to develop policy based on well derived data, with this data being easily and widely available to independent parties. The articles by Cheng et al. (2014), Chattopadhyay (2014), and Hao et al. (2014) all further underscore critically important facets of the continued utilization of antibiotics in animal husbandry. Looft and colleagues detail their research on how the use of the in-feed antibiotic carbadox cases dramatic short- and long-term effects on the composition of porcine gut microbiota (Looft et al., 2014). Diarra and Malouin specifically describe the impact of antibiotics in Canadian poultry production and describe the use of alternatives, such as bioactive molecules from cranberries, that should not drive antibiotic resistance (Diarra and Malouin, 2014). Similarly, Rendondo et al. provide thoughtful insights into the use of tannins in lieu of antibiotics for improving health in poultry (Redondo et al., 2014). Lin details that the effective of antibiotics as growth promoters is linked to decreased activities of bile salt hydrolase, which thus makes targeting this enzyme directly a promising method for removing antibiotics for use as growth enhancers (Lin, 2014).

You and Silbergeld critically discuss the effects of antimicrobials as drivers of resistome expansion (You and Silbergeld, 2014), a major secondary effect due to environmental pollution. The effects of antibiotics permeating our environment are highlighted by Conro and colleagues who present their findings that the presence of antibiotics in aquatic environments can induce co-aggregation of bacterial species as an effective mechanism to combat the effects of the antimicrobials (Corno et al., 2014), which can lead to extensive resistance through the transfer of resistance genes among these aggregated bacteria. It is a small leap for these microbes to then impact humans and other organisms. Aminov provides the example of the rampant use of tetracyclines for non-medical purposes as driving the penetration of *tet*(X) into pathogenic microbial communities (Aminov, 2013b). Chowdhury and colleagues eloquently discuss the import of surveillance strategies for critically elucidating the emergence of drug resistant pathogens in the context of low-dose antibiotic use in animal husbandry (Roy Chowdhury et al., 2014).

In summary, the articles within this Research Topic serve as a "call to arms" for scientists, policy makers and the public to be increasingly vigilant about the use of antimicrobials, particularly in low-dose or where they can become widespread in the environment, in order to maintain our capacity to effectively care for individuals with infectious diseases. The articles also provide new concepts for approaches for the development of antimicrobials as well as for novel growth enhancers for the use in animal husbandry.

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The intrinsic resistome of bacterial pathogens

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Intrinsically resistant bacteria have emerged as a relevant health problem in the last years. Those bacterial species, several of them with an environmental origin, present naturally low-level susceptibility to several drugs. It has been proposed that intrinsic resistance is mainly the consequence of the impermeability of cellular envelopes, the activity of multidrug efflux pumps or the lack of appropriate targets for a given family of drugs. However, recently published articles indicate that the characteristic phenotype of susceptibility to antibiotics of a given bacterial species depends on the concerted activity of several elements, what has been named as intrinsic resistome. These determinants comprise not just classical resistance genes. Other elements, several of them involved in basic bacterial metabolic processes, are of relevance for the intrinsic resistance of bacterial pathogens. In the present review we analyze recent publications on the intrinsic resistomes of *Escherichia coli* and *Pseudomonas aeruginosa*. We present as well information on the role that global regulators of bacterial metabolism, as Crc from *P. aeruginosa*, may have on modulating bacterial susceptibility to antibiotics. Finally, we discuss the possibility of searching inhibitors of the intrinsic resistome in the aim of improving the activity of drugs currently in use for clinical practice.

Keywords: intrinsic resistance, MDR efflux pump, biofilms, swarming, phenotypic resistance, persistence

Clinical definition of antibiotic resistance is mainly based on the bacterial response to treatment. One microorganism is considered as resistant if there exists a high likelihood of therapeutic failure upon antibiotic treatment. Consequently, resistance has been operationally defined in the basis of breakpoints of the minimal inhibitory concentrations (MICs). More recently, an ecological definition of antibiotic resistance is emerging, which is based in the MIC value identifying the upper limit of the wild-type population (Turnidge et al., 2006). This is defined as the ecological cut off (ECOFF) value and all strains presenting MICs above ECOFF are considered resistant from an ecological point of view, even if they are classified as susceptible in the basis of clinical breakpoints. When defining the intrinsic resistome, we are facing the same situation. Bacteria are classically considered intrinsically resistant in the basis of the clinical definition, in other words, if their infections cannot be treated with a given antibiotic. Three are the most relevant causes of this intrinsic resistance: lack of the target, activity of chromosomally encoded antibiotic-inactivating enzymes and reduced uptake of the antibiotic, the later includes reduced permeability of the cellular envelopes and activity of efflux pumps (Nikaido, 1989, 1994; Li et al., 1994; Fernandez and Hancock, 2012). More recently, in the same line of the ecological definition of resistance, the "intrinsic resistome" has been defined as the set of elements that contributes directly or indirectly to antibiotic resistance, and whose presence is independent of previous antibiotic exposure and is not due to horizontal gene transfer (HGT; Fajardo et al., 2008; Wright, 2010).

This definition encompasses all the chromosomally encoded elements that have not been recently acquired as the consequence of the recent human use of antibiotics for therapy and farming purposes. Consequently, for any bacterial species an intrinsic resistome can be defined, irrespective on whether or not this species is classified as intrinsically resistant in the basis of clinical breakpoints. From the studies on the intrinsic resistome, two categories of genes have emerged (Fajardo et al., 2008). Those which inactivation make bacteria more resistant to antibiotics and those which inactivation make bacteria more susceptible. The first ones define elements that are relevant for the acquisition of resistance. For instance, the mutation in a transcriptional repressor of a multidrug (MDR) efflux pump turns the microorganism more resistant to antibiotics (Alonso and Martinez, 1997, 2000, 2001). Mapping these elements is important to define the capability of an organism to evolve toward resistance by mutation (Martinez and Baquero, 2000) and is thus relevant for predicting the evolution of resistance (Martinez et al., 2007, 2011a). The other group of elements define the determinants that contribute to the natural phenotype of susceptibility to antibiotics of a given species, and constitute the *bona fide* intrinsic resistome. Inactivation of these elements make bacteria more susceptible to antibiotics, which may be useful for improving efficacy of current drugs (Martinez, 2012). This has been the situation of inhibitors of plasmid-encoded β-lactamases, which have been demonstrated to be efficient drugs to be used in combination with β-lactams (Reading and Cole, 1977). Similarly, the inhibition of a MDR efflux pump (or another mechanism of intrinsic resistance) might also improve the efficacy of antibiotics currently in use or allow the utilization of others (Lomovskaya et al., 1999; Renau et al., 1999; Lomovskaya and Watkins, 2001). For instance, macrolides are not used for treatment of Gram-negative infections because these organisms are intrinsically resistant to this family of antibiotics. However,

the major *Escherichia coli* efflux pump AcrAB extrudes macrolides and its inactivation might increase the susceptibility of *Escherichia coli* to these antibiotics (Chollet et al., 2004). This evidence indicates that macrolides might be useful for treating Gram-negative infections if they are used in combination with an inhibitor of MDR efflux pumps.

As stated above, the main causes of intrinsic resistance from a clinical viewpoint are lack of the target and the inactivation, low uptake and efflux of the antibiotic. However, all bacterial species harbor in their genomes genes encoding MDR efflux pumps, and several present also chromosomally encoded antibiotic-inactivating enzymes, even though they are not classified as intrinsically resistant from the clinical point of view (Saier et al., 1998; Webber and Piddock, 2003; Piddock, 2006; Poole, 2007; Vila and Martinez, 2008; Nikaido, 2009). The study of the intrinsic resistome of bacterial pathogens has shown that in addition to these elements, several others contribute to the phenotype of resistance. Among them, there are the aforementioned classical resistance genes, but there exist also several other elements belonging to all functional categories, including elements of the bacterial general metabolism (Fajardo et al., 2008). These results indicate that the specific phenotype of susceptibility to antibiotics of a given bacterial species is an emergent property consequence of the concerted action of several elements (Girgis et al., 2009). The large functional diversity of the elements of the intrinsic resistome indicates this has not evolved to specifically counteract the activity of the antibiotics. Together with the proposal that antibiotics might be molecular signals at the low concentrations they are likely present in natural ecosystems (Davies, 2006; Linares et al., 2006; Yim et al., 2006, 2007; Fajardo and Martinez, 2008), this situation allows a complementary view to the traditional weapons/shields roles that antibiotics and their resistance genes may have at natural ecosystems (Martinez, 2008; Aminov, 2009, 2010; Fajardo et al., 2009; Martinez et al., 2009a; Allen et al., 2010; Davies and Davies, 2010; Sengupta et al., 2013).

Studying the intrinsic resistome is of relevance for predicting evolution of resistance (Martinez et al., 2007, 2011a), for understanding the linkage between resistance and other bacterial processes as virulence (Martinez and Baquero, 2002; Martinez et al., 2009a,b) or metabolism (Martinez and Rojo, 2011), and for defining novel targets which inactivation make bacteria more susceptible to antibiotics (Martinez et al., 2011b; Martinez, 2012). In this article we present information of those organisms (*Escherichia coli* and *Pseudomonas aeruginosa*) for which more information on their intrinsic resistome is available. We discuss as well some issues concerning transient phenotypic resistance, which also depends on the intrinsic capabilities of the bacteria for evading antibiotics action (**Figure 1**). Finally, we present updated information on studies on the development of inhibitors of resistance.

METHODS FOR ANALYZING THE INTRINSIC RESISTOME

Genome-wide analysis of the intrinsic resistome of a given microorganism requires using high-throughput technologies. Among them, the use of insertion or deletion libraries, allows determining the contribution of each single gene to the

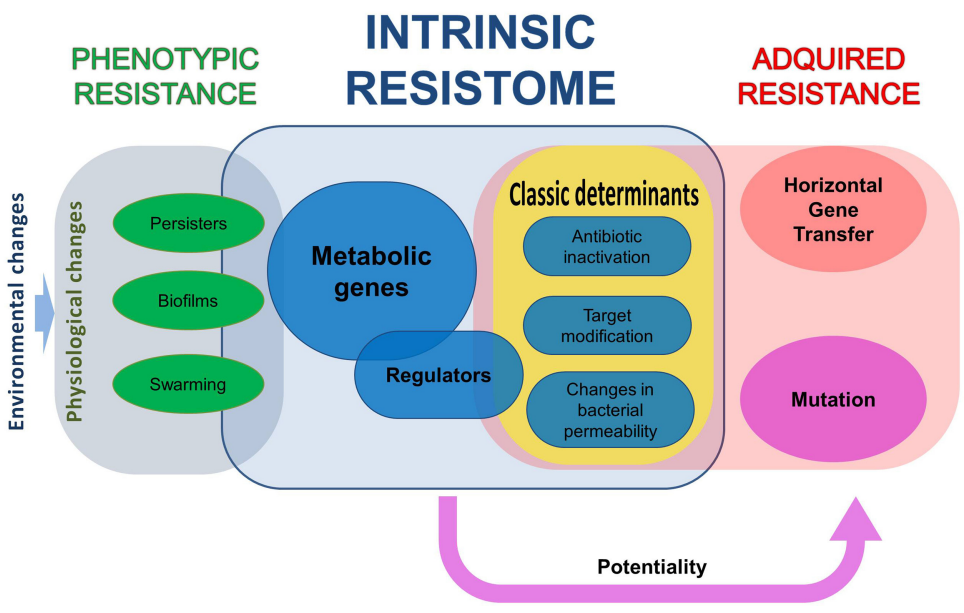

FIGURE 1 | The different elements in bacterial resistance to antibiotics. All bacteria have a repertoire of elements that contribute to their characteristic phenotype of susceptibility to antibiotics, what has been dubbed as intrinsic resistome. Some of the elements of this resistome are classical resistance elements, as antibiotic-inactivating enzymes, whereas others belong to all functional categories. The mutation of some of these elements makes bacteria more susceptible to antibiotics, whereas for some others increased resistance is acquired. Nevertheless, acquisition of a phenotype of increased resistance to antibiotics not always implies a genetic change, either because of mutation or as the consequence of the acquisition of a resistance gene by horizontal gene transfer. Phenotypic, non-inheritable resistance can be achieved by different processes that include, among others, growth in biofilms, swarming adaptation, and development of persistence.

characteristic phenotype of a given bacteria (Liu et al., 2010). This method is the best suited to determine if the inactivation of a gene changes bacterial susceptibility. However, it does not allow analyzing the effect of mutations that do not fully inactivate a given determinant but changes its activity. Alternatively, the use of plasmid libraries containing each open reading frame of a given genome allows to establish the contribution to resistance of each gene when is overexpressed or when it is transferred to a heterologous host (Soo et al., 2011; Spindler et al., 2013). This method is only useful for analyzing acquired resistance, but not for studying genes which inactivation alters the bacterial susceptibility to antimicrobials. The use of high-throughput sequencing and microarray technologies is also useful for studying intrinsic resistance. These methodologies can be used for comparing populations grown in the absence and in the presence of antibiotics. These populations can be formed by the type of libraries above mentioned (transposon-tagged of expression libraries), in which case the enrichment of mutants or clones in the presence of antibiotics allow defining genes contributing to resistance (Girgis et al., 2009; Zhang et al., 2012; Spindler et al., 2013). While these methods are faster and cheaper than methods based in analyzing the susceptibility of each single mutant/clone, their main drawback is that among all potential determinants that contribute to resistance, only those with lower fitness costs will be enriched and several of the elements that may be detected by classical susceptibility tests would not be detected using enrichment-based technologies. This drawback may be a benefit when the analysis is not made on a library, but using a wild-type strain. In these circumstances, sequencing the evolving population allows in a single step defining the mutations that produce resistance and present the smallest fitness costs among those analyzed, as well as to determine potential compensatory mutations (Gullberg et al., 2011; Toprak et al., 2012). These mutations are the most likely found in clinical isolates (Martinez et al., 2007, 2011a; Shcherbakov et al., 2010).

THE *Escherichia coli* INTRINSIC RESISTOME

Although *Escherichia coli* is traditionally considered a susceptible organism, acquired resistance to antibiotics was first detected in enteric bacteria, *Escherichia coli*, *Shigella*, and *Salmonella*, in the late 1950s to early 1960s (Watanabe, 1963). Nowadays, *Escherichia coli* accounts for 17.3% of clinical infections requiring hospitalization and is the second most common cause of infection behind *Staphylococcus aureus* (18.8%). Among outpatient infections, *Escherichia coli* is the most common organism (38.6%).

The antibiotic intrinsic resistome of *Escherichia coli* has been studied by testing the susceptibility to several antibiotics of mutants from gene knockout collections (Tamae et al., 2008; Liu et al., 2010) or transposon-tagged mutant libraries (Girgis et al., 2009). The results from these screenings showed that several genes participate in the phenotype of susceptibility to antibiotics in this species. Among them, some are classical resistance genes. Indeed, this bacterium has different known resistance mechanisms; such as the AmpC β-lactamase and MDR efflux systems like AcrAB–TolC (Lindberg and Normark, 1986; Ma et al., 1993; Lubelski et al., 2007; Jacoby, 2009). In addition, *Escherichia coli* harbors several genes that might be of relevance for its susceptibility to antibiotics as those involved in the repair of DNA damage or cell membrane synthesis and integrity. In addition to these comprehensive analysis, other studies based on the whole-genome sequence of strains evolving in the presence of antibiotics (Toprak et al., 2012) or on the enrichment of specific mutants of transposon-tagged libraries grown with antibiotics have been used to track genes relevant for the development of resistance (Zhang et al., 2012). However, in this type of analysis, mutants are competing one to each other and only those with the highest fitness will be selected.

Escherichia coli as a Gram-negative bacterium presents two cell membranes, and both are non-specific barriers preventing drug influx into the cell. The lipopolysaccharide (LPS) of the outer membrane protects the cell against hydrophobic antibiotics and polycationic compounds such as aminoglycosides and polymyxins. LPS presents anionic groups and it is the first barrier where some antibiotics bind (Hancock, 1984). Because of this, changes in the LPS can alter the susceptibility to antibiotics. For instance, the loss of *rffA*, a gene encoding an enzyme implicated in the LPS synthesis, increases susceptibility to gentamicin (Tamae et al., 2008). The *Enterobacteriaceae* common antigen (ECA) is also an intrinsic resistance element that provides protection against organic acids (Barua et al., 2002). In a similar way as happens with the LPS, changes in ECA may alter the susceptibility to antibiotics. In line with this reasoning is the finding that inactivation of the ECA biosynthesis protein WzxE increases *Escherichia coli* susceptibility to nalidixic acid and amikacin (Girgis et al., 2009). Mechanisms that control the negative charge of the outer membrane, as the Yrb system and the Pmr regulon, modulate the bacterial susceptibility to neutral or negatively charged compounds, such as nalidixic acid, lomefloxacin, and doxycycline (Girgis et al., 2009).

The gates of entry of several nutrients through the outer membrane are the porins, which are also used by antibiotics for entering into the cell (Nikaido, 1994). Many *Escherichia coli* strains have developed resistance to antibiotics as β-lactams and quinolones by mutations in the genes that encode porins or regulate their expression (Hirai et al., 1986; Adler et al., 2013). In addition, transient down-regulation of the expression of the porins can also trigger phenotypic resistance (Fernandez and Hancock, 2012). A general mechanism that prevents the cellular accumulation of drugs is their active extrusion from the cell or from the cytoplasmic membrane through MDR efflux pumps (Nikaido, 1998a,b). The AcrAB–TolC system of *Escherichia coli* is one of the best-characterized MDR transporters (Ma et al., 1995). This system consists of AcrA, a membrane fusion protein; AcrB, a transporter of the RND family; and TolC, an outer membrane protein. AcrAB–TolC is a major determinant for drug intrinsic resistance in *Escherichia coli* (Sulavik et al., 2001). Furthermore, its expression is increased in the presence of bile salts, which are present in the habitat of *Escherichia coli*, the gut (Thanassi et al., 1997; Rosenberg et al., 2003). This means that *Escherichia coli* might present a lower susceptibility to antibiotics when is growing inside its host as the consequence of the overexpression of the MDR efflux pump AcrAB–TolC. Several other MDR transporters provide resistance to a narrow range of compounds. *Escherichia coli* contains five putative ABC-type MDR-like transporters. However,

none of these systems provides an appreciable drug resistance to *Escherichia coli*, excepting YbjYZ (macrolide-specific ABC-type efflux carrier), which confers resistance to macrolides composed of 14- and 15-membered lactones, as erythromycin (Kobayashi et al., 2001; Nishino and Yamaguchi, 2001). The EmrAB is another MDR pump that protects the cell from several chemically unrelated antimicrobial agents, e.g., the protonophores carbonyl cyanide *m*-chlorophenylhydrazone and tetrachlorosalicyl anilide and the antibiotics nalidixic acid and thiolactomycin (Lomovskaya et al., 1995).

In addition to MDR efflux pumps, *Escherichia coli* harbors antibiotic-inactivating enzymes. It is worth mentioning that whilst MDR efflux pumps usually confer low-level resistance to a wide range of compounds, antibiotic-inactivating enzymes confer frequently high-level resistance to antibiotics belonging to a single structural family. All *Escherichia coli* strains have the chromosomally encoded β-lactamase AmpC. Although it contributes to resistance to antibiotics, it is difficult to assume that this is its original functional role if we take into consideration that the natural habitat of *Escherichia coli* is the gut, which microbiota does not include β-lactam producers. AmpC β-lactamases hydrolyze broad and extended-spectrum cephalosporins but are not inhibited by β-lactamases inhibitors such as clavulanic acid (Jacoby, 2009). Considering that β-lactams inhibit peptidoglycan transpeptidation, determinants involved in this process might be important for the susceptibility to these antibiotics. This is the case for *mltB* and *slt*, which encode membrane-bound lytic murein transglycosylases (von Rechenberg et al., 1996) and of *ampG*, which encodes a transporter involved in the recycling of murein (Jacobs et al., 1994). These genes are not only relevant for β-lactam susceptibility. They are also important for the regular physiology of *Escherichia coli*, since the loss of *mltB*, *slt*, *ampG*, and *ampC* is deleterious (Girgis et al., 2009).

Among those determinants involved in the intrinsic resistance of *Escherichia coli*, some deal with the general response to stress, including the repair of damaged DNA. It is known that quinolones produce DNA damage and induce the SOS repair system (Howard et al., 1993). However, other antibiotics with a different mechanism of action as nitrofurantoin and metronidazol can also damage DNA, and the RecF pathway of DNA repair of single-strand breaks and gaps (*recF*, *recO*, *recR*, *recQ*, and *recJ*; Amundsen and Smith, 2003) is important to prevent damages caused by these drugs. It has been reported that the generation of hydroxyl radicals leading to double-strands breaks contributes to cell death caused by bactericidal antibiotics (Kohanski et al., 2008). In this sense DNA repair mechanisms involved in the maintenance of DNA integrity as the RecBCD system or genes involved in the response of the cell to damaging agents (*dinG*, *xseA, xseB*, and *gshB*) constitute a valuable battery of defense mechanisms against antibiotics (Liu et al., 2010). A similar response to stress might be in the basis of the role of ribosomal proteins on intrinsic resistance. Indeed, several genes that encode ribosomal proteins (*rplA*, *rpmE*, *rpmJ*) or proteins involved in their modification (*rimK*) have a role in the susceptibility to a set of antibiotics belonging to different structural families (Liu et al., 2010).

Resistance is also provided by some genes that encode elements that are involved in the regulation of gene expression (*dksA*, *fur*, *hfq*, *hns*, *mfd*, *nusB*, *rseA*, *xapR*, *yciT*; Liu et al., 2010). These genes act in different ways, some of them involved in the stress response. For instance the DksA transcription factor regulates genes involved in double-strand break repair (Meddows et al., 2005). However, other determinants are global regulators of basic processes of the bacterial physiology. This is the case of Fur, that is the master regulator in the response to iron availability (Bagg and Neilands, 1987), an environmental cue with relevance for infection (de Lorenzo and Martinez, 1988; Martinez et al., 1990). This indicates that bacterial physiology and the bacterial response to environmental inputs are cornerstones for the phenotype of susceptibility to antibiotics. In addition to genes with known functions, there are several other determinants of the *Escherichia coli* intrinsic resistome which functional role is not known. This is the case of *yecR* and *yfgC*. Removal of *yfgC* decreases the MIC of vancomycin, rifampicin, and ampicillin (Tamae et al., 2008). *yfgC* has homology to peptidases, and apparently it is located in the bacterial inner membrane (Gardy et al., 2005). The existence of elements of unknown function conferring resistance to several antibiotics bear witness regarding the complexity and the variety of intrinsic resistance mechanisms encoded in the *Escherichia coli* genome.

THE INTRINSIC RESISTOME OF *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the most metabolically versatile bacteria described so far (Lister et al., 2009). This particular feature allows it to colonize multiple environments, being isolated from seawater (Levin and Cabelli, 1972), soil (Green et al., 1974), interacting mutualistically with plant roots (Green et al., 1974; Walker et al., 2004), as a plant pathogen (Walker et al., 2004; Records, 2011) and infecting animals (Petersen et al., 2002), including humans (Stover et al., 2000). In addition of its role in infections, *P. aeruginosa* has been isolated at hospitals in different inorganic surfaces, from ventilation and intubation equipments, contact lens and even in hydrotherapy pools (Morales et al., 2012). This versatility and ability to survive on minimum nutritional requirements (Favero et al., 1971) have made this bacterium one of the most successful nosocomial opportunistic pathogens (Lister et al., 2009).

Not only is this ability to survive anywhere the cause of the ecological success of this organism. This bacterium also shows high levels of antibiotic resistance which often makes impossible its eradication. Evolutionary forces have built its high resistance, and countless elements contribute to intrinsic and acquired resistance of this bacterium, considered a model organism to study mechanisms of resistance (Poole et al., 1993).

Although the recent acquisition of antibiotic resistance genes has been extensively described in *P. aeruginosa* (Woodford et al., 2011), these acquired elements are not the unique cause of antibiotic resistance in *P. aeruginosa*; multiple elements contribute substantially to the resistance of this bacterium. Many works have been performed in order to unravel the intrinsic resistome of *P. aeruginosa.* Transposon-tagged libraries have demonstrated that the inactivation of several genes cause changes in antibiotic susceptibility. The majority of these genes are not related with the cell envelope or efflux pumps and many of them are involved in bacterial metabolism (Breidenstein et al., 2008; Fajardo et al., 2008; Schurek et al., 2008; Alvarez-Ortega et al., 2010; Fernandez et al.,

2013). Mutants with changes in susceptibility to ciprofloxacin, aminoglycosides, β-lactams, and polymyxin B have been identified using this method.

Ciprofloxacin is a broad-spectrum fluoroquinolone that target the bacterial enzymes DNA gyrase and topoisomerase IV (Drlica and Zhao, 1997). Thirty-five and 79 mutants with increased and decreased susceptibilities to this antibiotic, respectively (Breidenstein et al., 2008), were identified in a screening of a PA14 mutant transposon library (Liberati et al., 2006). The majority of these mutants demonstrated only twofold changes in susceptibility compared with the respective isogenic strain; just the mutant in *ftsK* was eightfold more susceptible. Mutants in four genes involved in DNA replication and repair (Holliday junction helicase *ruvA*, the ATP-dependent RNA helicase *recG*, recombinase *xerD*, and the site specific recombinase *sss*) present increased susceptibility to ciprofloxacin. Additionally, *ruvA*, *xerD*, and *fstK* grow slower compared with the wild-type. On the contrary, mutants in genes *nuoBDGHIJLN* that encode a dehydrogenase are less susceptible to ciprofloxacin. These mutants in the *nuo* operon present decreased susceptibility to tobramycin also (Schurek et al., 2008) supporting the idea that some of the elements involved in intrinsic resistance have not evolved to counteract the activity of a specific antibiotic (Fajardo et al., 2008; Martinez and Rojo, 2011).

Despite this apparent lack of specificity, there is not a common response to all antibiotics, even for those belonging to the same structural family. In a screening using imipenem, meropenem and ceftazidime just one mutant (PA0908) presented reduced susceptibility to all three antibiotics and two (*glnK* and *ftsK)* showed increased susceptibility to all three antibiotics (Alvarez-Ortega et al., 2010). Even more, the mutant in *ftsK*, which encodes a protein involved in cell division (Lewenza et al., 2005), presents an increased susceptibility to ciprofloxacin too (Breidenstein et al., 2008). In the same way, mutants in many genes that were more susceptible to β-lactams (PA0401, *pyrB* and *pyrD*; Alvarez-Ortega et al., 2010) presented decreased susceptibility to polymyxin B (Fernandez et al., 2013). However, this is not a general trend, since mutants in *galU*, *ampR*, *lptc*, *aroB*, *wapR*, and *ssg* showed decreased susceptibility to β-lactams and they are more susceptible to polymyxin B also (Fernandez et al., 2013). The majority of these genes participate actively in the central metabolism of *P. aeruginosa* (Stover et al., 2000) reinforcing the idea that genes involved in metabolism can play an important role in the intrinsic resistance to antibiotics of the microorganisms.

The cell membrane is considered one of the principal contributors to intrinsic resistance (Nikaido, 1989). In this way mutations in many genes that take part in the LPS synthesis in *P. aeruginosa* produce changes of the susceptibility against β-lactams and tobramycin. A mutant in *wapR*, which encodes a protein involved in the biosynthesis of the LPS core (Poon et al., 2008), is less susceptible to ceftazidime and meropenem; mutations in adjacent genes (PA5001, PA5002, PA5003, and PA5005) which participate in the LPS synthesis also present reduced susceptibility to ceftazidime and some mutants present cross resistance with meropenem (Alvarez-Ortega et al., 2010). A similar effect was observed in mutants of genes involved in the O-antigen synthesis (*wbpZ, wbpY, wzt, wzm*, and *wbpW*). These mutants presented decreased susceptibility to tobramycin, demonstrating the importance of the cellular membrane as a barrier to avoid the entrance of antibiotics inside the cell (Schurek et al., 2008).

Efflux pumps contribute considerably to antibiotic resistance in *P. aeruginosa.* They are involved in the extrusion of toxic substances, including antibacterial compounds, from inside the cell to the external environment (Webber and Piddock, 2003). In this bacterium, 12 different RND-type efflux systems (Blair and Piddock, 2009) that can eventually contribute to antibiotic resistance have been described. However, only MexAB-OprM has shown to play a relevant role on the intrinsic resistance of *P. aeruginosa* to antibiotics (Kohler et al., 1997). Expression of the other efflux pumps under standard growing conditions is too low to achieve resistance, an issue that is also frequently described for other bacteria (Grkovic et al., 2001, 2002). However, MDR efflux pumps can be overexpressed, and hence render resistance, either because of mutations in their regulatory elements or because of the presence of effectors that trigger their expression (Grkovic et al., 2001, 2002; Blair and Piddock, 2009; Hernandez et al., 2009, 2011).

Since intrinsic resistance is a multifactorial phenomenon, it might be modulated by global regulators. This is the case of the *P. aeruginosa* "catabolite repression control" regulator Crc (MacGregor et al., 1991). Crc is a post-transcriptional repressor that regulates the use of preferred carbon sources in nutrient-complex ecosystems (Morales et al., 2004; Moreno et al., 2009). Recent work has shown that inhibition of Crc makes *P. aeruginosa* less virulent and more susceptible to different antibiotics (Linares et al., 2010), the latter being a consequence of increased expression of transporters and changes in the LPS composition. This shows the existence of networks connecting antibiotic resistance, bacterial virulence and metabolism. The hubs of these networks may be good targets in the search of novel antimicrobials targeting simultaneously resistance, virulence, and bacterial physiology.

PHENOTYPIC RESISTANCE

Most studies on antibiotic resistance are based in the analysis of resistant organisms that have acquired the phenotype of resistance as the consequence of genetic, inheritable, changes, which can be mutations (including gene rearrangements) of the acquisition, through HGT, of resistance genes (Walsh, 2000). However, there are other situations, grouped under the name of phenotypic resistance, in which bacteria present a non-inheritable situation of resistance to the antibiotics (Levin, 2004; Levin and Rozen, 2006). Phenotypic resistance is thus defined as a transient situation in which a bacterial population, usually susceptible to antibiotics, is transiently resistant (**Figure 1**). The elements contributing to this phenotype are a part of the intrinsic resistome that are only unveiled under specific growing conditions (Martinez et al., 1994). Below, some examples of phenotypic resistance are described.

PERSISTENCE

Persistence is defined as a situation in which a bacterial subpopulation is not killed by a given antibiotic under conditions in which the bulk of the population is inhibited. Once antibiotic is removed and growth resumes, persistent cells behave as antibiotic susceptible as

the original population, which means that persistence is not the consequence of a genetic change. The existence of persister cells into a population is known since 1944, when this phenomenon was first reported for staphylococcal infections treated with penicillin (Bigger, 1944). Currently, persistence has been described for many other bacterial species and antibiotics. As stated, persisters are phenotypic variants that present increased resistance to antibiotics and are genetically identical to the wild-type. The percentage of persisters in a given culture can be as high as 1% of stationary-phase cells for several microorganisms (Kim and Wood, 2010). Establishment of a persistent subpopulation does not require previous antibiotic exposure.

The presence of persister cells in infections increase the chance of survival of the bacteria in the presence of antibiotics, an issue particularly relevant in the case of chronic infections. Because of this, the analysis of the genes and mechanisms responsible for the development of a persister phenotype is of relevance to implement novel therapeutic approaches based on the eradication of antibiotic resistant persistent cells.

The first gene described to be involved in the development of persistence was *hipA* (Moyed and Bertrand, 1983) in *Escherichia coli*. HipA is a toxin that belongs to the *hipBA* toxin/antitoxin system (TA systems; Correia et al., 2006), which overexpression inhibits cell growth and induces antibiotic resistance by persister formation. Other TA systems as MqsRA (Kim and Wood, 2010) or TisAB (Dorr et al., 2010; Lewis, 2010) have been also associated to the formation of persister cells, indicating that a misbalance in the production of toxin and antitoxin may be in the basis of this phenotype.

Toxin/antitoxin systems are highly distributed among different bacterial species. Usually, these systems are formed by two components, a toxin that inhibits cell growth and an antitoxin that impedes the activity of the toxin. At the moment, three general TA systems have been described. The antitoxins of TA types I and III are small RNAs, whereas the toxins of the type II TA systems are inhibited by protein antitoxins (Gerdes et al., 1997, 2005; Blower et al., 2011). Recent work has shown that type II TA systems are highly relevant for developing persistence in *Escherichia coli* and that the simultaneous deletion of 10 TA loci from the chromosome of *Escherichia coli* reduced the fraction of persistent cells by at least 100-fold (Maisonneuve et al., 2011; Gerdes and Maisonneuve, 2012).

In addition to TA systems, other factors modulate the development of persistence. Among them, elements involved in the regular bacterial metabolism have shown to be relevant. This is the case of *phoU*, which encodes a negative global regulator that suppresses the cellular metabolic activity, altering expression of several elements with relevance for bacterial physiology, ranking from flagella-encoding genes to genes encoding energy production enzymes (Li and Zhang, 2007).

In depth analysis on the mechanisms of persistence has shown that there are two types of persistent cells (Balaban et al., 2004), which further supports the idea that there exist different routes toward the development of persistence. Type I persisters constitute a pre-existing population of resting cells that are generated at stationary phase. These cells when are inoculated into fresh medium from stationary phase switch back to growing cells with a characteristic extended time lag. An example of genes involved in this phenotype is *hipA7* (Moyed and Bertrand, 1983). Type II persisters constitute a subpopulation of slowly growing cells. An example of genes involved in this phenotype is *hipQ* (Wolfson et al., 1990).

In agreement with the potential role that both bacterial metabolism and TA systems may have on the establishment of persistence, transcriptomic analysis of persister cells have shown that persistence is associated with the down-regulation of biosynthetic genes and the up-regulation of several TA modules (RelBE, MazEF, DinJYafQ, YgiU; Lewis, 2010), Some of these TA systems are known to affect translation (Christensen et al., 2001; Pedersen et al., 2002), which explains their effect in dormancy and consequently on antibiotic resistance. The SOS response, which up-regulates DNA repair functions, also induces several TA genes in *Escherichia coli*, whose promoters contain a Lex box: *symER, hokE, yafN/yafO*, and *tisAB/istR* (Dorr et al., 2010). This means that TA systems, and hence the formation of persister cells, can be activated by factors that trigger the SOS system. It is known that some antibiotics, such as quinolones, induce the emergence of persister cells (Dorr et al., 2010). Since these antibiotics produce DNA damage and activate the SOS system, it is likely possible that the effect of quinolones on persistence is SOS-dependent. The role of other stresses on persistence has been studied. This is the case of oxidative stress that is important during infection due to the immune response. However, this stress only induces persistence against fluoroquinolones but not for other antibiotics such as kanamycin. Surprisingly, this phenotype is due to the overexpression of the MDR efflux pump AcrAB–TolC (Wu et al., 2012). This indicates that transient expression of classic antibiotic resistance mechanisms forming part of the intrinsic resistome may be of relevance for the establishment of persistence.

One important issue for avoiding persistence would be finding elements which mutation impede the development of persistent cells. However, the analysis of a transposon mutants library of *Escherichia coli* (Hu and Coates, 2005; Hansen et al., 2008) and of the comprehensive Kei collection (Baba et al., 2006), did not allow identifying any gene which mutation fully impede the phenotype of persistence. These data suggest a great degree of redundancy in the elements and pathways involved in the development of persistence. Even though TA systems could be good candidates to decrease the fraction of persister cells in a given population, their high number, with 671 TA systems already identified in 126 prokaryotic genomes (Pandey and Gerdes, 2005) makes difficult developing drugs directed to reduce persistence by targeting these systems.

Another approach for eliminating persisters can be by shifting their metabolic state. A recent report shows that this is feasible by specific metabolic stimuli that allow the recovery of the proton motive force of persistent cells, enabling their killing by aminoglycosides (Allison et al., 2011).

BIOFILM

Biofilm is a structured population of bacteria embedded in a matrix, which is composed by polysaccharides, proteins, and extracellular DNA. It has been shown than cells growing in biofilms are less susceptible to antibiotics than those growing planktonically

(Mah and O'Toole, 2001; Amini et al., 2011). This phenotypic resistance is relevant for the treatment of infections on intubated or catheterized patients, as well as in infections of prosthesis and some chronic infections as those of cystic fibrosis patients that involve the colonization of surfaces (Costerton et al., 1999) in which biofilm formation is frequent. The antibiotic resistance associated with biofilms depends on several causes, some due to the structure of the extracellular matrix, some other to the physiological state of biofilm-growing bacteria; which is different to that of planktonic cells. Even inside the biofilm, bacteria show different metabolic states, because there is a gradient of nutrients and oxygen between the surface of the biofilm and its deeper region.

The extracellular matrix may change the activity of the antibiotics by two different reasons; by diminishing the diffusion of the antibiotic or by sequestering it through its binding to the matrix. This is not a general trend, since in several occasions, slow diffusion of the antibiotic is not the most important element in the phenotypic resistance displayed by biofilms (Walters et al., 2003; Stewart et al., 2009; Singh et al., 2010). Another aspect in which the extracellular matrix may participate in the phenotype of resistance of biofilms is by triggering specific mechanisms of resistance. DNA, a macromolecule capable of chelate cations, is one of the components of the extracellular matrix. Since a reduced concentrations of divalent cations trigger expression of the regulator of resistance to cationic antimicrobials PhoP–PhoQ (Mulcahy et al., 2008), the extracellular matrix trigger itself resistance to these drugs.

When analyzing the role of the metabolic state of bacteria on the phenotypic resistance of biofilms, it has been shown that the degree of resistance depends on the region of the biofilm and on the antibiotic involved. Different regions of the biofilms contain subpopulations in different metabolic stages that mainly depend on the oxygen and nutrients availability (Huang et al., 1995; Sternberg et al., 1999). It has been described than oxygen-rich regions of *P. aeruginosa* biofilms are highly susceptible to quinolones whilst cells in these regions are phenotypically resistant to cationic peptides, and the opposite occurs at regions of the biofilms with low oxygen tension (Rani et al., 2007). Altogether, these results indicate that the phenotypic resistance of bacterial biofilms depend on several factors that operate simultaneously.

SWARMING

Swarming is a specific type of movement of bacterial populations. It is characterized by the formation of hyper-flagellated cells in nutrient-rich environments. It is supposed that this type of motility allows the colonization of such environments. Consequently, swarming is just the most visible phenotype of a complex physiological adaptation process that is dependent on cell–cell signaling [quorum-sensing (QS)] and on nutrients availability (Fraser and Hughes, 1999). The capability of swarm has been described in different microorganisms, including *Escherichia coli, Serratia marcescens, Burkholderia thailandensis, Bacillus subtilis, Salmonella enterica* serovar Typhimurium, and *P. aeruginosa* (Kim et al., 2003; Overhage et al., 2008a; Lai et al., 2009), and it might be of relevance for the colonization of surfaces during infection, as for instance in the lungs of cystic fibrosis patients. A relevant characteristic of swarmer cells consists on their reduced susceptibility to different antibiotics (Kim et al., 2003; Overhage et al., 2008b; Lai et al., 2009).

Transcriptomic analyses of *P. aeruginosa* have shown that several genes change their expression during swarm cell differentiation. Some of these genes encode porins and efflux pumps, such as MexGHI-OpmD. A differential expression of these genes in swarmer cells might be involved in their phenotype of reduced susceptibility to antibiotics (Overhage et al., 2008a). Nevertheless, the reasons for this transient phenotype of antibiotic resistance are still far to be fully understood, since there are other elements that might be involved in the phenotype. For instance, several proteases (Lon, AsrA, PfpI, ClpS, and ClpP) that affect swarming motility are also relevant for the formation of biofilms, and therefore for phenotypic antibiotic resistance (Marr et al., 2007; Kindrachuk et al., 2011; Fernandez and Hancock, 2012).

Quorum-sensing seems to play a key role in swarming differentiation. It has been shown that PvdQ, an acylase that hydrolyzes the QS signal 3-oxo-C12-HSL [*N*-(3-oxo-dodecanoyl)-l-homoserine lactone] is involved in swarming. The analysis of cells either overexpressing or lacking PvdQ showed that PvdQ reduced *P. aeruginosa* outer membrane permeability, thereby elevating antibiotic resistance under swarming conditions, upon which this protein is up-regulated (Wang et al., 2013). A similar effect of reduced permeability has been shown for *Salmonella enterica* swarmer cells. In particular the expression of the porin OmpA, which is used for the entrance of nutrients and some antibiotics is low in swarming cells (Sugawara and Nikaido, 1992; Kim and Surette, 2004), suggesting that changes in the permeability of the cellular envelopes, in response to nutrients' availability, might be in the basis of the antibiotic resistance phenotype displayed by swarmer cells.

There are some common factors that might induce the different situations of phenotypic resistance above described. Changes in the environment as the lack of nutrients or low oxygen levels, which reduce the growth rates, occur in stationary phase and this induces persisters, which are much more abundant in this growth phase. These nutritional cues also take place in some regions of bacterial biofilms, mainly at their deepest zone, and are relevant for their reduced susceptibility to antibiotics. QS signaling is a relevant system in the basis of biofilm formation, which is also involved in triggering the bacterial physiological reprogramming that occurs in swarmer cells. Expression of TA systems occurs in response to external signaling, and is involved in the generation of persister cells, but also are differentially expressed during biofilm formation, directly or *via* QS signaling. Cellular damage produced by toxic compounds, antibiotics included, may induce the SOS response, which triggers expression of reparation systems and usually also reduces metabolic activity, a situation that induces the formation of persister cells and is also foreseen in biofilms.

It thus seems that bacterial populations have developed a battery of mechanisms to respond to stress and these mechanisms are also useful to transiently resist the activity of antibiotics.

INHIBITORS OF RESISTANCE DETERMINANTS

One of the areas under development in the search for novel drugs to fight infections is the study of inhibitors of resistance (Baquero

et al., 2011; Martinez et al., 2011b; Martinez, 2012). The use of these drugs in combination with those antibiotics against which the mechanisms of resistance operate, increases bacterial susceptibility to the antimicrobials. The first inhibitors of resistance were developed against mechanisms acquired through HGT, in particular plasmid-encoded β-lactamases (Bush, 1988). However, the definition of intrinsic resistome offers the possibility of developing inhibitors that increase susceptibility to all isolates of a given species (Garcia-Leon et al., 2012). For instance, the inhibition of MDR efflux pumps, which makes Gram-negative bacteria susceptible to macrolides, will allow the use of these compounds for treating infections by Gram-negative microorganisms.

Although clavulanic acid, the first inhibitor of β-lactamases was described more than 40 years ago (Reading and Cole, 1977), few other inhibitors, all of them inhibiting the same type of β-lactamases, are already in the market. Main efforts have focused on the inhibitors of other β-lactamases (Drawz and Bonomo, 2010) and MDR efflux pumps (Lomovskaya and Watkins, 2001). In the case of aminoglycoside-inactivating enzymes, the development of inhibitors is problematic due to the large number and variability of families of these resistance elements.

Based on the Ambler's classification (Ambler et al., 1991), the β-lactamases are divided into four classes: class A, C, and D being serine β-lactamases, and class B being metallo-β-lactamases (MBLs). The commercially available β-lactamase inhibitors (BLIs), clavulanic acid, sulbactam and tazobactam, are only effective against class A β-lactamases. However, their combinations with β-lactam antibiotics are still effective, despite the emergence of resistance, either because of gene-dosage effect (Martinez et al., 1987, 1989; Reguera et al., 1991), either because of mutations that make β-lactamase resilient to inhibition (Blazquez et al., 1993; Canica et al., 1998; Salverda et al., 2010; Frase et al., 2011; Li et al., 2012).

The discovery that carbapenems may inhibit some β-lactamases opened the possibility of finding antibiotics with dual activities, antimicrobial and inhibitor of resistance (Buynak, 2006). However, this can be a difficult task, since antibiotics as cefoxitin may either inhibit or induce expression of β-lactamases depending on their concentration (Tsuey-Ching et al., 2012). Because of this, mathematical models are being implemented to establish reliable dosage regimes (Bhagunde et al., 2012) and novel derivatives are being developed to surpass these problems. Among them, *N*-acyl β-sultams obtained, by sulfonylation of β-lactams and addition of an acyl group, inhibit *Enterobacter cloacae* class C β-lactamase (Page et al., 2003). Penicillin and cephalosporin sulfone derivatives are also part of the pipeline of BLIs. LN-1-255 is a penicillin sulfone that is active against OXA-type β-lactamases and is being used as model for further improvements in this type of inhibitors (Pattanaik et al., 2009; Drawz et al., 2010).

In addition to β-lactams that inhibit β-lactamases, there are also non-β-lactams able to inhibit these enzymes. One of the families receiving more attention is the one formed by boronic acids (Kiener and Waley, 1978), which are potential inhibitors of all types of serine β-lactamases (Tan et al., 2010), and seem to be effective even against penicillin-binding proteins (PBPs) resistant to β-lactams (Zervosen et al., 2012). Nevertheless clinical use of these compounds is compromised because of the toxicity of boron. Another class of non-β-lactam BLIs is formed by the diazabicyclooctanes (DBOs). There are two compounds of this family under clinical trials: MK-7655 and NXL104 (avibactam) (Coleman, 2011). MK-7655 is a potent inhibitor of class A and C β-lactamases, that can be used in combination with imipenem to kill AmpC and KPC (*Klebsiella pneumoniae* carbapenemase) producers (Hirsch et al., 2012). Avibactam is a promising BLI that inhibits class A and C β-lactamases, including BlaC from *Mycobacterium tuberculosis* (Xu et al., 2012). In combination with ceftazidime, avibactam protects β-lactams from hydrolysis in β-lactamase-producing *Enterobacteriaceae* and *P. aeruginosa* (Dubreuil et al., 2012; Levasseur et al., 2012). More recently it has been proposed that a triple combination avibactam, ceftazidime, and metronidazole will be useful for treating complicated intra-abdominal infections, which may present a mixed population of *Enterobacteriaceae* and anaerobes (Dubreuil et al., 2012; Lucasti et al., 2013).

Finding common inhibitors for all MBLs is a difficult task, due to the diversity of class B β-lactamases (Drawz and Bonomo, 2010). However, different structural families of inhibitors are under development, including tetrahydropyrimidine-2-thione and pyrrole derivatives (Hussein et al., 2012), 3-mercapto-1,2,4-triazoles and *N*-acylated thiosemicarbazides (Faridoon et al., 2012), *N*-heterocyclic dicarboxylic acids and pyridylmercaptothiadiazoles (Feng et al., 2012), 2-substituted 4,5-dihydrothiazole-4-carboxylic acids (Chen et al., 2012), or mercaptoacetate (Wachino et al., 2012).

Although most inhibitors of β-lactamases target a specific family of these enzymes, efforts have been made to develop drugs capable to inactivate a large range of β-lactamases. Among them single molecules as mercaptomethylpenicillinates (Buynak et al., 2004) or reverse hydroxamates and oximes (Ganta et al., 2009) are under development as well as combinations of compounds as BAL30376, which is a mixture of BAL19764, a siderophore monobactam active to class B MBLs, BAL29880, a bridged monobactam active to class C β-lactamases, and clavulanate (Livermore et al., 2010; Page et al., 2011).

In addition to traditional methods in searching enzyme's inhibitors, information derived from other studies may help in developing β-lactamases inhibitors. In this regard, the finding of a small protein, produced by the same producer of clavulanic acid, *Streptomyces clavuligerus* (Reading and Cole, 1977; Yuan et al., 2011) and capable to inhibit class A β-lactamases opens the possibility of developing peptides or haptamers capable to inhibit β-lactamases. Similarly, the finding that the active site structures and the catalytic mechanisms of N-terminal nucleophile hydrolase (a component of the bacterial proteasome) and β-lactamases are similar, allows discovering cross-inhibition of both enzymes by compounds as *O*-aryloxycarbonyl hydroxamates (Pelto and Pratt, 2008) or 1,3,4-oxathiazol-2-ones (Adediran et al., 2012). This opens the possibility of testing already known inhibitors of the bacterial proteasome as inhibitors of β-lactamases.

Beta-lactamases inhibitors will be very useful, but only for treating infections by those organisms presenting a β-lactamase. A wider spectrum of activity might have the efflux pumps inhibitors (EPIs). MDR efflux pumps are present in all bacterial species contributing to intrinsic and acquired (when overexpressed) resistance to all family of drugs. Since any single efflux pump

can extrude a wide range of antibiotics belonging to different structural families, its inhibition will simultaneously increase the bacterial susceptibility to several antibiotics (Vila and Martinez, 2008). *In vitro* work has shown that inhibition of efflux pumps makes bacteria more susceptible to antibiotics and also reduces the probability of emergence of antibiotic resistant mutants (Lomovskaya et al., 2001). In addition, since some efflux pumps contribute to the virulence of bacterial pathogens, their inhibition will also impair their capability for producing infections (Hirakata et al., 2009), including the formation of biofilms (Baugh et al., 2012).

Theoretically, there are different alternatives for inhibiting MDR determinants (Fernandez and Hancock, 2012). One that could serve for inhibiting all efflux pumps would be the inhibition of energy sources required for pumps activity. Unfortunately activity of MDR efflux pumps is coupled either to the membrane potential, either to ATP, both of which are key element for any eukaryotic of prokaryotic cell. This means that most of these potential inhibitors will be too toxic to be used. There are, however, some elements that are specific for bacteria and could be used as potential targets. This is the case of TonB, a protein involved in the activity of *P. aeruginosa* MDR efflux pumps by coupling the energized state of the membrane to the operation of bacterial transporters (Zhao et al., 1998).

Other ways of inhibiting the activity of efflux pumps include the interference with the pump assembly or the blockage of its activity, for instance using antibodies as has been described for SmrA from *Stenotrophomonas maltophilia* (Al-Hamad et al., 2011), the development of effectors precluding the release of the MDR repressor from its operator DNA, and the competition with antibiotics transported by the efflux pump.

One of the first EPIs with potential therapeutic use is the synthetic dipeptide amide phenylalanine-arginyl-β-naphthylamide, which inhibits several Gram-negative efflux pumps (Lomovskaya et al., 2001; Mahamoud et al., 2006), although not all of them (Sanchez et al., 2003). This molecule is a competitive inhibitor that binds to the same site used by the pump to bind the antibiotic. Some efforts have been made in the optimization of diamide-containing EPIs (Watkins et al., 2003). However, it has been shown that the moieties that are responsible for their unfavorable toxicological properties, are also essential for their activity, a situation that impedes their therapeutic use (Lomovskaya and Bostian, 2006).

Pyridopyrimidines and arylpiperazines have also been assayed as EPIs of MDR pumps and major efforts have been performed for their optimization (Nakayama et al., 2004a,b; Yoshida et al., 2006a,b, 2007). Differing to the previously described dipeptide amides, that only impede the action of the antibiotics they compete, pyridopyrimidines increase the susceptibility to all substrates of the efflux pumps, indicating a different mechanism of action (Lomovskaya and Bostian, 2006).

Although some efforts on the development of EPIs against efflux pumps for Gram-positive organisms, as *Staphylococcus aureus* NorA, have been made (Markham et al., 1999; Holler et al., 2012b; Sabatini et al., 2012), most EPIs so far described inhibit efflux pumps from Gram-negative organisms with some degree of specificity. Among them, pyridopyrimidines inhibit MexAB-OprM of *P. aeruginosa* (Yoshida et al., 2007), 1-(1-naphthylmethyl)-piperazine reversed multidrug resistance in *A. baumannii* but not in *P. aeruginosa* (Pannek et al., 2006), or 13-cyclopentylthio-5-OH tetracycline (13-CPTC), a semisynthetic tetracycline analogue that binds TetB pump of *Escherichia coli* (Nelson and Levy, 1999).

The finding that plant-produced compounds are substrates and inducers of efflux pumps (Matilla et al., 2007) suggests that these compounds may also inhibit MDR determinants. Indeed, it has been shown that plant extracts contain a variety of EPIs (Tegos et al., 2002; Musumeci et al., 2003; Lewis and Ausubel, 2006; Stavri et al., 2007), which may be useful for increasing the susceptibility to antibiotics of different bacterial species (Groblacher et al., 2012a,b; Holler et al., 2012a; Roy et al., 2012; Zhou et al., 2012).

In addition of inhibitors targeting specifically classical resistance elements, the study of the intrinsic resistome opens the possibility of looking for inhibitors of targets that contribute to resistance despite they are not classical resistance determinants. This is the case of the *P. aeruginosa* cyanide-insensitive terminal oxidase. Mutants defective in this gene are hypersusceptible to antibiotics (Tavankar et al., 2003). Consequently, inhibition of this component of the respiratory chain will increase the overall susceptibility of *P. aeruginosa* to antibiotics. Same situation happens with global regulators as the *P. aeruginosa* Crc post-transcriptional repressor (Morales et al., 2004; Moreno et al., 2009). Mutants defective in this gene are hypersusceptible to different antibiotics (Linares et al., 2010); hence its inhibition will increase susceptibility to these antibiotics.

An interesting approach in the search of inhibitors of resistance is the screening of non-antibiotic compounds, which had been already tested for other diseases, and may be used as helper compounds to improve efficacy of antibiotics (Martins et al., 2008). The benefit of using these compounds is that their use has been already approved, so as novel long and costly toxicological trials are not needed. Among them some anesthetics, antihistaminic, and psychotherapeutic compounds have demonstrated to improve the activity of antibiotics (Kristiansen and Amaral, 1997). Within this group of compounds some of them change the permeability of the bacterial membrane (Martins et al., 2008), as the anti-inflammatory drug diclofenac (Dutta et al., 2007) or the anti-psychotic chlorpromazine (Blair and Piddock, 2009), whereas for others, the mechanism of action is not known.

A more recent approach for improving the activity of the antibiotics is by enhancing the cellular responses associated to the antibiotics induced cell death pathway. It has been proposed that bactericidal antibiotics induce a cell pathway that involves the generation of oxygen reactive species (Kohanski et al., 2007, 2010). Understanding this pathway may reveal targets for adjuvants that improve the efficacy of the antibiotics (Farha and Brown, 2013). By using *Escherichia coli* whole-genome metabolic models and further experimental validation of predicted targets, it has been shown that inactivation of some elements increase susceptibility to oxidants and antibiotics (Brynildsen et al., 2013), which opens the possibility of searching a new family of drugs capable to increase the activity of antibiotics.

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Novel resistance functions uncovered using functional metagenomic investigations of resistance reservoirs

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Rates of infection with antibiotic-resistant bacteria have increased precipitously over the past several decades, with far-reaching healthcare and societal costs. Recent evidence has established a link between antibiotic resistance genes in human pathogens and those found in non-pathogenic, commensal, and environmental organisms, prompting deeper investigation of natural and human-associated reservoirs of antibiotic resistance. Functional metagenomic selections, in which shotgun-cloned DNA fragments are selected for their ability to confer survival to an indicator host, have been increasingly applied to the characterization of many antibiotic resistance reservoirs. These experiments have demonstrated that antibiotic resistance genes are highly diverse and widely distributed, many times bearing little to no similarity to known sequences. Through unbiased selections for survival to antibiotic exposure, functional metagenomics can improve annotations by reducing the discovery of false-positive resistance and by allowing for the identification of previously unrecognizable resistance genes. In this review, we summarize the novel resistance functions uncovered using functional metagenomic investigations of natural and human-impacted resistance reservoirs. Examples of novel antibiotic resistance genes include those highly divergent from known sequences, those for which sequence is entirely unable to predict resistance function, bifunctional resistance genes, and those with unconventional, atypical resistance mechanisms. Overcoming antibiotic resistance in the clinic will require a better understanding of existing resistance reservoirs and the dissemination networks that govern horizontal gene exchange, informing best practices to limit the spread of resistance-conferring genes to human pathogens.

Keywords: functional metagenomics, antibiotic resistance, bifunctional resistance gene, environmental resistance, resistance reservoir, transferable resistance

OVERVIEW

It is estimated that infection with antibiotic-resistant pathogens incurs over $25 billion in societal and healthcare costs annually in the United States (CDC, 2011). More than 70% of hospital-acquired bacterial infections are currently resistant to at least one of the major antibiotics used as standard treatment (Stone, 2009), and patient deaths as a result of hospital-acquired infections have increased by over 675% during the past 20 years (NIAID, 2012). As the incidence and spectrum of antibiotic resistance increases (Arias and Murray, 2009), antibiotic development has slowed to a trickle (Spellberg et al., 2008), further hindering the effectiveness of antibiotics to treat infectious disease. The majority of acquired antibiotic resistance genes in bacterial pathogens are obtained via horizontal gene transfer (HGT) (Ochman et al., 2000), likely with environmental origins (Benveniste and Davies, 1973; Wright, 2010; D'Costa et al., 2011). Accordingly, an increasing impetus has been placed on cataloging antibiotic resistance reservoirs (Allen et al., 2009b, 2010), determining how resistance genes are most readily transferred to the clinic (Marshall and Levy, 2011; Smillie et al., 2011), and identifying resistance mechanisms heretofore unseen in clinical settings (Jeon et al., 2011; Tao et al., 2012).

TRADITIONAL MECHANISMS OF ANTIBIOTIC RESISTANCE

An antibiotic-resistant phenotype can be either an acquired trait or intrinsic to the bacterium in question. In the case of intrinsic resistance, antibiotic therapy is rendered ineffective due to a pre-existing physiological trait of the species, such as reduced accessibility to or absence of a drug target (Alekshun and Levy, 2007; Martinez, 2008; Davies and Davies, 2010; Dantas and Sommer, 2012). Examples of intrinsic antibiotic resistance include vancomycin tolerance in Gram-negative bacteria (the outer cell membrane reduces access to the peptidoglycan target) (Arthur and Courvalin, 1993) and biofilm formation in numerous organisms (perhaps providing resistance via reduced drug penetration and/or altered microenvironments) (Stewart and Costerton, 2001). Conversely, acquired antibiotic resistance stems from the expression of a specific resistance gene and is commonly the result of *de novo* mutation or the acquisition of resistance-conferring genes on mobile genetic elements (e.g., plasmids, transposons, integrons) (Walsh, 2003). The antibiotic resistance genes present

in a microbial community that are capable of transfer to a new host are collectively referred to as the "transferable resistome." Intrinsic resistance is, by definition, limited to the context of the parent organism, whereas acquired resistance represents a more flexible phenotype, and its prevalence is more immediately responsive to selection pressure (Martinez, 2008). As nearly all infectious bacteria were antibiotic-susceptible prior to the introduction of antibiotic therapy (Houndt and Ochman, 2000; Davies and Davies, 2010), the exceeding majority of resistance in human pathogens is acquired, either through mutation or HGT (Alekshun and Levy, 2007). This resistance represents a diversity of biochemical mechanisms that break down into three general categories (Walsh, 2000, 2003): (1) inactivation of the antibiotic, (2) reducing intracellular antibiotic concentration through efflux or permeability barriers, and (3) altering the cellular target of the antibiotic, reducing their association.

Perhaps the most intuitive of resistance mechanisms, antibiotic inactivation, is sub-categorized into two groups: enzymes that inactivate drugs via degradation (e.g., the β-lactamases) vs. those that function via chemical modification. The β-lactamases are characterized by their ability to cleave the four-membered ring present in all β-lactam antibiotics and are some of the best-studied and widely-distributed antibiotic resistance genes (for review, see Jacoby and Munoz-Price, 2005). These enzymes confer high-level antibiotic resistance and are found associated with mobile DNA elements and integrated into bacterial chromosomes. β-lactamases function via either a serine active site or metal cation cofactor (Jacoby and Munoz-Price, 2005) and can be found across bacterial phyla. Antibiotic-modifying enzymes are also phylogenetically widespread, as well as mechanistically diverse. These enzymes can confer tolerance toward numerous drugs, including the aminoglycoside (Davies and Wright, 1997), tetracycline (Yang et al., 2004), amphenicol (Schwarz et al., 2004), and macrolide-lincosamide-streptogramin (Weisblum, 1998) antibiotics, typically functioning via covalent modification of the drug with some functional moiety (e.g., acetyl, phosphoryl, nucleotidyl, glycosyl, and hydroxyl groups) (Alekshun and Levy, 2007).

The intracellular concentration of any given antibiotic can be reduced by either efflux mechanisms to remove the drug from the cytosol or permeability barriers that limit the drug's uptake. Many antibiotics have poor activity against Gram-negative pathogens due to efflux systems (Levy, 1992), most notably the RND superfamily transporters (Li and Nikaido, 2004, 2009). Other major families of efflux systems include the MFS, SMR, and ABC superfamily transporters, which are present in both Gram-negative and -positive organisms (Li and Nikaido, 2004, 2009). Although commonly chromosomal, many efflux systems are found on plasmids and other mobile elements and can confer drug-specific, class-specific, or multidrug resistance (Poole, 2005). Some permeability barriers, such as the Gram-negative outer membrane (Arthur and Courvalin, 1993), represent intrinsic antibiotic resistance, while in other instances, permeability barriers are acquired. Examples include multidrug-resistance via the altered expression of Gram-negative porin proteins (e.g., OmpF in *Escherichia coli* and OprD in *Pseudomonas*) (Delcour, 2009) and glycopeptide resistance due to thickened Gram-positive cell walls (Cui et al., 2006).

Antibiotic resistance via cellular target modification often occurs via chromosomal mutation and represents a common means by which the fluoroquinolone, sulfonamide, and trimethroprim antibiotics, among others, are tolerated (Alekshun and Levy, 2007). In the case of the fluoroquinolones, mutations to a variety of residues within the quinolone-resistance-determining-region (QRDR) of the DNA gyrase GyrA or topoisomerase IV ParC/GrlA prevent the interaction of the synthetic antibiotic with its target, facilitating resistance (Hooper, 1999). Importantly, resistance to fluoroquinolones via mutation is typically recessive, suppressing the acquisition of a resistant phenotype in the presence of wild-type GyrA or ParC/GrlA and thus preventing widespread horizontal dissemination of resistant gene variants (Wolfson and Hooper, 1989; Soussy et al., 1993). However, a plasmid-borne GyrA protection protein, Qnr, has been discovered that confers low-level fluoroquinolone resistance (Tran and Jacoby, 2002) and can potentiate the incidence of QRDR mutations, which combined provide high levels of resistance (Jacoby, 2005). Both the sulfonamides and trimethroprim competitively inhibit enzymes within the folate biosynthesis pathway: mutations to the dihydropteroate synthase and dihydrofolate reductase (DHFR) enzymes can reduce affinity for sulfonamides and trimethroprim, respectively, and provide tolerance to the antibiotics (Huovinen et al., 1995). In addition to arising *de novo*, both sulfonamide- and trimethroprim-resistant enzymes are present on mobile DNA elements, providing resistance to numerous bacteria via HGT (Alekshun and Levy, 2007). Other highly mobile mechanisms of target-modification include vancomycin resistance via the modification of peptidoglycan precursors (Bugg et al., 1991), aminoglycoside resistance via methylation of the 16S rRNA subunit (Galimand et al., 2003), and macrolide resistance from 23S rRNA methylases (Zhanel et al., 2001).

RESERVOIRS OF TRANSFERABLE ANTIBIOTIC RESISTANCE

Research on antibiotic resistance over the past 70 years has focused on traditionally pathogenic bacteria isolated in a clinical setting and the role of antibiotic resistance genes already present in those species (Sommer et al., 2009; Davies and Davies, 2010). The resistance phenotype, however, is an ancient function of environmental bacteria (D'Costa et al., 2011), despite being largely absent from human pathogens prior to the antibiotic age (Hughes and Datta, 1983; Houndt and Ochman, 2000), with estimates that β-lactamases have existed for over 2 billion years (Hall and Barlow, 2004; Hall et al., 2004). Moreover, diverse mechanisms of antibiotic resistance have been discovered in nearly all environments (D'Costa et al., 2006; Allen et al., 2010; Davies and Davies, 2010; Wright, 2010); seemingly each new metagenomic study uncovers numerous examples of resistance genes previously unreported in public databases (Allen et al., 2009b; Sommer et al., 2009; Donato et al., 2010; Forsberg et al., 2012). In short, the diversity and abundance of antibiotic resistance in commensal microbiota and environmental settings dwarfs that which is seen in the context of human pathogens.

Importantly, environmental antibiotic resistance is not only widespread, but also represents the likely origins of the resistance seen in human pathogens. It has been known for 40 years that

environmental bacteria share the same resistance mechanisms as those seen in pathogens (Benveniste and Davies, 1973), with documented examples of environmental resistance genes moving from natural settings into human pathogens (Poirel et al., 2002, 2005). It is becoming increasingly evident that human pathogens and environmental organisms share antibiotic resistance genes; a recent study described seven resistance genes from non-pathogenic soil organisms, conferring tolerance to five antibiotic classes, with perfect identity to genes from phylogenetically and geographically diverse pathogens (Forsberg et al., 2012). Given the staggering diversity of environmental resistance, high adaptability of bacteria, and strong selection pressure for antibiotic resistance, the question of antibiotic resistance is "not a matter of if but only a matter of when" (Walsh, 2000).

To understand *when* novel resistance will appear in human pathogens and perhaps diminish its impact, one must understand *how* new resistance genes are most frequently acquired by pathogenic bacteria. Since the answer is, most commonly, *via HGT* (Hughes and Datta, 1983; Ochman et al., 2000; Alekshun and Levy, 2007), understanding the complement of resistance genes most likely to be transferred to pathogens is crucial to predicting resistance acquisition. Although cataloging the repertoire of resistance genes on Earth remains a prohibitively large undertaking, techniques for interrogating the resistance properties of complex microbial communities exist and are being applied toward the identification of diverse and novel resistance from numerous settings. Importantly, these studies are focused not only on environmental locales, but also on the resistomes associated with human and animal microbiota (Shoemaker et al., 2001; Sommer et al., 2010b; Sommer and Dantas, 2011). Although antibiotic resistance may have its origins in the environment (Benveniste and Davies, 1973; Wright, 2010; D'Costa et al., 2011), the commensal resistome shares many resistance genes with both pathogens (Sommer et al., 2010b) and environmental organisms (Forsberg et al., 2012) and represents a likely route through which these populations exchange resistance genes (Smillie et al., 2011; Sommer and Dantas, 2011). Focused efforts to understand how pathogens acquire antibiotic resistance will require a greater appreciation for the diversity of resistance genes and in which environments and under what conditions these resistance genes are most accessible to human pathogens.

INTERROGATING ANTIBIOTIC RESISTANCE PROPERTIES IN COMPLEX MICROBIAL COMMUNITIES

Traditionally, either culture-based (D'Costa et al., 2006) or PCR-based approaches (Perez-Perez and Hanson, 2002) have been used to study antibiotic resistance properties from microbial communities. While both techniques have led to major discoveries (Galan et al., 2013), both have inherent limitations that have contributed to an under-sampling of resistance genes from diverse microbial habitats. The majority of bacteria remain recalcitrant to culturing (Daniel, 2005) and are therefore not interrogated when culture-dependent techniques are employed. Additionally, linking a resistance phenotype to a causal genotype is a time-consuming process, often necessitating experimental scope to be limited to a small number of organisms, rather than whole communities. PCR screens are an effective means to identify or quantify resistance genes of known sequence, circumventing the need for culture (Knapp et al., 2010), but are only able to detect previously described genes and often require expression cloning and subsequent experimentation to verify function. In addition, annotation of antibiotic resistance genes in shotgun-sequenced microbial communities has proven challenging, as homology-based functional gene comparisons often fail due to low sequence similarity to previously discovered resistance genes, and *in silico* analyses are unable to confirm resistance function.

In contrast with standard techniques, *functional metagenomics* is a culture- and sequence-independent means of identifying transferrable antibiotic resistance in complex metagenomes. This method (**Figure 1**) involves shotgun-cloning total community DNA into an expression vector and transforming the library into an indicator host (commonly the model organism *E. coli*). The resulting transformants are then selected for the desired function (e.g., antibiotic resistance), and metagenomic DNA fragments are sequenced and annotated to identify causal survival-conferring genes (Allen et al., 2009b; Sommer et al., 2009). Functional metagenomics offers three classical advantages for the unbiased interrogation of complex resistomes (Daniel, 2005; Sommer and Dantas, 2011): (1) no need to culture organisms, (2) no required knowledge of resistance gene sequence, and (3) direct association between a genotype and a demonstrated resistance phenotype. Additionally, functional metagenomic selections specifically identify those genes within a metagenome capable of conferring antibiotic tolerance to the indicator host when expressed exogenously (i.e., they distinguish transferrable resistance from intrinsic resistance) (Dantas and Sommer, 2012). Recent improvements to the throughput of functional metagenomics (Forsberg et al., 2012) unlock the potential for the experiments of scale needed identify the specific sequences, and environments, most readily able to confer resistance to human pathogens, frequently represented by the opportunistic pathogen *E. coli*.

NOVEL RESISTANCE MECHANISMS UNCOVERED BY FUNCTIONAL METAGENOMIC SELECTIONS

The following sections review novel resistance mechanisms and genes uncovered by functional metagenomic selections; these studies are summarized in **Table 1**.

GENES HIGHLY DIVERGENT FROM KNOWN RESISTANCE GENES

PCR-based screens for known resistance genes are able to detect novel variants with minor sequence differences, but due to the requirement for conserved primer binding sites, they are often unable to detect genes that have diverged significantly from the canonical example. In comparison, functional metagenomic selections for antibiotic resistance frequently identify genes that are less than 65% identical at the amino acid level to known resistance genes. In this way, functional metagenomics expands our knowledge of what sequence variants are tolerated in a resistance gene while preserving the resistance mechanism, as well as what mutations lead to expanded specificity profiles.

β-lactamases are one of the largest and best studied classes of resistance determinants, and yet, novel β-lactamases are regularly uncovered with sequence-independent techniques (Allen et al., 2009b; Sommer et al., 2009; Donato et al., 2010; Cheng

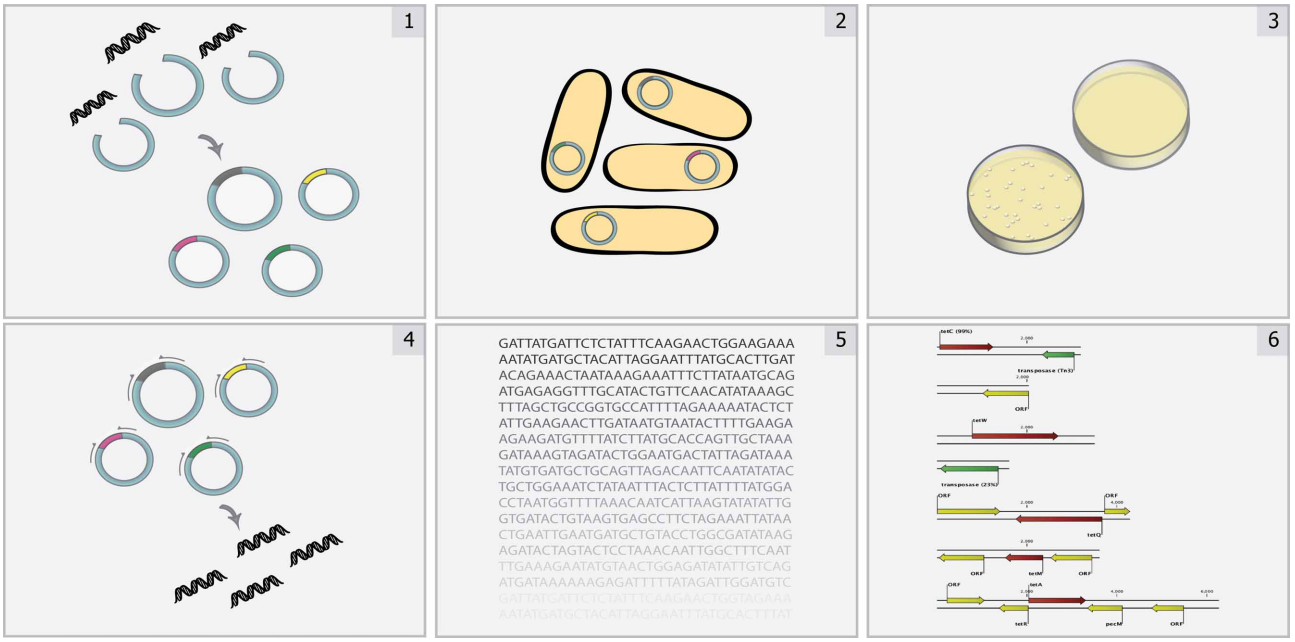

FIGURE 1 | Overview of functional metagenomic selections. Total metagenomic DNA is extracted from a microbial community sample, sheared, and ligated into an expression vector (Step 1) and is subsequently transformed into a suitable library host (Step 2) to create a metagenomic library. The library is then plated on media containing antibiotics inhibitory to the wild-type host (Step 3) to select for metagenomic fragments conferring antibiotic resistance. Metagenomic fragments present in colonies growing on antibiotic selection media are then PCR-amplified (Step 4) and sequenced using either traditional Sanger sequencing or next-generation sequencing methods (Step 5). Finally, reads are assembled and annotated in order to identify the causative antibiotic resistance genes (Step 6).

et al., 2012; Forsberg et al., 2012). In the first functional metagenomic selection for antibiotic resistance in the human gut microbiome, Sommer et al. identified ten novel β-lactamase families whose eleven members were only 35–61% identical to known genes (Sommer et al., 2009). Based on phylogenetic analysis with PhyloPythia, (McHardy et al., 2007) these genes originated primarily from the Firmicutes and Bacteroidetes phyla, which comprise the majority of the human gut microbiota but have been undersampled because they are less readily culturable in aerobic environments than Proteobacteria. Functional selection for β-lactam resistance in metagenomic libraries constructed from remote Alaskan soil revealed 13 novel β-lactamases of all four Ambler classes that were ≤67% identical to any known, functionally characterized gene (Allen et al., 2009b). In the case of the class B β-lactamases, several genes appear to be more closely related to the ancestral gene of that class than to clinically isolated genes, providing context for the evolution of clinical isolates. The study also confirmed that overall sequence similarity does not necessarily confer similar drug susceptibility profiles, supporting discovery techniques that confirm the function of identified genes.

Functional metagenomic selections also enable the detection of highly diverse classes of resistance genes, defined by substrate specificity more than shared sequence identity. For instance, aminoglycoside 6′-N-acetyltransferases [AAC(6′)s] are difficult to identify via PCR because of their high sequence diversity (Riesenfeld et al., 2004). Sommer et al. identified six AAC(6′)s with less than 48% amino acid identity to known genes and a methyltransferase conferring resistance to sisomycin with only 26.3% shared identity. Riesenfeld et al. and other studies have identified many aminoglycoside resistance genes less than 70% identical to previously reported sequences (Riesenfeld et al., 2004; Donato et al., 2010; Torres-Cortes et al., 2011; Cheng et al., 2012; McGarvey et al., 2012). Similarly, two novel bleomycin resistance genes were isolated from an activated sludge metagenomic library (Mori et al., 2008). Bleomycin resistance proteins act primarily by sequestering the antibiotic through electrostatic interactions, so their sequences tolerate an extreme amount of divergence while still maintaining the resistance function. Nevertheless, the study confirmed that all known bleomycin resistance proteins contain a proline near the N-terminus and have an acidic pI of <5, improving computational annotation of this class. Finally, McGarvey et al. identified an ADP-ribosyltransferase that was much longer any previously characterized using functional metagenomic selections (McGarvey et al., 2012). This full-length gene may not have been identified by PCR-based screens, but its discovery, enabled by functional metagenomics, expands our knowledge of the sequence diversity tolerated within this resistance class.

RESISTANCE GENES THAT WOULD NOT HAVE BEEN PREDICTED BY SEQUENCE

A major limitation of sequence-based metagenomics is its restriction to antibiotic resistance genes that are recognizable as members of a previously characterized class. BLAST, the most

Table 1 | Functional metagenomic investigations of antibiotic resistance reservoirs.

Resistance reservoir	Date	Novel resistance function identified	Total DNA queried*	References
FUNCTIONAL METAGENOMIC INVESTIGATIONS OF ENVIRONMENTAL RESERVOIRS				
Soil (remnant oak savannah)	2004	First use of functional metagenomic selections to investigate environmental antibiotic resistance; Nine novel aminoglycoside-resistance genes	5.4 GB	Riesenfeld et al., 2004
Activated sludge	2008	Two novel bleomycin-resistance genes	3.2 GB	Mori et al., 2008
Remote Alaskan soil	2009	13 novel β-lactamases; First discovery of bifunctional β-lactamase	12.4 GB	Allen et al., 2009b
Gypsy moth larvae midgut isolates	2009	*ramA* (AraC transcriptional regulator)	0.3 GB	Allen et al., 2009a
Remote Alaskan soil	2010	*pexA* (novel chloramphenicol transporter)	13.2 GB	Lang et al., 2010
Activated sludge	2010	Six novel chloramphenicol-modifying resistance genes; Novel aminoglycoside-resistance gene	1.9 GB	Parsley et al., 2010
Soil (apple orchard)	2010	Nine novel β-lactamases; Three novel aminoglycoside-resistance genes; A novel tetracycline efflux pump	13.4 GB	Donato et al., 2010
Alluvial soil	2011	Est136, chloramphenicol acetate esterase	1.6 GB	Tao, 2011; Tao et al., 2012
Soil (agricultural, nature reserve)	2011	*Tm8-3* (novel dihydrofolate reductase)	3.6 GB	Torres-Cortes et al., 2011
Wetland soil	2011	EstU1, family VIII carboxylesterase	0.3 GB	Kim et al., 2008; Jeon et al., 2011
Gull gut microbiome	2011	31 β-lactam resistance genes of undetermined mechanism	20.5 GB	Martiny et al., 2011
Urban soil	2012	Novel MFS and ABC transporters; Five novel aminoglycoside-resistance genes; ADP-ribosyltransferase longer than any previously discovered	2.8 GB	McGarvey et al., 2012
Multidrug-resistant soil isolates	2012	Novel β-lactamases; D-cycloserine efflux pump	2.6 GB	Forsberg et al., 2012
FUNCTIONAL METAGENOMIC INVESTIGATIONS OF HUMAN-ASSOCIATED RESERVOIRS				
Human oral microbiome	2003	Tetracycline-inactivating protein *tet(37)*	0.4–1.4 MB	Diaz-Torres et al., 2003
Human oral microbiome	2006	Tetracycline-resistance genes of undetermined mechanism	27.8 MB	Diaz-Torres et al., 2006
Human gut and oral microbiome	2009	10 new β-lactamase classes; Seven novel aminoglycoside-resistance genes	9.3 GB	Sommer et al., 2009
Pig gut microbiome	2009	Tetracycline-resistance genes *galE1* and *galE2* of undetermined mechanism	0.1 GB	Kazimierczak et al., 2009
Human gut microbiome	2012	Confirmed bifunctional aminoglycoside-resistance gene; Novel β-lactamase; Novel aminoglycoside-resistance gene	12.5 GB	Cheng et al., 2012

**For comparison, the genome size of E. coli K-12 substrain MG1655 is 4.64 MB.*

commonly used annotation tool, requires a threshold of shared sequence identity for a gene to be considered a member of an established gene class. Therefore, by definition, this method limits the ability to identify novel resistance genes. In addition, while profile hidden Markov model (HMM)-based annotation provides a sensitive, statistically sound analysis method capable of identifying remote homologs, these models still rely on underlying multiple sequences alignments of previously characterized proteins. In addition, current profile HMM databases provide high-level classification of antibiotic resistance genes (e.g., "Beta-Lactamase,"

rather than "TEM Beta-Lactamase"), providing little functional information about the gene and its potential resistance profile. Therefore, while these techniques have the potential to identify genes that have low sequence identity to known resistance genes, in the absence of additional functional confirmation, they cannot identify novel resistance mechanisms or verify that sequence variants are functional.

Functional metagenomics identifies resistance-conferring elements without prior knowledge of the sequence, circumventing this limitation. For instance, in a selection for tetracycline resistance encoded by the human oral metagenome, Diaz-Torres et al. discovered a novel tetracycline resistance gene, *tet(37)* (Diaz-Torres et al., 2003). *In vitro* analysis indicated that *tet(37)* inactivates tetracycline, making it one of only three tetracycline resistance proteins to utilize this mechanism (Thaker et al., 2010). *tet(37)* resembles flavoproteins, oxidoreductases, and NAD(P)-requiring enzymes in sequence and conserved motifs, but has no identity to *tet(X)*, the first tetracycline-inactivating gene identified. It is therefore unlikely that computational annotation would have identified it as a resistance gene (Diaz-Torres et al., 2003).

Similarly, a study of soil microbiota identified a dihydrofolate-reducing gene, *Tm8-3*, that resembles 3-oxoacyl-(acyl-carrier-protein) reductases but not *dhfr*, the target of trimethoprim (Torres-Cortes et al., 2011). A screen of an activated sludge metagenome also identified six resistance genes that appear to inactivate chloramphenicol through enzymatic modification, but which share no significant identity with known chloramphenicol acetyltransferases (Parsley et al., 2010). Although these genes may become important for clinical resistance in the future, their existence would likely have been overlooked without high-throughput functional selections independent of previous sequence knowledge.

Drug efflux is a widespread resistance mechanism common to many antibiotic classes, but individual transporters identified through computational annotation methods cannot be assigned antibiotic efflux properties without functional validation. Small changes in protein structure can change the drug specificity profile of an efflux pump and confer or eliminate resistance. Therefore, functional metagenomic selections are an attractive alternative for high-throughput characterization of transporters with tentative resistance annotations (Torres-Cortes et al., 2011). For instance, in their study of soil isolate metagenomes, Forsberg et al. identified a novel gene with only low identity to a drug/metabolite transporter. Were it not for functional selection of D-cycloserine resistance, this putative transporter would never have been identified as an antibiotic resistance gene (Forsberg et al., 2012). McGarvey et al. identified five novel MFS transporters and two ABC transporters from an urban soil that conferred resistance to tetracycline, chloramphenicol, or trimethoprim (McGarvey et al., 2012). Lang et al. successfully identified *pexA*, a novel amphenicol MFS transporter, despite its low identity (33%) to other drug resistance transporters, including any known chloramphenicol exporters (Lang et al., 2010).

BIFUNCTIONAL ENZYMES

Broad-spectrum resistance as a result of bifunctional resistance genes, the fusion of two complementary enzymatic functions into a single gene, is recognized as an increasing occurrence in many pathogens (Kim et al., 2006; Perez et al., 2007; Chandrakanth et al., 2008). Functional metagenomics provides an opportunity to identify fusion genes that are functionally active against multiple classes of antibiotic agents from natural resistance reservoirs and commensal organisms, and which have the potential to appear in clinically relevant bacteria.

Recently, functional metagenomic selections of a remote Alaskan soil revealed a novel bifunctional β-lactamase (Allen et al., 2009b). The 609-amino-acid protein is nearly double the length of a typical β-lactamase and is a natural fusion of genes for two different β-lactamase subclasses. The C-terminal domain (356 residues) aligns with class C β-lactamases, while the N-terminal domain (253 residues) aligns with class D β-lactamases, each contributing to the resistance profile of the full-length gene. The class C homologue confers resistance to cephalexin, while the class D homologue is responsible for resistance to amoxicillin, ampicillin, and carbenicillin. The bifunctional fusion gene therefore expands the resistance profile of the full-length gene beyond what either domain is responsible for alone (Allen et al., 2009b).

While this was the first bifunctional β-lactamase to be discovered, bifunctional resistance has been documented in the past, particularly against the aminoglycoside antibiotics (Daigle et al., 1999; Centrón and Roy, 2002; Dubois et al., 2002; Mendes et al., 2004; Robicsek et al., 2006). The most common mechanism of aminoglycoside resistance is deactivation of the molecule through modification by cytoplasmic enzymes: aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs), or aminoglycoside nucleotidyltransferases (ANTs). Four bifunctional enzymes, combining two complementary aminoglycoside-modifying enzymes as separate domains into a single open reading frame, significantly broadening the resistance profile, are known. Ferretti et al. reported the first bifunctional enzyme, AAC(6′)/APH(2″), demonstrating that it is capable of both acetylation (ACC activity found in N-terminal domain) and phosphorylation (APH activity found in C-terminal domain) of aminoglycosides (Ferretti et al., 1986). This bifunctional enzyme was also discovered in a functional metagenomics study of the human gut microbiota (Cheng et al., 2012). The ability to doubly modify aminoglycosides enables this enzyme to confer resistance to nearly all clinically relevant aminoglycosides except streptomycin and spectinomycin (Daigle et al., 1999). Similarly, the bifunctional resistance enzymes characterized as an AAC(3)-Ib/AAC(6′)-Ib′ (Dubois et al., 2002), an ANT(3″)-Ii/AAC(6′)-IId (Centrón and Roy, 2002), and an AAC(6′)-30/AAC(6′)-Ib′ (Mendes et al., 2004) all exhibit an expanded resistance profile by combining two different aminoglycoside-modifying enzymes into a single gene.

AAC(6′)-Ib-cr is the only bifunctional resistance enzyme identified capable of conferring resistance to two different structural classes of antibiotics, aminoglycosides and fluoroquinolones (Vetting et al., 2008). A variant of a common aminoglycoside acetyltransferase AAC(6′)-Ib, AAC(6′)-Ib-cr is also capable of N-acetylation of fluoroquinolones. Only two codon changes in the original enzyme are responsible for the fluoroquinolone-resistance phenotype (Vetting et al., 2008).

The merger of two genes with complementary enzymatic activities represents a novel mechanism for overcoming the increasing challenge by antibiotics, resulting in extremely broad resistance to entire antibiotic structural classes. It is likely that more examples of bifunctional resistance exist in the environment, and functional metagenomics provides a powerful technique for the discovery of functionally active bifunctional enzymes that may have great clinical implications.

UNUSUAL MECHANISMS OF RESISTANCE

Functional metagenomic selections have the potential to identify another important class of resistance genes, those that have a primary role in the cell other than antibiotic resistance. This includes housekeeping genes whose overexpression provides resistance, general stress response transcription factors, and enzymes with promiscuous activity.

Selections for trimethoprim resistance, for example, frequently identify genes encoding DHFR, the cellular target of trimethoprim (Torres-Cortes et al., 2011; McGarvey et al., 2012). Cloning a *dhfr* homolog into a high copy-number plasmid results in overexpression of DHFR, which sequesters the antibiotic (Flensburg and Skold, 1987). McGarvey et al. identified 19 highly divergent DHFRs from a soil metagenome. The proteins shared only 7–44% amino acid identity in pairwise alignments, but a subset selected for further investigation conferred similar levels of trimethoprim resistance on the host when cloned into the same position of a high-copy number vector (McGarvey et al., 2012). Although clinical isolates of *E. coli* that massively overproduce DHFR as a result of promoter mutations and intrinsically resistant *dhfr* homologs have been identified (Flensburg and Skold, 1987), in most instances, resistance conferred by overexpression of a heterologous gene is likely independent of its ability to replace the host homolog. Rather, the introduced gene may sequester enough of the antibiotic to allow the native homolog to continue functioning. Selections for D-cycloserine resistance that identify D-alanine-D-alanine ligase, the target of the antibiotic, likely fall under the same class (Cheng et al., 2012). Creating and screening libraries in low-copy number vectors is essential in these instances to identify genes that are naturally resistant to the antibiotic, rather than simply selecting for overexpression.

In contrast, antibiotic resistance conferred by transcriptional regulators represents the successful interaction of a heterologous gene with existing host cellular pathways. The AraC transcriptional stress response regulators *marA*, *soxS*, and *rob* share a highly overlapping regulon and mediate low-level antibiotic resistance in *E. coli* and its close relative *E. fergusonii* (Alekshun and Levy, 1997; Martin and Rosner, 2002). As might be expected, these genes are often identified in functional metagenomic selections against chloramphenicol, tetracyclines, and other antibiotics when *E. coli* is the indicator host. A homolog of the AraC transcriptional regulator *ramA*, from Enterobacter sp., was also identified from a gypsy moth larvae gut isolate metagenomic library as conferring resistance to *E. coli* against multiple antibiotics of different classes when overexpressed (Allen et al., 2009a). These results provide valuable information about the ability of horizontally acquired genes from closely related phyla to incorporate into a cell at the regulatory level, a mechanism of antibiotic resistance that has been previously under-considered.

Certain genes identified by functional metagenomic selections are surprising in that they more closely resemble protein families that perform unrelated functions in the cell than previously identified resistance genes. They may represent instances of expanded substrate specificity, where mutation to a gene with an unrelated function confers resistance. By first screening a soil metagenomic library for esterase activity, Jeon et al. identified a family VIII carboxylesterase that hydrolyzes both esters and the amide bond of β-lactams, apparently utilizing the same catalytic site residues for both reactions (Jeon et al., 2011). The protein changes that enable it to also hydrolyze β-lactams are currently unidentified. A novel chloramphenicol acetate esterase, EstDL136, isolated from a soil metagenome, hydrolyzes the amide linkage of both chloramphenicol and its synthetic derivative florfenicol (Tao, 2011; Tao et al., 2012). Many of the surrounding genes in the metagenomic fragment are most closely related to Sphingomonadaceae, which are frequently considered for bioremediation for their ability to degrade many compounds (Stolz, 2009). EstDL136 may have originally evolved to detoxify another compound, later expanding its substrate specificity to chloramphenicol.

FUTURE DIRECTIONS AND CHALLENGES

Functional metagenomic selections have proven to be an excellent technique for the discovery of novel antibiotic resistance mechanisms and genes encoded by varied environmental and human-associated microbial communities. As library sizes continue to increase, diverse indicator hosts are established, and techniques for improved functional selection and mechanistic determination are developed, functional metagenomics will only become better powered to explore the transferable resistome of complex microbial communities.

All of the metagenomic libraries discussed above were constructed in *E. coli*. The commercial availability of competent *E. coli* with extremely high transformation efficiencies has made this organism the preferred host for library construction, and these studies show that *E. coli* permits the heterologous expression of genes from many phyla. However, there are many clinically important classes of antibiotics that are intrinsically inactive against this Gram-negative species (e.g., glycopeptides, macrolides, oxazolidinones), prohibiting functional metagenomic selections in this host from identifying antibiotic resistance genes active against these classes. Development of a Gram-positive indicator host will represent a major step forward for the field (Riesenfeld et al., 2004; Sommer et al., 2009). This host must also exhibit a high transformation efficiency to enable the creation of metagenomic libraries large enough to permit the discovery of novel resistance genes and should lack restriction-modification systems or other host defenses that would prevent the expression of foreign DNA. *E. coli* mutants that mimic Gram-positive species through the alteration of cell membrane and wall structure are another possible alternative (Tamae et al., 2008).

The taxa from which antibiotic resistance genes can be expressed in *E. coli* are also limited by the ability of the host to recognize gene promoters and ribosome binding sites and translate them into functional proteins. Engineering *E. coli* to

produce tRNA that recognize rare codons and alternative sigma factors will expand the range of taxa interrogated (Riesenfeld et al., 2004; Sommer et al., 2009, 2010a).

Finally, genes of unknown function should be further investigated to determine their mechanisms of resistance. Functional metagenomic selections that uncover a multitude of confirmed resistance genes in a high-throughput manner often require complementary biochemical experimentation to understand novel mechanisms of antibiotic resistance. Many metagenomic functional selections have identified resistance-conferring inserts whose mechanisms remain obscure because they cannot be determined based on sequence alone (Riesenfeld et al., 2004; Diaz-Torres et al., 2006; Kazimierczak et al., 2009; Martiny et al., 2011; McGarvey et al., 2012). These genes are excellent candidates for future functional characterization.

ALTERNATIVE STRATEGIES TO COMBAT ANTIBIOTIC RESISTANCE

The depth and diversity of antibiotic resistance genes uncovered by functional metagenomic selections, many of which are associated with mobile genetic elements, brings to light the need for novel strategies to combat antibiotic-resistant pathogens. Alternative approaches that modulate the host immune response (e.g., immunomodulatory peptides, vaccination, therapeutic antibodies) or target the pathogen (e.g., anti-virulence initiatives, phage therapy, antibiotic potentiators) have been explored with some success (Planson et al., 2011; Pieren and Tigges, 2012).

For instance, recent studies have suggested engineered bacteriophage as an adjuvant to enhance the bactericidal activity of antibiotics and avoid evolution of resistance to either (Lu and Collins, 2009; Parracho et al., 2012). In this case, bacteriophage is engineered to repress non-essential genes (e.g., the SOS system) not directly targeted by antibiotics, weakening the cell and potentiating antibiotic activity. Other adjuvant compounds that inhibit intrinsic repair pathways or cell tolerance mechanisms are being explored (Fischbach, 2011).

Several permeabilizing products capable of disturbing the cell membrane have been identified, thus allowing antibiotics to penetrate the cells more efficiently. One such product is the low molecular-weight oligosaccharide nanomedicine OligoG, which is able to disturb multidrug-resistant bacteria (Khan et al., 2012).

Drug efflux pumps, both specific and multidrug-resistant, may be inhibited through a variety of strategies. These include interfering with the expression of a functional transporter at different stages between transcription and final assembly, interfering with the assembly of channel proteins, designing inhibitors that compete with the antibiotic for efflux, disrupting the energy source, and blocking the efflux channel (Pages and Amaral, 2009). Deletion of genes involved in expression of porins has been shown to increase antibiotic resistance (Rodrigues et al., 2011). Therefore, strategies combining overexpression of porins used by the antibiotic to penetrate the cell with downregulation of efflux pumps may allow the antibiotic to overcome existing resistance mechanisms.

CONCLUSION

Functional metagenomic selections are a powerful technique for high-throughput characterization of the transferable resistome encoded by environmental and human-associated microbial communities, which have the potential to provide human pathogens with resistance genes through HGT. The advantages of this technique over traditional culture- and sequence-based screens are underlined when considering novel resistance mechanisms. Because resistance genes are selected on the basis of function regardless of sequence, the functional metagenomic selections reviewed above identified numerous genes with sequences highly divergent from other members of their class, as well as novel resistance mechanisms that would not have been recognized as resistance genes based on sequence alone. Conversely, they confirm antibiotic resistance function in putative drug transporters, which are frequently annotated as such regardless of true resistance ability, and permit the accurate annotation of resistance genes typically associated with other cellular functions. They have also led to the discovery of confirmed bifunctional enzymes, with broad resistance spectrums resulting from the fusion of two resistance genes. By providing a broader understanding of the resistance mechanisms currently in existence, functional metagenomic selections will aid the production of new antibiotics less susceptible to existing resistance mechanisms, as well as alternative strategies for combating antibiotic resistance.

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The antibiotic resistance "mobilome": searching for the link between environment and clinic

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Antibiotic resistance is an ancient problem, owing to the co-evolution of antibiotic-producing and target organisms in the soil and other environments over millennia. The environmental "resistome" is the collection of all genes that directly or indirectly contribute to antibiotic resistance. Many of these resistance determinants originate in antibiotic-producing organisms (where they serve to mediate self-immunity), while others become resistance determinants only when mobilized and over-expressed in non-native hosts (like plasmid-encoded β-lactamases). The modern environmental resistome is under selective pressure from human activities such as agriculture, which may influence the composition of the local resistome and lead to gene transfer events. Beyond the environment, we are challenged in the clinic by the rise in both frequency and diversity of antibiotic resistant pathogens. We assume that clinical resistance originated in the environment, but few examples of direct gene exchange between the environmental resistome and the clinical resistome have been documented. Strong evidence exists to suggest that clinical aminoglycoside and vancomycin resistance enzymes, the extended-spectrum β-lactamase CTX-M and the quinolone resistance gene *qnr* have direct links to the environmental resistome. In this review, we highlight recent advances in our understanding of horizontal gene transfer of antibiotic resistance genes from the environment to the clinic. Improvements in sequencing technologies coupled with functional metagenomic studies have revealed previously underappreciated diversity in the environmental resistome, and also established novel genetic links to the clinic. Understanding mechanisms of gene exchange becomes vital in controlling the future dissemination of antibiotic resistance.

Keywords: antibiotic resistance, horizontal gene transfer, environmental resistome, evolution

INTRODUCTION

Griffith (1928) demonstrated the transformation of non-virulent *Streptococcus pneumoniae* with a heat-stable substance from a virulent strain. The "transforming principle" was later identified as DNA (Avery et al., 1944), and the notion that genetic material can flow both vertically (from parent to offspring), and horizontally (from siblings or peers) in bacteria continues to form the basis of modern-day molecular genetics. Also in 1928, Alexander Fleming discovered the antibiotic penicillin: arguably one of the most important discoveries in modern medicine, but one that is threatened today by the mechanisms discovered by Griffith and Avery. In this review, we highlight recent advances in our understanding of horizontal gene transfer of antibiotic resistance genes in the environment. The development and use of functional metagenomic techniques coupled with advances in sequencing technologies have revealed new diversity in the antibiotic resistome, and also established novel genetic links between the environment and the clinic.

ON THE ORIGINS OF RESISTANCE: EXPLORING THE ENVIRONMENTAL RESISTOME

The antibiotic "resistome" is the collection of all genes that directly or indirectly contribute to antibiotic resistance, both in the environment and the clinic. Antibiotic-producing bacteria of the genus *Streptomyces* are found abundantly in soils around the globe, and encode resistance genes against their own antibiotics [the so-called "producer hypothesis" (Cundliffe, 1989; Wright, 2007)]. Importantly, these organisms are often multi-drug resistant (D'Costa et al., 2006), which may reflect their ability to produce more than one antibiotic, or be a by-product of their evolution in proximity to antibiotic-producing neighbors in the soil. It is clear that the environmental "resistome" is a substantial source of resistance genes, and it has been theorized that antibiotic-producing organisms in the environment are the source of resistance genes found in clinical pathogens. Davies and colleagues demonstrated almost 40 years ago that the biochemical activity of aminoglycoside resistance enzymes encoded by producing organisms was identical to those found in pathogens (Benveniste and Davies, 1973). However, direct evidence of gene transfer between the environmental resistome and the clinic are rare as resistance genes may undergo many rounds of evolution between the soil and the clinic (Aminov and Mackie, 2007). The most cited examples of recent exchange between the environmental and clinical resistomes include the class-A extended-spectrum β-lactamase CTX-M, found on plasmids carried by major global pathogens and traced to environmental *Kluyvera* spp. (Humeniuk et al., 2002), and the quinolone resistance gene *qnr* (found on a broad-host

range conjugative plasmid from a ciprofloxacin-resistant strain of *Klebsiella pneumoniae* and traced to several environmental waterborne species (Poirel et al., 2005a,b; Baquero et al., 2008). Still, the environmental resistome is so vast (and underexplored) that further links to the clinic are sure to surface.

SAMPLING THE ENVIRONMENT FOR RESISTANCE GENES

Two main approaches are used to study the environmental resistome: culture-based and metagenomic searches. Culture-based approaches involve growing all microorganisms resistant to a given antibiotic in the lab, and subsequently analyzing their genetics and associated biochemistry for resistance determinants. While the major disadvantage to this technique is that it is estimated that only a small fraction (∼1%) of environmental organisms are readily grown in the lab (Amann et al., 1995), culture-based approaches allow detailed studies of the genomic context of resistance (including identification of host species, identification of multiple resistance determinants in a single genome, identification of antibiotic production capabilities of the host, etc.) that is not possible using culture-independent methods. Metagenomic studies are performed by culture-independent extraction of total microbial DNA from the environment, and subsequent analysis using either polymerase chain reaction (PCR)-based methods with specific primers or deep sequencing technologies. While a metagenomic approach has the obvious advantage of overcoming culture-based bias, it can be difficult to design oligonucleotide primers with both enough specificity and flexibility to capture sequences with minor deviation from the reference sequence. Metagenomic datasets can be difficult to assemble, and using bioinformatics alone to search for homologs of known resistance determinants may miss novel resistance determinants that are not sufficiently homologous to known genes. Functional metagenomic approaches (in which a metagenomic library is expressed in a heterologous host and screened for resistance) offer significant advantages over culture-based and metagenomic/PCR-based screens and are discussed in detail in the final section of this review.

NOVEL RESISTANCE DETERMINANTS AS A RESULT OF GENE MOBILIZATION

Perhaps one of the main reasons why resistance genes are missed in traditional metagenomic screens is that genes may not present as resistance genes in their native context. Often, resistance genes are expressed in low copy number in their native host because they form part of a tightly coordinated network (Martinez, 2008). However, if these genes are mobilized into a new genetic context where regulation is absent (or expression is strongly favored), they many function as novel resistance determinants. An excellent example of this type of resistance gene "repurposing" is the efflux pumps belonging to the resistance/nodulation/cell division (RND) family (reviewed in Dantas and Sommer, 2012). This family of small molecule pumps is found ubiquitously in all kingdoms of living organisms, where they function as transporters of toxic compounds. However, when mobilized and expressed at high levels in pathogens like *Campylobacter jejuni, Escherichia coli, Salmonella enterica* serotype Typhimurium or *Pseudomonas aeruginosa,* they are capable of conferring high level antibiotic resistance via drug efflux (reviewed in Piddock, 2006a,b; Dantas and Sommer, 2012). Gene mobilization can therefore contribute to the formation (as well as the dissemination) of antibiotic resistance.

THE ENVIRONMENTAL "MOBILOME"

Bacterial evolution occurs on an accelerated time scale compared to plant and animal species, due to the ease with which genes move between organisms. Gene transfer allows a bacterium to build on existing adaptations in order to invade a new niche or to be more successful in its current niche; the abundance of naturally transformable bacteria, transducing phage, and conjugative elements present in the environment are testament to the importance of horizontal gene transfer to bacterial evolution (Ochman et al., 2000).

WHAT DRIVES GENE TRANSFER IN THE ENVIRONMENT?

The modern environmental resistome is under selective pressure from human activities such as the use of antibiotics in agriculture, which may influence the composition of the local resistome and lead to gene transfer events. However, rough calculations suggest that 1 g of soil can contain ∼580 different species of actinobacteria, which have the genetic capacity to produce 11,600 bioactive small molecules (Wright, 2010). Since these organisms also encode resistance elements to counteract the effects of the molecules they produce, the networks of selection and resistance that exist in soil are vast, and have evolved over millennia. In a carefully controlled study of 30,000-year old Beringian permafrost, D'Costa et al. (2011) showed that genes conferring resistance to β-lactams, tetracyclines, and glycopeptides existed in the environment well before the use of these antibiotics in the clinic. Structural and functional assays on the glycopeptide resistance element VanA demonstrated that the ancient soil resistance determinant was similar to the modern clinical resistance element. While this study showed that the origin of resistance to several natural product antibiotics is in the environment, a quantitative temporal study of resistance genes in agricultural soil from the Netherlands revealed that levels of all resistance genes investigated rose over time, from the pre-antibiotic era (1940s) to the present (Knapp et al., 2010). Moreover, plasmids isolated from pathogenic bacteria pre-dating the antibiotic era do not contain resistance genes (Hughes and Datta, 1983). While resistance to natural product antibiotics is undoubtedly ancient, anthropomorphic factors are clearly contributing to the mobilization, fixation, and dissemination of resistance genes in the present day. Although there is no direct evidence that antibiotics reach concentrations in the soil that approach the highly selective concentrations used therapeutically, antibiotic resistance genes may be necessary if that niche is affected by agricultural animals or humans frequently treated by antibiotics (Wiedenbeck and Cohan, 2011).

REQUIREMENTS FOR SUCCESSFUL GENE EXCHANGE

Regardless of mechanism or environment, the success of gene transfer depends first and foremost on the proximity of donor and recipient – they must be in physical proximity, and are generally assumed to share the same niche. Genetic exchange communities are variable in taxonomic composition but usually share vector types (plasmid or transposon) and do not contain

strong restriction/modification systems such that genetic exchange is more frequent (Wiedenbeck and Cohan, 2011). DNA transferred via transformation is typically short (often the length of one to several genes), which limits the chances a restriction enzyme target sequence will occur, and increases the chances of recombination in deeply divergent bacterial species (Wiedenbeck and Cohan, 2011). Plasmids transferred via conjugation are often able to carry several resistance genes in tandem (reviewed in Barlow, 2009). Once inside the recipient cell, new DNA must replicate autonomously or integrate into the chromosome of the recipient (by homology or via insertion sequences). Recombination is heavily favored if the sequence is flanked by insertion sequences as integration is therefore independent of sequence homology (Vo et al., 2010).

Integrons and transposons are now widely recognized as playing important roles in the dissemination of antibiotic resistance. Integrons are composed of three key elements: the *intI* integrase, a specific recombination site *attI* and a promoter (expertly reviewed in Stalder et al., 2012). Integrons are found chromosomally, on plasmids and transposons, and are found widely distributed in both the clinic and the environment, especially in Gram-negative bacteria. Interestingly, a class 1 integron found in an isolate of *Pseudomonas* from 15,000- to 40,000-year-old Siberian permafrost contained all the elements characteristic of modern-day clinical class 1 integrons, including being located on a transposon and containing an antibiotic resistance gene (*aadA2*, encoding resistance to streptomycin and spectinomycin; Petrova et al., 2011).

The exchange of DNA can occur passively, but may also be actively controlled. Many naturally competent species of *Streptococcus* undergo autolysis to liberate cellular DNA using the same signaling molecule that triggers DNA uptake in the surviving population (Guiral et al., 2006; Perry et al., 2009). Some genera can actively secrete DNA, including *Acinetobacter*, *Alcaligenes*, *Azotobacter*, *Pseudomonas*, *Micrococcus*, *Bacillus*, and *Flavobacterium* (reviewed in Thomas and Nielsen, 2005). On the flip side, bacteria also express defense mechanisms to prevent invasion by foreign DNA elements. Viral infections are prevented at the level of adsorption, injection, or by abortive infection, while restriction–modification systems and the use of sugar-non-specific nucleases target invading nucleic acids. An area of intense research is immunity mediated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas motifs, present in most archeal and many bacterial genomes (reviewed in Horvath and Barrangou, 2010).

GENE TRANSFER IN THE SOIL

While gene exchange occurs in all environments, it has been best studied in the soil. The physical properties of soil (temperature, pH, concentration of nutrients and oxygen, etc.), combined with biological factors (diversity, nature, and total microbial biomass) dictate the frequency and nature of gene transfer in this environment (Aminov, 2011). Soil bacteria typically undergo higher rates of gene transfer in areas of higher nutritional content like the rhizosphere, the phyllosphere, decaying plant and animal tissues, and manure-applied soil (van Elsas and Bailey, 2002), and are capable of transformation, transduction, and conjugation.

Transformation is the uptake and incorporation of extracellular DNA from the environment (Chen et al., 2005). Not all bacteria can become "competent" for genetic transformation [although recent reports put the number of naturally transformable strains at more than 60 (Johnsborg et al., 2007)], but both environmental organisms and clinical pathogens use this method of gene acquisition. Environmental bacteria that undergo natural transformation include species of *Pseudomonas* (most studied of which is *P. stutzeri*), species of *Acinetobacter* (notably *A. baylyi*), and the plant pathogens *Ralstonia solanacearum* and *Xylella fastidiosa* (reviewed in Seitz and Blokesch, 2012). The mineral/particulate composition of soil has been implicated in protecting naked DNA against degradation on several occasions, making transformation a viable method of gene transfer in the environment (Aminov, 2011). Transformation may also play a role in the dissemination of integron-based resistance both in the environment and in the clinic: in a study using the naturally competent environmental organism *Acinetobacter baylyi*, Domingues et al. (2012) found that DNA from integron-carrying strains of *Acinetobacter, Citrobacter, Enterobacter, Escherichia, Pseudomonas*, and *Salmonella* could confer antibiotic resistance as well as transfer integrons and transposons in a 24 h period. Furthermore, the transformation of *Acinetobacter baylyi* occurred with equal efficiency using the supernatants from heat-killed bacterial cultures; DNA purity was not important for transformation. This study is important in that it demonstrates that environmental bacteria have the capacity to acquire and replicate genes from bacteria found in clinical settings; although it does not show an historical association between antibiotic producers and the origins of clinical resistance, it demonstrates the capacity for genetic exchange between these two communities.

Transduction involves the transfer of genes via bacteriophages, and is likely an important mechanism of HGT in the environment. Bacteriophage found in soil display local tropisms, and become highly adapted to the bacteria present in their immediate environment (Vos et al., 2009). Lysogenic phage are present in approximately 30% of cultivable soil bacteria, and estimates of prevalence range from 4 to 68% in culture-independent assessments of soil (Ghosh et al., 2008). Generalized transducing phages can be isolated from *Streptomyces* sp. (Burke et al., 2001), and Ghosh et al. (2008) demonstrated that viral preparations from soil carry hybrid 16S ribosomal ribonucleic acid (rRNA) genes indicative of horizontal transfer and recombination within the community. A study of the fecal virome of swine fed the common agricultural antibiotics carbadox and ASP250 revealed that in-feed antibiotics induced prophages from gut bacteria, and induced population shifts in both the bacterial and viral populations. However, metagenomic sequencing revealed that most viromes harbored few antibiotic resistance genes (0.01% of total reads; Allen et al., 2011). In a study of bacteriophage DNA isolated from environmental water samples (including both urban sewage and river water), Colomer-Lluch et al. (2011) found a relative abundance of *bla*(TEM) and *bla*(CTX-M). Interestingly, transduction has been linked to the transfer of pathogenicity islands and virulence traits more often than resistance genes in the clinic: sub-inhibitory concentrations of ciprofloxacin have been reported to promote the mobilization and transfer of the SaPIbov1 pathogenicity island

in *Staphylococcus aureus* via SOS-response-mediated transduction (Ubeda et al., 2005), and fluoroquinolones trigger the expression and dissemination of prophage genes including Shiga toxin in *Escherichia coli* H57:0157, also via SOS (Zhang et al., 2000). Given the enormous diversity of environmental phage and the relatively few viromes that have been sequenced thus far, further metagenomic exploration will provide many more examples of phage-mediated transfer of resistance in both the environment and the clinic.

The most frequent mechanism of horizontal gene transfer for antibiotic resistance genes is on plasmids and integrative conjugative elements (ICEs or conjugative transposons) via conjugation. Conjugative transfer of DNA requires physical contact between donor and recipient cells, and the formation of a pore through which DNA can pass (Thomas and Nielsen, 2005). Plasmids are classified according to their replication and partitioning systems into specific incompatibility (Inc) groups, where two plasmids belonging to the same Inc group cannot co-exist in the same bacterial cell (Shintani et al., 2010b). Antibiotic resistance genes have been found on plasmids belonging to Inc groups P, Q, N, and W, all of which are characterized by a wide host range, including both environmental and pathogenic bacteria (Popowska and Krawczyk-Balska, 2013). Plasmids of subgroup IncP-1 are highly efficient in their ability to spread via conjugation, and are also able to replicate in virtually all Gram-negative bacteria (Shintani et al., 2010a; Popowska and Krawczyk-Balska, 2013). Plasmids belonging to this Inc group often also carry genes conferring resistance against heavy metals (including Ni, Cd, Co, Cu, Hg, Pb, Zn), which can co-select for antibiotic resistance genes. Due to all these factors combined, it has been suggested that spread of multidrug resistance in soil, water and wastewater treatment plants is mainly due to IncP-1 plasmids (Popowska and Krawczyk-Balska, 2013).

Integrative conjugative elements include all self-transmissible integrative and conjugative elements, regardless of their mechanism of integration or conjugation (Wozniak and Waldor, 2010). Unlike plasmids, however, ICEs must integrate into the chromosome to be maintained. They require little sequence specificity for integration, however, and are therefore considered capable of both intracellular and intercellular transfer (Wozniak and Waldor, 2010). Since ICEs are known carriers of antibiotic resistance genes [the first known mobile element with ICE-like properties was Tn*916* carrying tetracycline resistance (Franke and Clewell, 1981)] and have broad-host range, they are potentially important links between resistance in the environment and the clinic. For example, ICE elements have recently been characterized in the genus *Frankia*, a member of the actinobacteria (Ghinet et al., 2011). Members of the actinobacteria are prolific antibiotic producers, and usually also resistant to multiple antibiotics (D'Costa et al., 2006). Gene exchange across genera on broad-host range plasmids has been demonstrated to occur in nutrient rich environments like the rhizosphere: an IncP-1 plasmid was shown to undergo high frequency conjugal transfer in bacteria belonging to the alpha, beta, and gamma Proteobacteria, as well as to *Arthrobacter* sp., a member of the actinobacteria (Molbak et al., 2007). Several low %G+C conjugative plasmids conferring resistance to sulfonamides were recently discovered in manure-spread soil (Heuer et al., 2009). This study showed not only conjugative transfer to *Escherichia coli* from the soil-based host (postulated to be *Acinetobacter* sp.), but highlights gene transfer in an environment in which the soil mobilome mixes frequently with human and animal microbiota. Furthermore, *Acinetobacter baumannii* is an environmental organism and an emerging clinical pathogen (Howard et al., 2012). Plasmids found in environmental species that can also be found in the hospital setting offer a further potential link between the environment and the clinic.

DIVERSITY OF ANTIBIOTIC RESISTANCE ELEMENTS IN WATER ENVIRONMENTS

Antibiotic resistant marine bacteria have been found as far as 522 km offshore and at depths as extreme as 8,200 m (reviewed in Aminov, 2011). Not surprisingly, antibiotic resistance and the presence of plasmids in marine bacteria tends to correlate with the degree of pollution in the environment (Aminov, 2011). Marine environments have been studied both *in situ* using metagenomic sampling methods and under laboratory conditions simulating the natural environment, and undergo all three methods of gene exchange like their counterparts in the soil (for an excellent review, see Aminov, 2011). The importance of water environments for gene exchange is that they are mixing grounds for environmental and clinical organisms. In a study examining the diversity of integron-based resistance genes in freshwater floc, Drudge et al. (2012) used a combination of PCR-based amplification (targeting the conserved regions of type I integrons) and microarray-based detection of resistance genes from the amplicons. This protocol allowed the authors to increase the sensitivity of the assay as well as to provide genomic context to resistance. The effluent from wastewater treatment plants is well-studied for similar reasons, as environmental and clinical organisms mix in this environment in the presence of high levels of pollutants like pesticides, detergents, heavy metals, and antibiotics. Szczepanowski et al. (2009) used a PCR-based approach to identify 140 clinically relevant plasmid-based antibiotic resistance genes in the metagenome of a wastewater treatment plant. In a similar study of wastewater, Parsley et al. (2010) used sequence- and function-based metagenomic approaches to identify resistance determinants from bacterial chromosomes, on plasmids and in viral metagenomes found in activated sludge from a treatment plant. Although these authors did not identify as many resistance genes as Szczepanowski et al. (2009), the diversity of sources of resistance elements emphasizes the broad range of mobile genetic elements present in these environments, and the corresponding broad reach of target organisms.

A WORD ABOUT FITNESS COSTS

Acquiring resistance genes can incur fitness costs, such that a resistance gene may not become fixed in a population unless a positive selective pressure is present (Martinez, 2012). In many soil and water environments, selective pressures are abundant due to regrettable anthropomorphic activities. However, several studies have shown that the introduction of a resistance gene does not necessarily impose a metabolic burden (reviewed in Martinez, 2012). For example, AmpC β-lactamases are usually accompanied

by repressors of their expression on *Salmonella* plasmids (Verdet et al., 2000) which minimize their fitness cost. Antibiotic resistance genes are often found in conjunction with other resistance genes, such that selection for one will maintain the others in the population. Heavy metal resistance genes, and genes coding for the production of siderophores, toxin/antitoxin systems, or bacteriocins are often found on the same mobile elements as resistance genes, which become fixed in the population due to the co-selection of their beneficial counterparts, even in the absence of antibiotic selection.

LINKING THE SOIL TO THE CLINIC

The mobility of resistance genes is well-documented in both the environment and in the clinic, but tangible links between the two remain elusive. The above-mentioned examples of aminoglycoside resistance (Benveniste and Davies, 1973), CTX-M in *Kluyvera* spp. (Humeniuk et al., 2002) and *qnr* in waterborne *Vibrio*, *Shewanella*, and *Aeromonas* (Poirel et al., 2005a,b; Baquero et al., 2008) are conclusive examples of recent exchange between the environmental and clinical resistomes. Comparing the amino acid sequence of the aminoglycoside phosphotransferase APH(3′) between transposons found in Gram-negative and Gram-positive pathogens and from environmental bacteria (*Bacillus circulans* and *Streptomyces fradiae*) also indicates that they have diverged from a common ancestor (Trieu-Cuot and Courvalin, 1986). These examples provide powerful evidence that resistance is able to move from the environment into pathogens, but given the prevalence of resistance genes in environmental reservoirs, certainly more examples of transfer exist?

The acquisition of antibiotic resistance by clinic pathogens from the environment must follow the same rules as all gene transfer events: the most likely place for gene exchange is a niche shared by pathogens and environmental organisms (Wiedenbeck and Cohan, 2011). Sommer et al. (2009) used functional genomics to clone and express DNA from the human microbiome and select for resistance to various antibiotics. The healthy human microbiome is presumably in constant contact with environmental organisms, but is also exposed to pathogenic microorganisms. This approach identified several new resistance factors, but importantly also identified a number of resistance genes harbored in the aerobic gut flora of healthy individuals that are also found in major pathogens (Sommer et al., 2009). Wiedenbeck and Cohan (2011) propose screening for resistance genes in the metagenomes of other organisms from which we frequently acquire pathogens, like agricultural animals, mice, ticks, and mosquitoes, as commensal microorganisms could provide an important link to pathogens.

The most recent evidence for transfer of resistance genes between the environment and the clinic is provided by Forsberg et al. (2012). Using an innovating approach, the authors cultured multi-drug resistant Proteobacteria from the soil to enrich for resistance genes, and a metagenomic library was constructed from the enriched cells (Forsberg et al., 2012). This library was transformed into *Escherichia coli*, and selected on media containing 1 or 12 antibiotics representing sulfonamides, aminoglycosides, phenicols, β-lactams, and tetracyclines at inhibitory concentrations. Resistance was detected against all 12 antibiotics, and 110 resistance genes were identified via homology. Of the genes identified, 18 had 100% identity to entries in GenBank, and a further 32 had ≥90% homology. Importantly, 54% of resistance genes identified by this method were previously unknown, and many of which would not have been predicted to encode resistance genes by sequence alone. Moreover, seven sequences were identified with 100% identity to resistance genes in clinical pathogens, including resistance against β-lactams, tetracyclines, aminoglycosides, sulfonamides, and chloramphenicol. Although the authors could not show definitively that these genes originated in soil organisms due to the nature of their metagenomic approach, these results emphasize the importance of the soil resistome regardless of the direction of gene flow (from soil to clinic, or vice versa).

CONCLUSION

Regardless of methodology, numerous studies have confirmed the fact that antibiotic resistance genes have an environmental reservoir. However, the number and diversity of resistance genes that are found in clinical pathogens is relatively small compared to the diversity in the environment (Martinez, 2012). Why is there such a diversity of resistance genes in the environment? How are they maintained (and what role do they play) outside the clinic? What is the bottleneck preventing transfer of environmental genes to clinical pathogens (and how can we maintain it)? Due to horizontal gene transfer, the microbial world should be considered in a pan-genomic sense, where the selective pressures applied on the environmental microbiome can result in the recruitment and dissemination of resistance genes in clinical pathogens. Mapping both the environmental resistome and the associated mobilome are important steps in slowing the rise of resistant pathogens in the clinic.

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Rise and dissemination of aminoglycoside resistance: the *aac(6′)-Ib* paradigm

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Enzymatic modification is a prevalent mechanism by which bacteria defeat the action of antibiotics. Aminoglycosides are often inactivated by aminoglycoside modifying enzymes encoded by genes present in the chromosome, plasmids, and other genetic elements. The AAC(6′)-Ib (aminoglycoside 6′-*N*-acetyltransferase type Ib) is an enzyme of clinical importance found in a wide variety of gram-negative pathogens. The AAC(6′)-Ib enzyme is of interest not only because of his ubiquity but also because of other characteristics, it presents significant microheterogeneity at the N-termini and the *aac(6′)-Ib* gene is often present in integrons, transposons, plasmids, genomic islands, and other genetic structures. Excluding the highly heterogeneous N-termini, there are 45 non-identical AAC(6′)-Ib related entries in the NCBI database, 32 of which have identical name in spite of not having identical amino acid sequence. While some variants conserved similar properties, others show dramatic differences in specificity, including the case of AAC(6′)-Ib-cr that mediates acetylation of ciprofloxacin representing a rare case where a resistance enzyme acquires the ability to utilize an antibiotic of a different class as substrate. Efforts to utilize antisense technologies to turn off expression of the gene or to identify enzymatic inhibitors to induce phenotypic conversion to susceptibility are under way.

Keywords: antibiotic resistance, aminoglycoside, inhibition, acetyltransferase, mobile elements, integron, transposon

AMINOGLYCOSIDES AND RESISTANCE

Aminoglycosides are bactericidal antibiotics that affect translation fidelity and, according to recent data, they may also stimulate the production of highly deleterious hydroxyl radicals (Vakulenko and Mobashery, 2003; Magnet and Blanchard, 2005; Jana and Deb, 2006; Kohanski et al., 2007; Majumder et al., 2007). Aminoglycosides are used to treat infections caused by gram-negative bacilli and, in combination with β-lactams or vancomycin, to treat some gram-positive pathogens, mainly staphylococci (Yao and Moellering, 2007). Since a step in the uptake process requires functional respiration, the spectrum of action of aminoglycosides is limited to aerobic bacteria (Muir et al., 1984). In addition to their most common uses, aminoglycosides can be utilized to treat diseases such as tuberculosis (Menzies et al., 2009; Brossier et al., 2010), plague, tularemia, brucellosis, endocarditis caused by enterococci, and others (Vakulenko and Mobashery, 2003; Yao and Moellering, 2007; Ramirez and Tolmasky, 2010). The fact that aminoglycosides also cause a decrease in eukaryotic translational fidelity permitted to initiate efforts to developed them as drugs to treat nonsense mutation related genetic disorders such as cystic fibrosis and Duchenne muscular dystrophy (Rich et al., 1990; Kellermayer, 2006; Hermann, 2007; Kondo et al., 2007; Zingman et al., 2007; Bidou et al., 2012; Kandasamy et al., 2012). A chemical labyrinthectomy using intratympanic injection of aminoglycosides is used when most treatments of Ménière's disease fail (Huon et al., 2012; Pacheu-Grau et al., 2012). Aminoglycoside-based drugs are also inhibitors of reproduction of the HIV virus, a property that could result in their utilization in the treatment of AIDS patients (Houghton et al., 2010).

The basic chemical structure of aminoglycosides is characterized by the presence of an aminocyclitol nucleus (streptamine, 2-deoxystreptamine, or streptidine) linked to amino sugars through glycosidic bonds. However, other compounds with different basic structures are also included within the aminoglycosides family, e.g., spectinomycin, an aminocyclitol not linked to amino sugars or compounds containing the aminocyclitol fortamine (Bryskier, 2005). They reach the cytoplasm of the bacterial cell in a three-step process, of which the first one is energy-independent and the following two are energy-dependent (Taber et al., 1987; Vakulenko and Mobashery, 2003; Ramirez and Tolmasky, 2010). At the molecular level, the action of aminoglycosides is characterized by interactions between the antibiotic molecule and the 16S rRNA. Although for all aminoglycosides the effect of this interaction is a change of conformation of the decoding A site producing one that resembles the closed state induced by interaction between cognate tRNA and mRNA, it must be noted that not all aminoglycosides seem to bind the same sites of the 16S rRNA. The consequence of the conformational change induced by the interaction 16S rRNA-aminoglycoside is the reduction of the proofreading capabilities of the ribosome, which in turns results in high levels of mistranslation (Bakker, 1992; Busse et al., 1992; Vakulenko and Mobashery, 2003; Vicens and Westhof, 2003; Magnet and Blanchard, 2005; Majumder et al., 2007; Zaher

and Green, 2009; Ramirez and Tolmasky, 2010). Other molecular effects of some aminoglycosides have been described but it is not clear if some of them are not secondary to protein mistranslation. They include inhibition of 30S ribosomal subunit assembly, induction of RNA cleavage, or interference with the action of RNase P (Mikkelsen et al., 1999; Mehta and Champney, 2003; Belousoff et al., 2009).

Aminoglycosides are powerful tools against infections (Labaune et al., 2001; Avent et al., 2011) but unfortunately the levels of resistance are growing and in consequence failure of treatments with aminoglycosides is becoming more common (Galani et al., 2002; van 't Veen et al., 2005; Tolmasky, 2007a; Soler Bistue et al., 2008). Bacteria have developed numerous mechanisms to resist the action of aminoglycosides and cells can possess the genetic determinants for several of them enhancing the levels of resistance and making it very difficult to overcome all of them. Enzymatic inactivation by acetylation, adenylylation, or phosphorylation at different locations of the aminoglycoside molecule is among the most clinically relevant strategies bacteria use to resist the action of these antibiotics (Shaw et al., 1993; Vakulenko and Mobashery, 2003; Tolmasky, 2007a; Ramirez and Tolmasky, 2010; Chen et al., 2011; Chiang et al., 2013). The enzymes that catalyze these reactions are collectively known as aminoglycoside modifying enzymes. Other well studied mechanisms are: (1) mutation of the 16S rRNA or ribosomal proteins modify the target eliminating or reducing the interaction with the antibiotic molecule (O'Connor et al., 1991); (2) methylation of 16S rRNA, a mechanism found in most aminoglycoside-producing organisms and in clinical strains (Schmitt et al., 2009; Wachino and Arakawa, 2012); (3) reduced permeability to the antibiotic molecule by modification of the permeability of the outer membrane or diminished inner membrane transport (Hancock, 1981; Taber et al., 1987; Macleod et al., 2000; Over et al., 2001); (4) export outside the cell by active efflux pumps (Hocquet et al., 2003; Morita et al., 2012; Wachino and Arakawa, 2012); (5) sequestration by tight binding to a low active aminoglycoside acetyltransferase (Magnet et al., 2003); and (6) extracellular DNA shielding in biofilms (Chiang et al., 2013).

The general characteristics of all known aminoglycoside modifying enzymes have been recently reviewed (Ramirez and Tolmasky, 2010). This review will focus on the aminoglycoside 6′-*N*-acetyltransferase type Ib [AAC(6′)-Ib], which is of great clinical relevance and it is found in over 70% of AAC(6′)-I-producing gram-negative clinical isolates (Vakulenko and Mobashery, 2003), and has been the subject of numerous studies (Tolmasky, 2007a; Cambray and Mazel, 2008; Ramirez and Tolmasky, 2010).

THE AAC(6′)-Ib PROTEIN

The aminoglycoside *N*-acetyltransferases (AAC) belong to the GCN5-related N-acetyltransferase superfamily, also known as GNAT. This is a large group of enzymes that includes about 10,000 proteins from all kinds of organisms that share the property to catalyze the acetylation of a primary amine in numerous acceptor molecules using acetyl CoA as donor substrate (Neuwald and Landsman, 1997; Dyda et al., 2000; Vetting et al., 2005). The AACs are subdivided in groups based on the position where the acetyl group is transferred in the acceptor aminoglycoside molecule. Known AACs catalyze acetylation at the 1 [AAC(1)], 3 [AAC(3)], 2′ [AAC(2′)], or 6′ [AAC(6′)] positions (Shaw et al., 1993; De Pascale and Wright, 2010; Ramirez and Tolmasky, 2010). AAC(6′) enzymes are the most numerous group of AACs, more than 40 have been described, and can be found in gram-negatives as well as gram-positives (Shaw et al., 1993; Miller et al., 1997; Wright, 1999; Tolmasky, 2007a; Ramirez and Tolmasky, 2010). AAC(6′) enzymes are subdivided in two groups, AAC(6′)-I and AAC(6′)-II, which are differentiated by the profile of the aminoglycosides inactivated. With a few exceptions, AAC(6′)-I enzymes specify resistance to several aminoglycosides plus amikacin and gentamicin C1a and C2 but not to gentamicin C1 (Shaw et al., 1993). On the other hand, AAC(6′)-II enzymes catalyze acetylation of all forms of gentamicin but not of amikacin (Rather et al., 1992). In addition, enzymes with extended spectrum that may merit addition of new subclasses of AAC(6′)-I enzymes have been recently described (Casin et al., 2003; Robicsek et al., 2006; Strahilevitz et al., 2009). Phylogenetic analyses divided the AAC(6′) enzymes into three clades. However, with the information available it is still not clear if all AAC(6′) enzymes evolved from a single origin or the three groups are less related and the 6′ acetylating activity has evolved independently at least three times (Salipante and Hall, 2003). According to the phylogenetic analyses recently communicated by Salipante and Hall (Salipante and Hall, 2003) the AAC(6′)-Ib is most closely related to AAC(6′)-IIa AAC(6′)-IIb, AAC(6′)-IIc, and AAC(6′)-IId.

There are numerous variants of AAC(6′)-Ib, many of them identified by modifications in the name of the enzyme such as the addition of subscripts or a prime symbol superscript (Cambray and Mazel, 2008; Ramirez and Tolmasky, 2010). However, a large number of versions of the protein, or predicted protein, have all been named AAC(6′)-Ib, which can be a source of confusion or indetermination. These variants mainly differ at the N-terminus, however one should be careful when considering these differences because not in all of them the N-terminus has been experimentally determined (Dery et al., 1997; Casin et al., 2003; Soler Bistue et al., 2006; Maurice et al., 2008). **Table 1** shows a list of the *aac(6′)-Ib* gene versions found in different genetic environments and bacterial species. Some variants differing at the N-termini such as AAC(6′)-Ib$_3$, AAC(6′)-Ib$_4$, AAC(6′)-Ib$_6$, and AAC(6′)-Ib$_7$ have been compared and it was found that they have similar behavior (Casin et al., 1998) but variations as small as one or two amino acids at key positions proved to be of high relevance (**Table 2**). For example, the AAC(6′)-Ib$_{11}$ found in *S. Typhimurium* has L and S residues at positions 118 and 119 as opposed to Q and L or Q and S, the amino acids present at these positions in all previously described enzymes, acquired an extended resistance spectrum that includes all three gentamicin forms (Casin et al., 2003). (Amino acid numbers throughout the text are based on the sequence corresponding to accession number AF479774.) Another example worth mentioning is the AAC(6′)-Ib', originally found in *Pseudomonas fluorescens* BM2687, but previously generated by site-directed mutagenesis in the laboratory (**Table 1**). This protein has a L to S substitution at amino acid 119 that confers the enzyme an AAC(6′)-II profile, i.e., the enzyme confers resistance to gentamicin but

Table 1 | AAC(6′)-Ib variants.

Number	AAC(6′)-Ib enzyme	Gene allele	Genetic localization	Species	Reference
1	NP_608307	*aac(6′)-Ib*	pJHCMW1::Tn*1331*, pKPN4, pMET1::Tn*1331.2*, pKlebpneu15S, pR23::Tn*1331*, pAAC154::ΔTn*1331*, pColEST258, pJHC-MW1, Tn*1332*, class 1 integron, pRMH712::Tn*1331*, SGI1-V::class 1 integron	*K. pneumoniae, En.* spp., *Pseudomonas putida, Proteus mirabilis*	NP_608307, YP_001338668, YP_001928078, YP_001928081, YP_002286819, YP_004455304, YP_006958960, YP_006959190, ZP_14492679 (contig), ZP_14498301 (contig), ZP_14503930 (contig), ZP_14509538 (contig), ZP_14515174 (contig), ZP_14520767 (contig), ZP_14526392 (contig), ZP_14531781 (contig), ZP_14537604 (contig), ZP_14543183 (contig), ZP_14548764 (contig), ZP_14554292 (contig), ZP_14559831 (contig), ZP_14565429 (contig), ZP_14571055 (contig), ZP_14576504 (contig), ZP_14581777 (contig), ZP_14587733 (contig), ZP_14593028 (contig), ZP_14598930 (contig), ZP_19010829 (contig), AAC6_KLEPN, AF479774_5, AAA69747, AAA98404, ABA54975, ABR80438, ACB55476, ACB55479, ACI63081, ACL36604, ADK35766, AED98720, AED99555, AEG74535, AEW43367, EJJ31842 (contig), EJJ31842 (contig), EJJ31884 (contig), EJJ31888 (contig), EJJ48672 (contig), EJJ48714 (contig), EJJ49532 (contig), EJJ65671 (contig), EJJ65889 (contig), EJJ68295 (contig), EJJ79581 (contig), EJJ81436 (contig), EJJ85464 (contig), EJJ96302 (contig), EJJ96595 (contig), EJK03278 (contig), EJK13161 (contig), EJK16025 (contig), EJK18863 (contig), EJK30890 (contig),

(Continued)

Table 1 | Continued

Number	AAC(6′)-Ib enzyme	Gene allele	Genetic localization	Species	Reference
					EJK33635 (contig), EKV58688 (contig)
2	YP_002286969	*aac(6′)-Ib*	p12::Tn*1331*	*K. pneumoniae, E. coli*	YP_002286969, ZP_16459764 (genomic scaffold), ZP_19016755 (contig), ACI63027, EGB78408 (contig), EKV58524 (contig)
3	AAA26550	*aac(6′)-Ib*	pAZ007	*Serratia marcescens*	AAA26550
4	AAR18814	*aac(6′)-Ib*	pKP31::class 1 integron	*K. pneumoniae*	AAR18814
5	CBI63199	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	CBI63199
6	CBI63201	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	CBI63201
7	CBI63203	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	CBI63203
8	ABG77519	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	ABG77519
9	CBL95252	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	CBL95252
10	CBL95256	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	CBL95256
11	CBI63204	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	CBI63204
12	CBI63202	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	CBI63202
13	YP_003937697	*aac(6′)-Ib*	pETN48::Δclass 1 integron	*E. coli*	YP_003937697, CBX36023
14	ADC80806	*aac(6′)-Ib*	pRYC103T24::class 1 integron In4-like, pKSP212::class 1 integron	*E. coli,* uncultured bacterium	ADC80806, AFR44153
15	YP_005797131	*aac(6′)-Ib*	Class 1 integron (Chromosome)	*A. baumannii*	YP_005797131, AEN92376
16	YP_005525242	*aac(6′)-Ib*	Class 1 integron (Chromosome)	*A. baumannii*	YP_005525242, YP_006289231, YP_006848983, ZP_11603605 (contig), ZP_16142456 (contig), ZP_16146111 (contig), EGK45756 (seq0044), AEP05746, AFI94936, EKE64317 (contig), EKE64588 (contig), AFU38752
17	NP_863005	*aac(6′)-Ib*	p1658/97::class 1 integron, class 1 integron (Chromosome), class 1 integron, plasmid In238a	*E. coli, A. baumannii, K. pneumoniae, K. oxytoca, En. cloacae*	NP_863005, YP_001844882, AAO49600, ACZ55927, ACZ64698, AFS33307
18	ADC80825	*aac(6′)-Ib*	pRYC103T24::class 1 integron	*E. coli*	ADC80825

(Continued)

Table 1 | Continued

Number	AAC(6′)-Ib enzyme	Gene allele	Genetic localization	Species	Reference
19	YP_002791392	*aac(6′)-Ib*	pEC-IMP::class 1 integron, pEC-IMPQ::class 1 integron, pb1004::class 1 integron, class 1 integron	*En. cloacae, Salmonella enterica subsp. enterica* serovar *Bredeney, P. aeruginosa*	YP_002791392, YP_002791702, ACF59628, ACO54016, ACO54326, ADF47469
20	AEO50496	*aac(6′)-Ib*	Class 1 integron	*Se. marcescens*	AEO50496
21	ACB41759	*aac(6′)-Ib*	Class 1 integron	*E. coli*	ACB41759
22	BAL45797	*aac(6′)-Ib*	pKPI-6::class 1 integron	*K. pneumoniae*	BAL45797
23	AAC46343	*aac(6′)-Ib*	Class 1 integron	*P. aeruginosa*	AAC46343
24	AAD02244	*aac(6′)-Ib9*	Class 1 integron	*P. aeruginosa*	AAD02244
25	YP_003108195	*aac(6′)-Ib-cr*	pEK516, pEK499, pEC_L8, pUUH239.2	*E. coli, K. pneumoniae*	YP_003108195, YP_003108338, YP_003829182, YP_005351453, ACQ41894, ACQ42045, ADL14076, AET17280
26	ZP_18354173	*aac(6′)-Ib-cr*		*K. pneumoniae*	ZP_18354173 (genomic scaffold), EKF76226
27	ACD56150	*aac(6′)-Ib-cr*	pHS1387::class 1 integron	*Escherichia coli*	ACD56150
28	ADY02579	*aac(6′)-Ib-cr*	Class 1 integron	*Aeromonas media*	ADY02579
29	NP_957555	*aac(6′)-Ib-cr*	pC15-1a, pKP96::class 1 integron, pNDM-MAR, pGUE-NDM, pKDO1, pHe96, pKas96, pECZ6-1::class 1 integron, Class 1 integron, pLC108::class 1 integron, pJIE101,	*Escherichia coli, K. pneumoniae, Kluyvera ascorbata,* mixed culture bacterium, *K. oxytoca, Se. rubidaea, En. cloacae, Aeromonas allosaccharophila, Providencia* spp., *Shigella* spp., *En. aerogenes*	NP_957555, YP_002332851, YP_005352168, YP_006953881, YP_006973732, AAR25030, ABC17627, ABM47029, ABY74389, ACD03312, ACD03322, ACM24788, ACT97328, ACT97332, ACT97345, ACT97681, ACV60575, ADA60222, ADE44336, ADP30789, ADU16107, ADU16118, ADY02556, AEC49701, AEC49704, AEL33522, AEO45791, AEO79936, AEO79967, AEP16466, AER36609, AEU10750, AEU10754, AFB82784, AFC38861, AFI72862, AFV52812, AFV70394
30	1V0C_A	*aac(6′)-Ib*		*Escherichia coli* Chain A, Structure	1V0C_A, 2BUE_A, 2VQY_A
31	YP_006501621		pKOX_R1::class 1 integron, class 1 integorn,	*K. oxytoca, K. pneumoniae*	YP_006501621, AFM57748, AFN35014

(Continued)

Table 1 | Continued

Number	AAC(6′)-Ib enzyme	Gene allele	Genetic localization	Species	Reference
32	ABC54722	*aac(6′)-Ib*	pAS1::InVC117	*Vibrio cholerae*	ABC54722
33	BAE66666	*aac(6′)-Ib*	Class 1 integron	*Vibrio cholerae* O1	BAE66666
34	YP_007232190	*aac(6′)-Ib*	pPC9	*P. putida*	YP_007232190
35	ZP_16084267	*aac(6′)-Ib*	Class 1 integron (Chromosome)	*A. baumannii*	ZP_16084267 (contig), ZP_16086960 (contig), ZP_16140385 (contig), EKA73751 (contig), EKK08901 (contig), EKK18976 (contig)
36	AFS51540	*aac(6′)-Ib9*	pKS208::class 1 integron	Uncultured bacterium	AFS51540
37	YP_006957899	*aac(6′)-Ib-cr4*	pMdT	*SS. enterica* subsp. *enterica serovar* Typhimurium	YP_006957899, AFU63391
38	ADZ96942	*aac(6′)-Ib-cr*	Plasmid	*K. pneumoniae*	ADZ96942
39	CAA42873	*aac(6′)-Ib3, aac(6′)-Ib5*	plasmid pCFF04	*P. aeruginosa*	CAA42873
40	AAB24284	*aac(6′)-Ib4*	pSP21::class 1 integron, pEI1573::class 1 integron	*Se.* spp., uncultured bacterium, *En. cloacae*	AAB24284, YP_006941442, YP_006965430
41	AAN41403	*aac(6′)-Ib11*	pSTI1::class 1 integron	*SS. enterica* subsp. *enterica* serovar Typhimurium	AAN41403
42	YP_006903338	*aac(6′)-Ib*	pNDM102337::class 1 integron, pNDM10505::class 1 integron	*Escherichia coli*	YP_006903338, YP_006953195
43	YP_006959139	*aac(6′)-Ib*	pNDM10469::class 1 integron	*K. pneumoniae*	YP_006959139
44		*aac(6′)-Ib7*	Plasmid	*En. cloacae, Citrobacter freundii*	Not available
45		*aac(6′)-Ib8*	Plasmid	*En. cloacae*	Not available

The pJHCMW1-encoded AAC(6′)-Ib variant (accession number NP_608307) was subjected to BLASTP and the identical proteins were identified. Those that were closely related but not identical were identified by numbers. The names given in the publications or GenBank entries are shown. Those that were named aacA4 were named aac(6′)-Ib here.

not amikacin (Rather et al., 1992; Lambert et al., 1994). A highly surprising effect occurred in the natural variant known as AAC(6′)-Ib-cr, which has the modifications W104R and N181Y (**Tables 1**, **2**). The substrate spectrum was expanded to include quinolone antibiotics, crossing the barrier from the aminoglycosides (Robicsek et al., 2006). Since the first detection of the AAC(6′)-Ib-cr variant there have been numerous reports of its presence, and variants of it, across the world in different genetic environments suggesting an extraordinary ability to disseminate (Quiroga et al., 2007; Cattoir and Nordmann, 2009; Strahilevitz et al., 2009; Rodriguez-Martinez et al., 2011; Ruiz et al., 2012; De Toro et al., 2013). Furthermore, there have been cases where a strain was found to simultaneously include genes coding for AAC(6′)-Ib and AAC(6′)-Ib-cr (Kim et al., 2011). AAC(6′)-Ib is also found fused to the C-terminal end of AAC(3)-Ib protein within a class I integron found in a *Pseudomonas aeruginosa* strain (Dubois et al., 2002) and to the C-terminus of the AAC(6′)-30 also within a *P. aeruginosa* class I integron (Mendes et al., 2004).

Subcellular localization studies of the AAC(6′)-Ib enzyme encoded by Tn*1331* showed that the enzyme is homogeneously distributed in the cytoplasmic compartment (Dery et al., 2003).

Table 2 | Phenotypes of representative mutants of AAC(6′)-Ib.

Mutation[a]	AAC(6′)-Ib variant name	Phenotype	References
Y80C		S	Panaite and Tolmasky, 1998
D117A		S	Pourreza et al., 2005
L119S	AAC(6′)-Ib′, AAC(6′)-Ib$_7$, AAC(6′)-Ib$_8^b$	Specificity, Gm^r Ak^s	Rather et al., 1992; Lambert et al., 1994; Casin et al., 2003
Q118L, L119S	AAC(6′)-Ib$_{11}$	Specificity, Gm^r Ak^r	Casin et al., 2003
L120A		S	Pourreza et al., 2005
Y166A		Specificity, Ak^s Km^r	Shmara et al., 2001
E167A		S	Shmara et al., 2001
F171A		S	Shmara et al., 2001
F171L		Thermosensitive for Ak and Nm	Panaite and Tolmasky, 1998; Shmara et al., 2001
W104R, D181Y	AAC(6′)-Ib-cr	Expanded substrate spectrum including quinolones	Robicsek et al., 2006

S, susceptible.

[a] Numbering from sequence in accession number AF479774.

[b] The proteins differ at the amino terminus.

AAC(6′)-Ib was one of three aminoglycoside modifying enzymes used in a study consisting of molecular dynamics simulations of the enzymes and aminoglycoside ribosomal RNA binding site, unliganded, and complexed with an aminoglycoside, kanamycin A. These studies concluded that the enzymes efficiently mimic the nucleic acid environment of the ribosomal RNA binding cleft (Romanowska et al., 2013). Extensive studies using mutagenesis showed some interesting phenotypes such as modifications in specificity, enhanced activity, or selective thermosensitivity (**Table 2**) (Panaite and Tolmasky, 1998; Chavideh et al., 1999; Shmara et al., 2001; Casin et al., 2003; Pourreza et al., 2005; Kim et al., 2007; Maurice et al., 2008). In addition, alanine scanning showed that several amino acid substitutions by A had minor effects. These mutagenesis studies together with structural and enzymatic analyses led to a deep understanding of features and characteristics of AAC(6′)-Ib proteins (Rather et al., 1992; Vetting et al., 2004; Maurice et al., 2008; Vetting et al., 2008; Ramirez and Tolmasky, 2010). The three dimensional structure of AAC(6′)-Ib and AAC(6′)-Ib$_{11}$ have been experimentally determined in various conditions. AAC(6′)-Ib was crystallized in complex with coenzyme A and also in complex with both coenzyme A and kanamycin. The structures were solved to 1.8 Å and 2.4 Å resolution, respectively (Maurice et al., 2008). The broad spectrum variant AAC(6′)-Ib$_{11}$ was crystallized in the absence of substrate and the structure was solved to 2.1 Å resolution (Maurice et al., 2008). These studies concluded that AAC(6′)-Ib exists as a monomer while AAC(6′)-Ib$_{11}$ shows monomer/dimer equilibrium (Maurice et al., 2008). This was a somewhat surprising finding considering that previous studies had shown that two other acetyltransferases, AAC(6′)-Ii and AAC(6′)-Iy, exist as dimers (Wright and Ladak, 1997; Wybenga-Groot et al., 1999; Draker et al., 2003; Vetting et al., 2004; Wright and Berghuis, 2007; Vong et al., 2012). Interestingly, analysis of these crystal structures showed the presence of a flexible flap in AAC(6′)-Ib$_{11}$ that may be the basis for its ability to utilize amikacin as well as gentamicin as substrates (Maurice et al., 2008). In another study a molecular model of AAC(6′)-Ib-cr has been generated (Maurice et al., 2008; Vetting et al., 2008), which led to postulate that the D181Y substitution is mainly responsible for modification in the strength of binding of the antibiotic substrate and that the substitution W104R stabilizes the positioning of Y181 (Robicsek et al., 2006; Strahilevitz et al., 2009).

Table 1 shows that there are 45 non-identical AAC(6′)-Ib related entries in the NCBI database, 32 of which have identical name in spite of not having identical amino acid sequence. The N-termini of these proteins show the highest degree of heterogeneity with high variations in length stretching up to 60 amino acids, but these differences were suggested to be irrelevant (Casin et al., 1998; Maurice et al., 2008). Therefore, we defined a highly conserved central region composed of 181 amino acids shared by all proteins, which were compared using the MAFFT alignment algorithm (Katoh and Standley, 2013). Pairwise comparisons show that the sequences have 1 to 8 amino acid differences and a total of 24 positions showed amino acid variations. Moreover, clustering using the UPGMA algorithm (Sneath and Sokal, 1973) defined 18 sequence clusters, 14 of which consist of a singleton, and 4 of which include 2–16 proteins (**Figure 1**). Different clusters can exhibit similar properties while others show substantial differences in their characteristics such as those cases in which there are significant specificity variations like extended substrate spectrum as described in the above paragraphs.

THE *aac(6′)-Ib* GENE

The *aac(6′)-Ib* genes are usually found as fully functional or deficient gene cassettes associated to class 1 integrons, insertion sequences such as IS*26*, and truncated or disrupted integrons (**Figure 2** and **Table 1**) (Sarno et al., 2002; Woodford et al., 2009; Ramirez and Tolmasky, 2010). These genetic elements may be part of plasmids, transposons, genomic islands, or other structures such as the KQ element (Rice et al., 2008), which together contribute to the gene's ability to disseminate at the cellular and molecular levels (Tolmasky, 2007b). When present in integrons, *aac(6′)-Ib* gene cassettes can be found located adjacent to the 5′-conserved region, i.e., flanked by *attI* and *attC*, or internal to the variable portion containing *attC* loci at both ends (**Figure 2**). In both cases, as expected, the gene cassette can be mobilized by the integrase IntI1 (**Figure 2**) (Cambray et al., 2010; Hall, 2012). In addition, a gene cassette-like structure containing *aac(6′)-Ib*, composed of a copy of *attI1** at

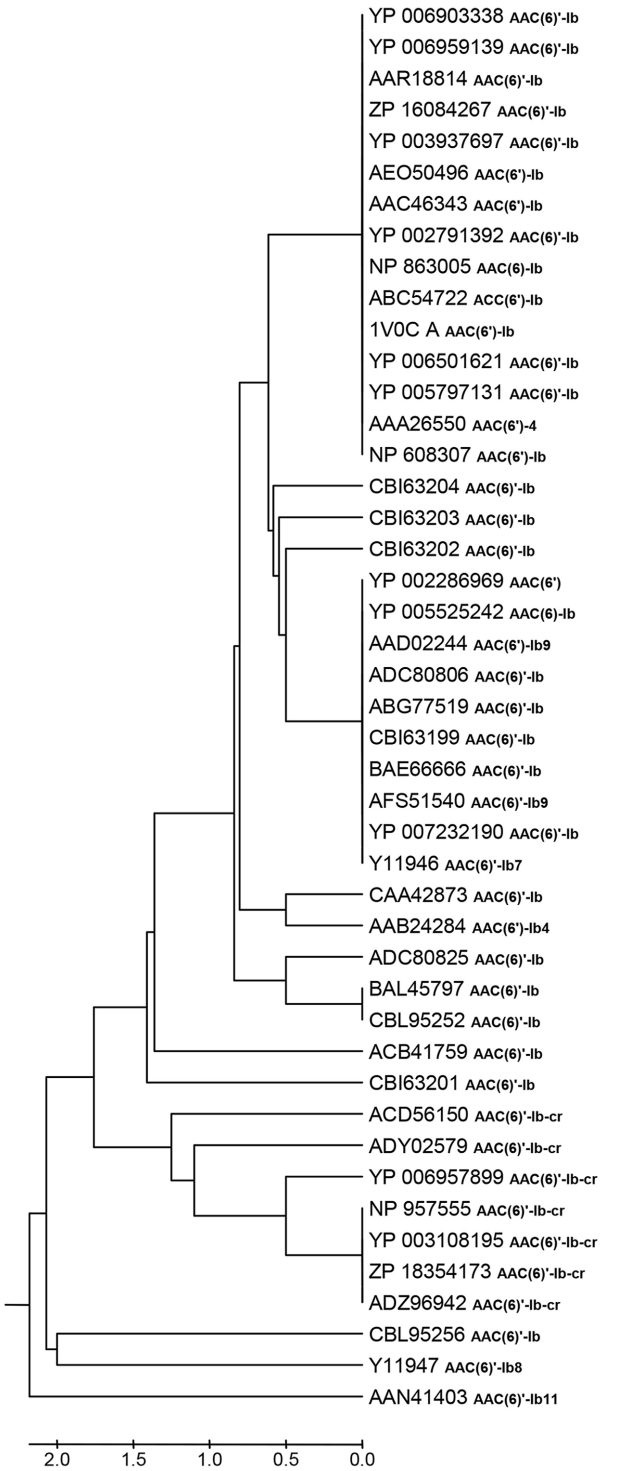

FIGURE 1 | UPGMA clustering analyses of 45 AAC(6′)-Ib protein sequences. The optimal tree with the sum of branch length = 20.70628249 is shown. The evolutionary distances were computed using the number of differences method and are in the units of the number of amino acid differences per sequence. All positions containing gaps and missing data were eliminated. There were a total of 181 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

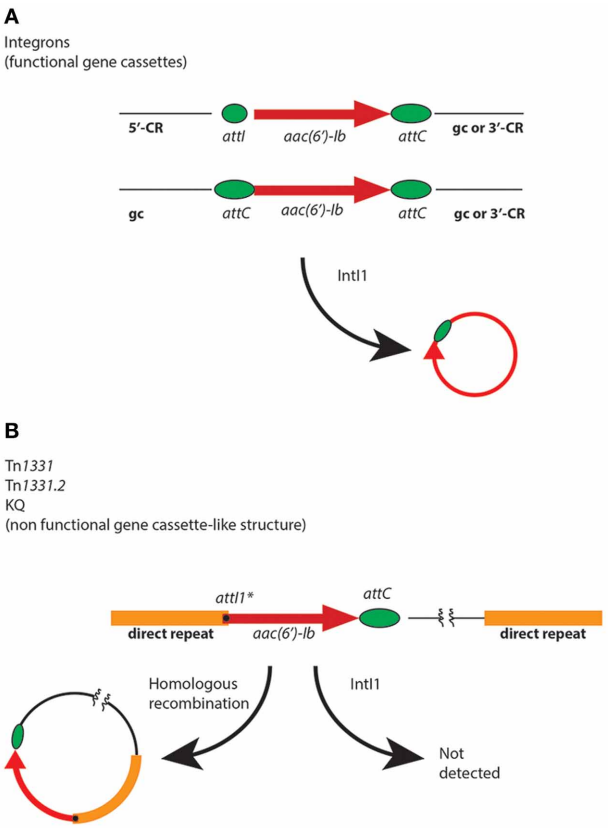

FIGURE 2 | Mobilization of *aac(6′)-Ib*. (A) Generic genetic maps of integrons in which an *aac(6′)-Ib* gene cassette is located immediately following the 5′ conserved region (5′-CR) (top map) or following one or more gene cassettes (gc) inside the variable portion, and followed by other gene cassettes or the 3′ conserved region (3′-CR) (bottom map). The small green ellipse represents *attI* and the big green ellipses represent *attC*. **(B)** Relevant portion of the Tn*1331*, Tn*1331.2*, and KQ elements (Tolmasky and Crosa, 1987; Tolmasky et al., 1988; Sarno et al., 2002; Rice et al., 2008). For clarity Tn*1332*, which has a more complicated structure in its direct repeats (Poirel et al., 2006), is not shown, but it could experience mobilization by homologous recombination as shown. The black dot represents *attI1**. The homologous recombination pathway for generation of an *aac(6′)-Ib*-containing circular molecule has been proposed by Zong et al. (2009).

the beginning of the structural gene and a regular *attC* downstream of it (see **Figure 2**), was found as part of a region resembling the variable portion of integrons in Tn*1331* (Woloj et al., 1986; Tolmasky et al., 1988; Tolmasky, 1990; Tolmasky and Crosa, 1993; Sarno et al., 2002), Tn*1331.2* (Tolmasky and Crosa, 1991), Tn*1332* (Poirel et al., 2006), the KQ element (Rice et al., 2008), a Tn*1331* derivative recently isolated from a clinical *Klebsiella pneumoniae* strain belonging to the ST512, which derived from the ST258, known to be spread worldwide (Chen et al., 2012; Garcia-Fernandez et al., 2012; Warburg et al., 2012), and a complex mosaic region present in the chromosome of *Proteus mirabilis* JIE273 (Zong et al., 2009). Assays overexpressing IntI1 in cells containing Tn*1331* were unable to detect any excision of the *aac(6′)-Ib* gene cassette-like structure,

suggesting that it is nonfunctional or it excises at an extremely low efficiency (Ramirez et al., 2008). Interestingly, despite the IntI1-mediated lack of mobility of this DNA region, the gene could be mobilized by means of a mechanism recently proposed by Zong et al. that occurs through homologous recombination between 520-bp direct repeats located upstream and downstream of the gene cassette-like structure (**Figure 2**) (Zong et al., 2009).

These multiple locations taken together with the ability of the genetic elements to spread at the molecular and cellular level provide *aac(6′)-Ib* genes with the capability to reach virtually all gram-negatives and other undetermined bacteria such as those that are still unculturable. The gene has also been found in plasmids harboring resistance genes of high importance such as the recently described *ndm-1* (Yong et al., 2009; Bonnin et al., 2012; Villa et al., 2012).

INHIBITION

The rise in drug resistance affects all known antibiotics and has been identified as one of the greatest threats to human health. Therefore, there is an immediate need for new agents with activity against multiresistant bacteria and at the present moment there is no evidence that this need will be fully met in the near future (Boucher et al., 2009). A group of pathogens that cause the majority of hospital infections, named ESKAPE (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, P aeruginosa,* and *Enterobacter*), is becoming highly resistant to antibiotics including aminoglycosides (Rice, 2008). They carry aminoglycoside modifying enzymes genes, and one of the most common in the gram-negative members is *aac(6′)-Ib* (Ramirez and Tolmasky, 2010; Shaul et al., 2011; Herzog et al., 2012). An obvious solution to this problem would be the development of new aminoglycosides, a strategy that is being pursued using numerous approaches (Green et al., 2010, 2011; Houghton et al., 2010). A variety of new aminoglycoside derivatives including chemical modification of existing aminoglycosides, aminoglycoside dimers, or aminoglycoside-small molecule conjugated are being produced and tested (reviewed in Houghton et al., 2010). In particular plazomicin (ACHN-490), a novel neoglycoside derived from sisomicin that carries a hydroxymethyl group at position 6′, has shown enhanced activity against multiresistance gram-negatives and gram-positives including strains carrying *aac(6′)-Ib* (Endimiani et al., 2009; Landman et al., 2011).

Others are approaching the problem in such a way that the existing aminoglycosides continue to be effective by designing enzymatic inhibitors that can act in combination with the antibiotic, mimicking the strategy successfully used to curb resistance to β-lactams (Williams and Northrop, 1979; Daigle et al., 1997; Haddad et al., 1999; Liu et al., 2000; Burk and Berghuis, 2002; Boehr et al., 2003; Draker et al., 2003; Gao et al., 2005, 2006; Welch et al., 2005; Lombes et al., 2008; Magalhaes et al., 2008; De Pascale and Wright, 2010; Drawz and Bonomo, 2010; Green et al., 2012; Vong et al., 2012). However, these efforts are still scarce when one compares them to those invested to discover and design β-lactamase inhibitors. Furthermore, the attempts to find inhibitors of AAC(6′)-Ib have only yielded a compound, synthesized using non-aminoglycoside-like fragments, with a rather modest level of inhibition of AAC(6′)-Ib (Lombes et al., 2008).

An alternative approach that is being explored is silencing expression of the resistance gene. Early attempts at interfering with expression of *aac(6′)-Ib* consisted of identifying regions available for interaction with antisense oligonucleotides in a monocistronic *in vitro* synthesized mRNA by RNase H mapping in combination with computer prediction of its secondary structure (Sarno et al., 2003). The selected sites were used as targets for a collection of oligodeoxynucleotides, of which some had the ability to induce RNase H-mediated *in vitro* degradation of the mRNA, inhibited *in vitro* synthesis of the enzyme in coupled transcription/translation assays, and upon delivery by electroporation significantly reduced the number of cells surviving after exposure to amikacin (Sarno et al., 2003). The mechanism of this *in vivo* inhibition is most probably through RNase H digestion of the mRNA, but other possibilities such as steric hindrance cannot be discarded at this time. Alternatively, modest but significant inhibition of expression of *aac(6′)-Ib* was achieved by applying EGS technology, in which short antisense RNA molecules, known as external guide sequences, are used to elicit RNase P-mediated degradation of a target mRNA (Guerrier-Takada et al., 1997; Lundblad and Altman, 2010). Initially, *E. coli* harboring *aac(6′)-Ib* were transformed with recombinant clones specifying the appropriate RNA oligonucleotide sequences under an inducible promoter. The transformed derivatives were then cultured in the presence of amikacin under conditions of expression of the external guide sequences. The results showed that in a few cases the external guide sequences induced a reduction of the minimal inhibitory concentration of amikacin (Soler Bistue et al., 2007). These results were considered proof of concept, but the strategy was not viable because antisense oligonucleotides must be added from the milieu and find their way inside the cells without being degraded. Thus, nuclease resistant oligonucleotide analogs that still induce inhibition of gene expression by RNase P activation had to be found. Out of a variety of oligoribonucleotide analogs including 2′-*O*-methyl oligoribonucleotides, phosphorodiamidate morpholino oligomers, phosphorothioate oligodeoxynucleotides, or locked nucleic acids (LNA)/DNA co-oligomers that were tested, LNA/DNA co-oligomers with certain configurations were found to be capable of eliciting RNase P-mediated cleavage of mRNA *in vitro* (Soler Bistue et al., 2009). Following this finding, a selected LNA/DNA co-oligomer was added to the hyperpermeable *E. coli* AS19 harboring *aac(6′)-Ib* and it was found that growth was inhibited in the presence of amikacin, indicating that the compound may have induced RNase P-mediated inhibition of expression of the gene (Soler Bistue et al., 2009). These results were encouraging but it must be noted that inhibition of expression of *aac(6′)-Ib* is still far from being a viable option to overcome aminoglycoside resistance in the clinical setting. Several problems remain to be solved like inducing penetration of the oligonucleotide analogs inside wild type cells in enough quantities to exert the biological activity or achieve enough inhibition levels in spite of the usual presence of multiple copies of the gene due to its inclusion in high copy number plasmids. Toward finding solutions to

these problems, recent experiments suggest that LNA/DNA co-oligomers may be able to reach the cytoplasm of untreated cells at low efficiency (Traglia et al., 2012). Strategies will have to be developed to increase the efficiency of delivery inside bacterial cells.

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Comparison of multi-drug resistant environmental methicillin-resistant *Staphylococcus aureus* isolated from recreational beaches and high touch surfaces in built environments

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Over the last decade community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a major cause of disease in the general population with no health care exposure or known classical risk factors for MRSA infections. The potential community reservoirs have not been well defined though certain strains such as ST398 and USA300 have been well studied in some settings. MRSA has been isolated from recreational beaches, high-touch surfaces in homes, universities, and other community environmental surfaces. However, in most cases the strains were not characterized to determine if they are related to community-acquired or hospital-acquired clinical strains. We compared 55 environmental MRSA from 805 samples including sand, fresh, and marine water samples from local marine and fresh water recreational beaches ($n = 296$), high touch surfaces on the University of Washington campus ($n = 294$), surfaces in UW undergraduate housing ($n = 85$), and the local community ($n = 130$). Eleven USA300, representing 20% of the isolates, were found on the UW campus surfaces, student housing surfaces, and on the community surfaces but not in the recreational beach samples from the Northwest USA. Similarly, the predominant animal ST133 was found in the recreational beach samples but not in the high touch surface samples. All USA300 isolates were multi-drug resistant carrying two to six different antibiotic resistance genes coding for kanamycin, macrolides and/or macrolides-lincosamides-streptogramin B, and tetracycline, with the majority (72%) carrying four to six different antibiotic resistance genes. A surprising 98% of the 55 MRSA isolates were resistant to other classes of antibiotics and most likely represent reservoirs for these genes in the environment.

Keywords: MRSA, environmental, recreational beaches, high touch surfaces, USA300

INTRODUCTION

Staphylococcus aureus is part of the normal flora and can be found in the anterior nares as well as on the skin, axilla, perineum, and pharynx. It has been estimated that 25–35% of healthy humans in the general community have *S. aureus* in their anterior nares (Grundmann et al., 2006). However the range varies depending on the population examined and when the study was done. It is now thought that ∼20% of population almost always carries *S. aureus*, another 20% rarely carries *S. aureus*, and the remaining 60% of the population is intermittently colonized (Kluytmans et al., 1997).

Methicillin-resistant *S. aureus* (MRSA) was first identified over 50 years ago and has become a major nosocomial and community pathogen. MRSA strains are *S. aureus* that have a *mecA* gene which codes for a unique penicillin-binding protein that has decreased affinity for β-lactams. This protein allows for cell growth in the presence of penicillins and other β-lactam antibiotics, which are the antibiotics of choice for staphylococcal skin and soft tissue infections (Eady and Cove, 2003; Grundmann et al., 2006). Over the last decade community-acquired MRSA (CA-MRSA) causing primarily skin and soft tissue infections has emerged as a major cause of disease in the general population with no health care exposure or known classical risk factors for MRSA infections around the world (King et al., 2006). In 2005 there were ∼19,000 deaths due to MRSA in hospitalized USA patients. More importantly three fourth of the US MRSA infections were in people that had not been previously hospitalized, had no contact with hospitals, and were part of the general community (Klevens et al., 2007). CA-MRSA morbidity and mortality per 100,000 people have been estimated at 4.6 and 0.5, respectively (Klevens et al., 2007). CA-MRSA strains initially from community patients are now entering the hospital environments to become the predominant nosocomial MRSA isolates (Popovich et al., 2008). In North America, most CA-MRSA disease is due to a single strain, USA300, which produces a number of toxins and has the ability to cause skin and soft tissue infections in otherwise healthy individuals and appears to be more robust on fomites (Desai et al., 2011). In other geographical locations, CA-MRSA can be a variety of different strains (Nimmo and Coombs, 2008; Bartels et al., 2009). Originally CA-MRSA causing disease

was less likely to be resistant to other antimicrobial agents than MRSA strains isolated from the high-antibiotic selection pressure of the hospital environment (Eady and Cove, 2003).

Staphylococcus aureus and MRSA can be transmitted from people-to-people, from fomites-to-people, and from air-to-people (Huang et al., 2006; Zuckerman et al., 2009). People colonized or infected with *S. aureus* or MRSA shed into their environments contaminating surfaces and fomites at concentrations sufficient for survival for extended periods of time in the environment which should allow for transfer to skin, clothing, and other fomites (Boyce et al., 1997; Otter et al., 2011).

Environmental reservoirs associated with healthcare settings and closed communities such as daycares, schools, prisons, and sport teams have been examined (Centers for Disease Control and Prevention, 2001; Eady and Cove, 2003; Nguyen et al., 2005; Roberts et al., 2011a,b,c). *S. aureus* and MRSA contamination in the marine environment have been examined including shedding from recreational bathers, untreated wastewater, and urban runoff (Selvakumar and Borst, 2006; Elmir et al., 2007; Börjesson et al., 2009; Plano et al., 2011). Over the past several years, *S. aureus* and MRSA have been isolated from marine water, stream water, and intertidal sand samples from beaches in California (Goodwin and Pobuda, 2009), Florida (Abdelzaher et al., 2010), Hawaii (Tice et al., 2010; Viau et al., 2011), and the Pacific Northwest (Soge et al., 2009). MRSA spatial distribution or strain characterization was not addressed in the studies by Goodwin and Pobuda (2009) in California and Tice et al. (2010) in Hawaii. A more recent study sampled fresh water streams draining into coastal beaches on O'ahu Hawaii found *S. aureus* (Viau et al., 2011). Temporal and spatial distribution of pathogenic microorganisms including *S. aureus* was examined in a Florida study (Abdelzaher et al., 2010), however none of the presumptive *S. aureus* isolates were biochemically confirmed as *S. aureus*. The study by Soge et al. (2009) identified MRSA and/or *S. aureus* isolated in 2008, from 6 of 10 marine beaches from the Pacific Northwest, however the number of samples taken was limited. That study found that the MRSA isolates were related to strains previously associated with hospital-acquired MRSA (five SSC*mec* type I, and one non-typeable and ST types 30, 45, 59, 1405), and the *S. aureus* isolates (ST15, ST30, ST59) suggested that they are related to hospital-acquired MRSA. ST5, ST30, ST45, ST59 were all major clonal complexes found in the United States (Schwalm et al., 2011).

In this current paper we compare 55 environmental MRSA isolates, including 11 USA300, collected from 805 samples taken from fresh and marine beaches, high touch surfaces within the community and the University of Washington. This represents the first comparison of characterized MRSA isolates from different natural and built environments.

MATERIALS AND METHODS

BACTERIAL ISOLATES

All samples from recreational marine or fresh water beaches, and swabs from the environmental surfaces were enriched in Bacto® m *Staphylococcus* broth (1.5×) (Difco Laboratories, Sparks, MD, USA) supplemented with a final concentration of 75 μg/L polymyxin B and 0.1% potassium tellurite (Sigma Co., St. Louis, MO, USA) and 50 ml of media was used for wash clothes used for sampling the washing machines as previously described (Roberts et al., 2011c; Levin-Edens et al., 2012). All samples were incubated in 5% CO_2 at 36.5°C until turbid and black. Positive samples were diluted and plated onto Bacto® *Staphylococcus* Medium 110 (Difco) supplemented with 10 μg/ml methicillin and 0.01% potassium tellurite and Bacto® Mannitol Salts Agar (Difco), and the resulting strains were biochemically verified as *S. aureus* and confirmed as *mecA* positive by PCR assay as previously described (Roberts et al., 2011c; Levin-Edens et al., 2012). Twenty-two MRSA isolated from fresh water draining into recreational marine or fresh water beaches, seven MRSA isolates collected from sand, and two MRSA isolates collected from marine water identified from sand, fresh, and marine water samples from local marine and fresh water recreational beaches ($n = 296$) in 2010 (Levin-Edens et al., 2012) were included in the study. An additional 24 MRSA isolates from frequently touched 509 non-hospital environmental surfaces taken at a large university ($n = 294$), student homes ($n = 85$), and local community sites ($n = 130$) collected 2009–2010 (Roberts et al., 2011c) were included in the comparison (**Table 1**).

CHARACTERIZATION OF MRSA STRAINS

The SCC*mec* typing was done by multiplex PCR assay for types I–V using positive controls. SCC*mec* typing classifies the SCC*mec* elements based on their structural differences and has been used to discriminate MRSA strains and define MRSA clones[1]. Those that were not SCC*mec* type I–V were labeled as non-typeable (NT) (Soge et al., 2009). The MLST was previously determined by PCR and sequencing of the PCR products for the seven housekeeping genes (Soge et al., 2009). Alleles were assigned by a comparison of their sequences with the corresponding loci in the *S. aureus* MLST database and combined into an allelic profile with unique sequence type (ST)[2]. Drug resistance genes including kanamycin resistance gene, *aadD*, macrolide-lincosamide-streptogramin B resistance genes *erm*(A), *erm*(B), *erm*(C), and macrolide-streptogramin B resistant *msr*(A) gene, and tetracycline resistance genes *tet*(M) and *tet*(K) were previously determined by PCR assays (Soge et al., 2009). These specific antibiotic resistance genes were selected because they have previously been identified in MRSA isolates or have been associated with SCC*mec* elements (Soge et al., 2009; McDougal et al., 2010; Roberts et al., 2011a,b,c; Levin-Edens et al., 2012). Pulsed-field gel electrophoresis (PFGE) analysis was previously done and isolates were compared to four clinical MRSA USA300 strains used as positive controls to determine whether the environmental isolates were related to USA300 clone (McDougal et al., 2010).

RESULTS AND DISCUSSION

DISTRIBUTION OF MRSA ISOLATES

Methicillin-resistant *Staphylococcus aureus* was isolated from 10/85 (11.8%) university student homes surfaces and five (63%) of the eight undergraduate student homes had MRSA positive samples. The percentage of MRSA positive samples, which include only samples that yielded MRSA isolates and was a portion of the

[1]http://www.staphylococcus.net/
[2]http://saureus.mlst.net/

samples which were contaminated with *S. aureus*, in our university study was 10-fold higher than the 1.1% MRSA positive found from 1,320 surface samples taken from 35 homes in the Boston area (Scott et al., 2009). Our study had higher number of MRSA positive homes compared to the 9/35 (25.7%) of the homes that were contaminated with MRSA in the Boston study. In the university study, eight (9.4%) methicillin-susceptible *S. aureus* (MSSA) isolates from 3 (37.5%) homes versus the Boston study which found MSSA contaminated surfaces in 34 (97.1%) of 35 Boston homes. However, the demographics of the household members did vary between the current and previous study as did the number of samples per home and the number of homes tested (Scott et al., 2009). Thirty-one (10.5%) of the 296 recreational beach samples were positive for MRSA with 22 isolates (71%) coming from fresh water streams running into the marine and freshwater beaches. Fourteen (4.7%) of the recreational beach samples were *S. aureus* positive with 12 (85.7%) of the isolates coming from the fresh water stream samples draining into the marine beaches. A recent study in Hawaii found *S. aureus* in 19 (86.4%) of 22 fresh water streams with 42 (48.8%) of the 86 samples positive for *S. aureus* and one (5.2%) sample positive for MRSA (Viau et al., 2011). The source of the isolates was not identified nor was potential environmental sources examined or isolates further characterized in the Viau et al. (2011) study. Ten (3.4%) out of 294 university surface samples were MRSA positive, while nine (3.1%) were MSSA. This compares to an earlier study which found no MRSA from 70 high touch surfaces in a large urban US university, though samples from keyboards, telephone mouthpieces, and an elevator button were positive for *S. aureus* (Brooke et al., 2009). Four (3.1%) out of 130 community samples were MRSA positive, and one (0.8%) sample was MSSA positive. The closest community studies for comparison have been from public transport system. A London study found 9 (8%) of the 112 samples taken from hand-touch surfaces in the public transport system and in public areas of a hospital were positive for *S. aureus* but no MRSA was isolated (Otter and French, 2009). A recent study from Japan where environmental samples were collected from 2008 to 2010 found 8 (2.3%) of the 349 trains examined were positive for MRSA (Iwao et al., 2012).

Eleven (20%) of the 55 isolates had PFGE patterns that indicated that they were USA300 strains, the most prevalent community-acquired MRSA in the United States (Tenover and Goering, 2009). However in other parts of the world other strains of CA-MRSA have been identified (Nimmo and Coombs, 2008; Bartels et al., 2009). The USA300 isolates were not evenly distributed in all the environments sampled since all 11 came from samples in built environmental high touch surfaces (student housing 6/10; university 4/10; and community 1/4). Three different MLST types were associated with the USA300 PFGE pattern including ST5, ST8, and ST30 (**Table 1**).

DRUG RESISTANCE CHARACTERIZATION

Originally, CA-MRSA isolates were less likely to be resistant to other classes of antibiotics, however over the last decade USA300 isolates have become increasingly resistant to fluoroquinolones with 54% resistant by 2006 (Eady and Cove, 2003; McDougal et al., 2010). An early collection of 187 MRSA strains identified 30 USA300 isolates that were multiple-drug resistant and carried macrolide [*msr*(A) gene], and/or macrolide, lincosamide-streptogramin B [*erm*(A), *erm*(C) genes], and/or tetracycline resistance gene [*tet*(K)], and/or levofloxacin resistance due to gyrase mutations (Tenover et al., 2006). While a more recent study of 823 USA300 clinical isolates from the Centers for Disease Control and Prevention (CDC) collection found that 43% of the isolates were resistant to erythromycin and carried the *msr*(A) gene and 43% were resistant to erythromycin due to the presence of the *msr*(A) gene plus levofloxacin resistance due to gyrase mutations, and 8.4% were resistant to tetracycline due to presence of the *tet*(K) gene of which some also carried the *msr*(A) gene and/or the gyrase mutations, leaving just 5.6% susceptible to other classes of antibiotics examined (McDougal et al., 2010). However few groups have characterized the antibiotic resistance patterns of MRSA isolates found in the environment besides the study of Soge et al. (2009). In that study, five MRSA SCC*mec* type I isolates were resistant to one to four classes of antibiotics and all five carried the *erm*(A) gene, one carried both *tet*(K) and *tet*(M) genes, and one carried the *tet*(K) and the other carried the *tet*(M) tetracycline resistance genes. One of the MRSA isolates was able to transfer the *tet*(M) and *erm*(A) genes in mating experiment suggesting that MRSA strains could be environmental reservoirs for antibiotic resistance genes. In addition, 17/21 (81%) of the methicillin-resistant coagulase negative *Staphylococcus* spp. (MRCoNS) were resistant to one to five other classes of antibiotics and carried combinations of *erm*(B), *erm*(C), *msr*(A), *tet*(K), and/or *tet*(M) genes suggesting that MRCoNS strains could also be environmental reservoirs for mobile antibiotic resistance genes. In contrast, none of the environmental MSSA isolates carried any of the resistance genes examined.

Fifty-two (95%) isolates in the current study carried a variety of other antibiotic resistance genes and were multi-drug resistant, including all 11 USA300 isolates. Only three (5.5%) of the 55 MRSA isolates were negative by PCR for all seven antibiotic resistance genes examined and represented three different MLST types (**Table 1**). The most commonly found gene *aadD* was identified in 45 (82%) isolates, 42 (76%) isolates were positive for *msr*(A), 46 (84%) isolates positive for either *tet*(K) and/or *tet*(M), 27 (49%) isolates positive for *erm*(C), and 7 (13%) isolates positive for *erm*(A). None of the MRSA isolates carried the *erm*(B) gene. Iwao et al. (2012) examined susceptibilities of eight MRSA isolates from the public trains to nine other non-β-lactam antimicrobial agents and all were resistant to two to seven of the agents tested though specific resistance genes present were not determined. Seven (87.5%) of the eight strains resistant to kanamycin likely carried the *aadD* gene, which is similar to the 82% found in the current study, while 4 (50%) were macrolide resistant and 3 (37.5%) were clindamycin resistant. Three (75%) of the four Japanese isolates identified as ST8 CA-MRSA were resistant to gentamicin and kanamycin.

Nineteen (35%) of the MRSA isolates were positive for one to three different antibiotic resistance genes, and included 8 (57%) of the 13 MLST types from all the different environments and five of the USA300 strains. While 33 (60%) of the MRSA isolates carried four to six of the genes analyzed and included isolates from all the different environments with all the 13 MLST types and 6 of the 11 USA300 included in this group (**Table 1**).

Table 1 | Genotypic characteristics of MRSA isolates from recreational beaches and built environmental high touch surfaces.

Isolate	Source	SCC*mec* type[a]	MLST[b]	PFGE type	USA300	Antibiotic resistance genes[c]
***n* = 31**	**Recreational beaches 2010**					
401	Fresh water	IV	5	L		*tet*(M), *tet*(K), *erm*(C), *msr*(A), *aadD*
PC3	Fresh water	NT	6			*tet*(M), *tet*(K), *msr*(A), *aadD*
1012	Fresh water	NT	6			*tet*(M), *tet*(K), *msr*(A), *aadD*
111	Sand	IV	8	K		*erm*(C), *msr*(A), *aadD*
3mid	Sand	IV	15	G		*tet*(M), *tet*(K), *msr*(A), *aadD*
118	Fresh water	IV	15			*tet*(M), *tet*(K), *erm*(C), *msr*(A), *aadD*
603	Sand	IV	30	H		*tet*(M), *tet*(K), *erm*(C), *msr*(A), *aadD*
1112 (2)	Fresh water	II	45	M		*erm*(A), *aadD*
Seep1	Marine sand	IV	88			*tet*(M), *tet*(K), *msr*(A), *aadD*
257	Marine sand	IV	97	N		*tet*(K), *erm*(C), *msr*(A), *aadD*
302	Marine water	IV	109	F		*tet*(M), *tet*(K), *erm*(A), *msr*(A), *aadD*
308	Marine water	IV	109	F		*tet*(M), *erm*(A), *erm*(C), *msr*(A), *aadD*
361	Sand	IV	109	F		*tet*(M), *erm*(A), *erm*(C), *msr*(A), *aadD*
1112	Fresh water	I	133	B		None
244	Fresh water	IV	133	A		*erm*(C), *msr*(A), *aadD*
248	Fresh water	IV	133	A		*tet*(K), *msr*(A), *aadD*
252	Fresh water	IV	133	B		*tet*(K), *erm*(C), *msr*(A), *aadD*
125	Fresh water	IV	133	C		*tet*(K), *erm*(C), *msr*(A), *aadD*
909	Fresh water	IV	133	D		*tet*(K), *erm*(C), *msr*(A), *aadD*
910	Sand	IV	133	D		*tet*(K), *erm*(C), *msr*(A), *aadD*
512	Fresh water	IV	133	E		*tet*(K), *erm*(C), *msr*(A), *aadD*
824	Fresh water	NT	133			*tet*(K), *erm*(C), *msr*(A), *aadD*
515	Fresh water	IV	1875			*tet*(K), *erm*(C), *msr*(A), *aadD*
526	Fresh water	IV	1875			*tet*(M), *tet*(K), *msr*(A), *aadD*
813	Fresh water	IV	1875			*tet*(M), *tet*(K), *erm*(C), *msr*(A), *aadD*
823	Fresh water	NT	1875			*tet*(M), *tet*(K), *erm*(C), *msr*(A), *aadD*
1113	Fresh water	I	1956	J		None
1124	Fresh water	I	1956			*aadD*
827	Fresh water	IV	1956	I		*tet*(K), *erm*(C), *msr*(A), *aadD*
1017	Fresh water	NT	2049			*erm*(C), *aadD*
1019	Fresh water	NT	2049			*tet*(K), *erm*(C), *msr*(A), *aadD*
***n* = 10**	**Student homes 2010**					
S3–23	TV remote	IV	5	3	Yes	*tet*(M), *tet*(K), *msr*(A), *aadD*
S9–18	Bathroom floor	IV	5	3	Yes	*tet*(M), *msr*(A), *aadD*
S5–28	Bathroom light switch	IV	5	3	Yes	*tet*(M), *tet*(K), *aadD*
T4–38	Washing machine	IV	5	5		*tet*(M), *tet*(K), *msr*(A), *aadD*
J-5	Dorm water fountain	NT	8	1		None
R501	Couch	IV	8	4	Yes	*tet*(M), *msr*(A), *aadD*
R502	Toilet flush handle	IV	8 (931)[d]	4	Yes	*tet*(M), *tet*(K), *msr*(A), *aadD*
R509	Bathroom door knob	IV	8 (931)[d]	4	Yes	*tet*(M), *tet*(K), *msr*(A), *aadD*
A3–6	Microwave touchpad	IV	45	2		*tet*(M), *msr*(A), *aadD*
A4–3	Couch	IV	45	2		*tet*(M), *msr*(A), *aadD*
***n* = 10**	**University campus 2010**					
3–36	Bathroom	IV	5	3	Yes	*erm*(C), *msr*(A)
20	ATM	IV	5	3	Yes	*tet*(M), *tet*(K), *erm*(A), *erm*(C), *msr*(A), *aadD*
3–22	Bathroom	NT	5	12		*tet*(M)
3–2	Elevator button	I	8	9		*tet*(M), *erm*(C), *msr*(A)
8–509	Locker handle	IV	8	8	Yes	*tet*(M), *tet*(K), *msr*(A), *aadD*
26	ATM	IV	30	6	Yes	*tet*(M), *tet*(K), *erm*(A), *erm*(C), *msr*(A), *aadD*
36	ATM	I	45	7		*tet*(M), *tet*(K), *erm*(A), *erm*(C), *aadD*

(Continued)

Table 1 | Continued

Isolate	Source	SCC*mec* type[a]	MLST[b]	PFGE type	USA300	Antibiotic resistance genes[c]
3–6	Bathroom floor	NT	97	11		*tet*(M)
3–8	Elevator button	NT	97	10		*tet*(M)
3–10	Study lounge floor	NT	97	10		*tet*(M), *erm*(C)
***n* = 4**	**Community 2009–2010**					
40	ATM	NT	5	3	Yes	*tet*(M), *msr*(A)
55	ATM	IV	8	13		*tet*(M), *erm*(C), *msr*(A), *aadD*
5–3	Library computer touch screen	NT	30	14		*tet*(K), *aadD*
5–4	Library computer touch screen	IV	30	14		*tet*(M), *tet*(K), *msr*(A), *aadD*

[a] *SCCmec I–V was tested. If not one of these types, listed as non-typeable (NT);* [b] *MLST typing using seven housekeeping genes;* [c] *Kanamycin resistance gene, aadD; macrolide, lincosamide-streptogramin B resistance genes erm(A), erm(B), erm(C), and msr(A) gene and tetracycline resistance genes tet(M) and tet(K) were tested;* [d] *ST931 has recently been reassigned as ST8 due to revision of the gmk locus (Larsen et al., 2012).*

Twenty-two (40%) isolates carried four different antibiotic resistance genes and were isolated from the recreational beaches ($n = 15$), student homes ($n = 4$), university sites ($n = 1$), and community sites ($n = 2$). Ten of the isolates carried *aadD*, *erm*(C), *msr*(A), and *tet*(K) genes and included ST97 ($n = 1$), ST133 ($n = 6$), ST1875 ($n = 1$), ST1956 ($n = 1$), and ST2049 ($n = 1$), and eight were SCC*mec* IV and two NT. Eleven isolates carried *aadD*, *msr*(A), *tet*(K), and *tet*(M) genes and included ST5 ($n = 2$), ST6 ($n = 2$), ST8 ($n = 3$), ST15 ($n = 1$), ST30 ($n = 1$), ST88 ($n = 1$), and ST1875 ($n = 1$). Four (36%) of the 11 MRSA were USA300 strains by PFGE analysis.

The highest number of resistance genes was found in two USA300 isolates, from the University of Washington sites. They were positive for 6 genes [*aadD*, *erm*(A), *erm*(C), *msr*(A), *tet*(K), and *tet*(M)], included ST5 and ST30, and both isolates carried the SCC*mec* IV element. Nine (16%) isolates which carried five different antibiotic resistance genes were isolated from the recreational beaches $n = 8$, and university sites $n = 1$. Five isolates were positive for *aadD*, *erm*(C), *msr*(A), *tet*(K), and *tet*(M) genes, included ST5 ($n = 1$), ST15 ($n = 1$), ST30 ($n = 1$), ST1875 ($n = 2$), and four of the five isolates carried the SCC*mec* IV element. Two SCC*mec* IV, ST109 MRSA isolates from the recreational beaches were positive for *aadD*, *erm*(A), *erm*(C), *msr*(A), and *tet*(M) genes. One other SCC*mec* IV, ST109 isolate from the beach was positive for *aadD*, *erm*(A), *msr*(A), *tet*(K), and *tet*(M) genes (**Table 1**). One SCC*mec* I, ST45 isolate from the university site was positive for *aadD*, *erm*(A), *erm*(C), *tet*(K), and *tet*(M) genes (**Table 1**).

COMPARISON OF SCC*mec* TYPING AND MLST OF ISOLATES ACROSS DIFFERENT WA ENVIRONMENTS

Of the 55 MRSA isolates, 35 (63.6%) carried the SCC*mec* IV, while 14 (25.5%) were non-typeable which meant they did not carry SCC*mec* I–V, five (9.1%) carried the SCC*mec* I and one (1.8%) carried the SCC*mec* II (**Table 1**). In the much smaller Japanese study (Iwao et al., 2012), the SCC*mec* IV was also the most common [4/8 (50%)] with three (37.5%) isolates carrying the SCC*mec* II and one (12.5%) the SCC*mec* I elements. Few other studies of environmental MRSA have SCC*mec* typed their isolates.

There were 13 different MLST types identified from the WA isolates. Nine (69%) of these MLST types have commonly been isolated from humans (see text footnote 2). All 13 of the MLST types were found in MRSA isolated from the WA recreational beaches, five MLST types were found in MRSA isolated from the university, three MLST types (ST5, ST8, and ST45) were found in MRSA isolated from student home surfaces, and three MLST types (ST5, ST8, and ST30) were found in MRSA from the community surfaces. Two new MLST types ST1875 ($n = 4$), three SCC*mec* IV and one NT, and ST2049 ($n = 2$) both NT, were identified in recreational beach samples. The ST2049 is closely related to ST1962 which has previously been identified in squirrels as has ST1956 (see text footnote 2), both of which were found in the recreational beach samples. Interestingly, two of the three ST1956 isolates carried SCC*mec* I, more commonly associated with hospital-acquired MRSA. ST1875 (SCC*mec* IV, $n = 3$; NT, $n = 1$) is closely related to recently identified human ST1176 (CC5) from Brazil (Carmo et al., 2011) and a hospital-acquired ST1176 MRSA strain 2929B reported from Boston, MA, USA and thus could be of human origin (see text footnote 2).

Twenty-six (47%) of the MRSA belonged to three of the 13 MLST types and included nine isolates of ST133 (CC133), nine isolates of ST5 (CC5), and eight isolates of ST8 (CC8). ST5 and ST8 were found in all four environments (**Table 1**). All nine ST133 isolates were from both the marine and fresh recreational beaches and surrounding fresh water streams draining into the recreational beaches at multiple time points (Levin-Edens et al., 2012). Based on difference in PFGE patterns the nine isolates represented at least five different strains (**Table 1**). Seven of the MRSA strains representing all five different PFGE patterns carried the SCC*mec* IV element, one carried SCC*mec* I element, and one carried an element that was different than the I–V. Smyth et al. (2009) stated that *S. aureus* ST133 is an ungulate-animal-specific genotype with no association with humans reported in the literature. The ST133 was the most common lineage reported in *S. aureus* isolates collected from Danish small ruminants (65% of 179 sheep and 55% of 17 goats) (Eriksson et al., 2013). ST133 has been isolated from German wild boars (Meemken et al., 2013), and Japanese cats and dogs (Sasaki et al., 2012). More recently a single MRSA ST133 has been identified from 66 MRSA isolates with seven major clonal complexes from nasal swabs collected from patients upon admission to intensive care units at Pennsylvania State University (Schwalm

et al., 2011). Thus it is likely that MRSA ST133 is only rarely associated with humans, which correlates with its absence from human built environment surfaces. It is possible that ST133 is associated with common wild and/or pet animals found in urban areas such as Seattle in addition to ungulates and thus explains the wide distribution among the recreational beach areas at multiple time points within the city but its absence from surfaces in built environments in the same geographical area.

The nine ST5 isolates were found at the beaches ($n = 1$), and on the high touch surfaces in the student homes ($n = 4$), university campus ($n = 3$), and community sites ($n = 1$) (**Table 1**), represented four different PFGE patterns, seven were SCC*mec* IV and two were NT. ST5 has been associated with humans, chickens, turkey, and pork (see text footnote 2). USA100 a common hospital MRSA is ST5 and is part of the major clonal complex which is widely distributed (Schwalm et al., 2011; Iwao et al., 2012; Verghese et al., 2012; Kawaguchiya et al., 2013). ST5 was found in all four WA environments studied (**Table 1**).

There were eight ST8 isolates and like ST5 found in all environments sampled (recreational $n = 1$; students homes $n = 4$; university $n = 2$; community $n = 1$). Six of the ST8 were SCC*mec* IV isolated at the beaches, student homes, university campus, and community, while ST8 SCC*mec* I was isolated on a university surface and the ST8 NT isolated from student dorm. ST8 has been associated with USA300 clonal type which is now being found in hospital setting (Kawaguchiya et al., 2013) and four of the isolates had PFGE patterns that indicated that they were USA300 strains. ST8 MRSA has also been isolated from chickens, cows, horses, turkey, beef, and pork samples (see text footnote 2). One of the eight isolates was the only MRSA (strain 8-509) in the study to be Panton-Valentine Leukocidin (PVL) positive while the remaining MRSA were all PVL-negative in the WA samples as compared to four of eight MRSA isolates PVL+ in the Japanese study (Iwao et al., 2012).

Other ST types found in humans ST109 ($n = 3$), humans and animals ST15 ($n = 2$), ST45 ($n = 4$), ST88 ($n = 1$), ST97 ($n = 4$), or humans and meat products ST6 ($n = 2$), ST30 ($n = 4$), and ST40 ($n = 4$). ST97 is rarely found in humans and ∼90% of the submissions to the MLST website lists cow milk, bovine mastitis, or pigs (see text footnote 2). ST30 and ST45 belong to major clonal complexes CC30 and CC45 respectively and are often associated with human disease (Simor et al., 2002; Moore et al., 2010; Isobe et al., 2012). The MRSA ST30 clone, found in three of the WA environments, has been predominantly identified in Japanese hospitalized patients since the 1980s (Isobe et al., 2012) and have also been reported in MRSA infections from other parts of the world (Hetem et al., 2012). MRSA ST45, also found in three of the WA environments, is associated with USA600 which has been reported as an epidemic strain in Central European and Canadian hospitals (Witte et al., 1997; Simor et al., 2002) and been implicated in outbreaks of bloodstream infections in Detroit, MI, USA (Abdel-Haq et al., 2009; Moore et al., 2010).

CONCLUSION

Unlike recent studies of clinical USA300, all the environmental USA300 isolates in the current study were multi-drug resistant carrying two to six different antibiotic resistance genes. The majority (72%) carried four to six different antibiotic resistance genes and ∼95% of all the other MRSA isolates were multi-drug resistant. Similar results were found in the Japanese study of MRSA isolates from 2008 to 2010 (Iwao et al., 2012). Why there was such a high level of antibiotic resistance in environmental MRSA isolates is certainly not clear but does correlate with our previous small study (Soge et al., 2009). It is unlikely that the level of resistance found in the environmental MRSA isolates is unique to the Seattle, WA area or to the selected environmental surfaces examined. It will also be of interest to know if the environmental MRSA isolates are acquiring their antibiotic resistance genes from other members of the environmental bacterial community or if some of these genes have originated from bacteria in the hospital setting. The data suggests that the environmental MRSA isolates collected in the current study are potential antibiotic resistance gene reservoirs and should be able to act as donors to the microbes and the environmental antibiotic resistance gene pool and potentially contribute to the human microbiome antibiotic resistance gene pool.

A major difference between MRSA isolates isolated from WA recreational beaches and freshwater streams versus other WA environments is that we found no isolates related to USA300 by PFGE, though MLST type associated with USA300 and SCC*mec* IV were present. In contrast, in the built environments, USA300 MRSA isolates comprised 25–60% of the total number of MRSA isolates recovered (**Table 1**). Another major difference was that ST133 which has until recently thought to be exclusively an animal clone was one of the most commonly isolated ST type from recreational beaches 9/31 (29%) but was absent from the built environmental sites.

From all environmental sites, SCC*mec* IV was the most common 36/55 (66%) which is comparable with what is occurring in the health care setting with SCC*mec* IV replacing other SCC*mec* types (Healy et al., 2004; D'Agata et al., 2009). The most diversity of MLST types were found in the recreational beaches but this could be due to the larger number of different MRSA isolates included. There are a limited number of studies that have characterized environmental MRSA. The recreational beach study of Seifried et al. (2007) identified 18 USA300 isolates from recreational seawater in Hawaii. These isolates represented eight different *spa* types, at least three different SCC*mec* types and a variety of PFGE types. We did not find USA300 in our recreational samples but did find significant diversity of the MRSA strains as was found in Hawaii. Another study in California suggested that beachgoers could be a possible source of MRSA and *S. aureus* isolated in sand and seawater (Goodwin et al., 2012) though the isolates were not characterized to verify this hypothesis. Thus the differences we have found between MRSA strains isolated at the three recreational beaches in the Seattle and the built environmental surfaces may vary geographically with less differences between recreational beaches isolates taken from California, Hawaii or where the water is warmer and many more people swim. However it is clear that more studies characterizing the MRSA isolated in environmental setting need to be done to allow better understanding of the potential risk involved with environmental contact with MRSA and/or *S. aureus* contaminated community high touch surfaces and recreational beaches.

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Sterol C-22 desaturase ERG5 mediates the sensitivity to antifungal azoles in *Neurospora crassa* and *Fusarium verticillioides*

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Antifungal azoles inhibit ergosterol biosynthesis by interfering with lanosterol 14α-demethylase. In this study, seven upregulated and four downregulated ergosterol biosynthesis genes in response to ketoconazole treatment were identified in *Neurospora crassa*. Azole sensitivity test of knockout mutants for six ketoconazole-upregulated genes in ergosterol biosynthesis revealed that deletion of only sterol C-22 desaturase ERG5 altered sensitivity to azoles: the *erg5* mutant was hypersensitive to azoles but had no obvious defects in growth and development. The *erg5* mutant accumulated higher levels of ergosta 5,7-dienol relative to the wild type but its levels of 14α-methylated sterols were similar to the wild type. ERG5 homologs are highly conserved in fungal kingdom. Deletion of *Fusarium verticillioides erg5* also increased ketoconazole sensitivity, suggesting that the roles of ERG5 homologs in azole resistance are highly conserved among different fungal species, and inhibition of ERG5 could reduce the usage of azoles and thus provide a new target for drug design.

Keywords: resistance to antifungal agents, ergosterol biosynthesis, azole, *Neurospora crassa*, *Fusarium verticillioides*

INTRODUCTION

Antifungal azoles are the most commonly used drugs for controlling fungal infections. Azoles inhibit ergosterol biosynthesis by disrupting essential P450 superfamily protein lanosterol 14α-demethylase CYP51 (syn. ERG11) (Bossche et al., 1984; Kelly et al., 1993). In addition, the inhibition of lanosterol 14α-demethylase also results in the accumulation of the toxic 14-α methylated sterols, like 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol (Kelly et al., 1995). Incorporation of such compounds into fungal membranes reduces their rigidity (Abe et al., 2009).

Biological processes of sterol biosynthesis are highly conserved in fungal kingdom (Ferreira et al., 2005). In response to azole treatments, significant transcriptional increases in several genes involved in ergosterol biosynthesis, such as *ERG2* (C-8 sterol isomerase), *ERG3* (C-5 sterol desaturase), *ERG5* (C-22 sterol desaturase), *ERG6* (C-24 sterol methyltransferase), *ERG25* (C-4 methyl sterol oxidase), and *ERG11*, are consistently observed in many fungal species such as *Saccharomyces cerevisiae* (Bammert and Fostel, 2000; Agarwal et al., 2003), *Candida albicans* (De Backer et al., 2001; Liu et al., 2005), *Aspergillus fumigatus* (da Silva Ferreira et al., 2006), *Trichophyton rubrum* (Yu et al., 2007), *Cryptococcus neoformans* (Florio et al., 2011), and *Fusarium graminearum* (Liu et al., 2010; Becher et al., 2011). Many reports have shown that increased expression of *ERG11* could reduce azole susceptibility (Hamamoto et al., 2000; Schnabel and Jones, 2001; Du et al., 2004; Ma et al., 2006). In addition to *ERG11*, *ERG3*, and *ERG5* have also been shown to be related to drug susceptibility. Many *ERG3* mutants of *S. cerevisiae* or *C. albicans* with defects in C5-6 desaturase showed increased resistance to azoles (Watson et al., 1989; Martel et al., 2010a; Morio et al., 2012; Vale-Silva et al., 2012). A clinical isolate of *C. albicans* with mutations in both *ERG11* and *ERG5* is cross resistance to azoles and amphotericin B (Martel et al., 2010b). However, due to lack of systemic analysis, except *ERG11*, contributions of most of these azole-responsive genes to azole resistance were not fully understood.

Since the non-pathogenic *Neurospora crassa* has less redundant genes in ergosterol biosynthesis relative to other filamentous pathogenic fungi and has knockout mutants available for most of ergosterol synthesis genes, it can be an excellent model for systemic analysis of the contributions of these azole-responsive genes to azole resistance. In this study, we demonstrate that ERG5 mediates the sensitivity to antifungal azoles in both *N. crassa* and *Fusarium verticillioides*.

MATERIALS AND METHODS

STRAINS AND CULTURE CONDITIONS

Neurospora crassa strains used in this study are listed in **Table 1**. All of them were obtained from the Fungal Genetics Stock Center[1] (University of Kansas Medical Center). Vogel's minimum medium (Vogel, 1956), supplemented with 2% (w/v) sucrose for slants or 2% glucose for plates and the liquid medium, was used for

[1] www.fgsc.net

Table 1 | *Neurospora* strains used in this study.

Strain	Genotype
FGSC#4200	Wild type, a
FGSC#18507	*erg2* (NCU04156), a, hetero
FGSC#13802	*erg5* (NCU05278), a
FGSC#13803	*erg5* (NCU05278), A
FGSC#12752	*erg6* (NCU03006), a
FGSC#12753	*erg6* (NCU03006), A
FGSC#18506	*erg24* (NCU08762), a, hetero
FGSC#17674	*erg25* (NCU06402), a

culturing strains. All cultures were grown at 28°C in the light. Antifungal compounds were added as needed.

DRUG SUSCEPTIBILITY TEST

Ketoconazole, fluconazole, and itraconazole were dissolved in dimethyl sulfoxide (DMSO) and then aseptically added to the autoclaved medium before making agar plates. The final concentrations of ketoconazole, fluconazole, and itraconazole in the agar plates were 2, 25, and 10 μg/ml, respectively. The final DMSO concentration was below 0.25% (v/v). Two microliters of conidial suspension were inoculated on plates ($\Phi = 9$ cm) with or without antifungal drugs and incubated at 28°C for 66 h.

MIC DETERMINATION OF KETOCONAZOLE, ITRACONAZOLE, AND FLUCONAZOLE

The MICs of three azoles for each strain were determined in 96-well microtiter plate according to National Committee for Clinical Laboratory Standards (CLSI, 2008). Briefly, 100 μl of 2× azole solution and 100 μl of conidial suspension were added into each well. The final conidial concentration was approximately 1×10^6 conidia/ml. The plates were incubated at 28°C for 24 h. The MIC was determined as the lowest drug concentration without growth.

RNA EXTRACTION AND DIGITAL GENE EXPRESSION PROFILING ANALYSIS

The transcriptional responses of ergosterol biosynthesis genes to ketoconazole treatment in the wild-type strain were detected by digital gene expression (DGE) profiling (Nielsen et al., 2006). Briefly, conidia of the wild-type strain was inoculated into 20-ml liquid medium in a plate ($\Phi = 9$ cm) and incubated for 24 h at 28°C in darkness to form mycelial mat on the surface of the liquid medium. Mycelial mat then was cut into small pieces ($\Phi = 10$ mm) and transferred to the liquid medium (2 pieces/100 ml). Cultures were incubated at 28°C with shaking at 180 rpm for 12 h. Ketoconazole was then added into the medium to reach a final concentration of 2.5 μg/ml. After 24 h of incubation, mycelia were harvested and total RNA was extracted and subjected to DGE analysis as described by Sun et al. (2011).

STEROL EXTRACTION AND ANALYSIS

Sterols of dried mycelium (0.3 g) was extracted into 1.5-ml chloroform under ultrasonication for 12 h. The extracts were dried and subsequently dissolved into 150 μl methanol under ultrasonication for 1 h. After filtration with a Millipore filter, the extracts were subjected to HPLC-MS analysis following the method reported by Cañabate-Díaz et al. (2007) with some modifications.

Liquid chromatography separation was performed on an Agilent Zorbax Extend-C18 1.8 μm 2.1 mm × 50 mm column using an Agilent 1200 Series system (Agilent, USA). Total flow rate was 0.4 ml min^{-1}; mobile phase A consisted of water with 0.01% acetic acid and mobile phase B consisted of acetonitrile. Total elution program was 27 min. Gradient began with 70% mobile phase B, changed to 100% B over 2 min, maintained a constant level from 2 to 18 min at 100% B and then decreased to 70% B over 1 min prior to re-stabilization over 8 min before the next injection. The column temperature was maintained at 40°C. The injection volume was 10 μl.

Mass spectra were acquired with an Agilent Accurate-Mass-Q-TOF MS 6520 system. Analytes were detected in the positive ionization mode with an APCI probe. For Q-TOF/MS conditions, fragmentor and capillary voltages were kept at 130 and 3500 V, respectively. Nitrogen was supplied as the nebulizing and drying gas. Temperature of the drying gas and vaporizer were both set at 350°C. The corona was set to 4 μA. The flow rate of the drying gas and the pressure of the nebulizer were 3 l min^{-1} and 50 psi, respectively. Full-scan spectra were acquired over a scan range of *m/z* 80–1000 at 1.03 spectra s^{-1}. The derived sterols were identified with reference molecular weight and fragmentation spectra for known standards.

PHYLOGENETIC ANALYSIS

Protein sequences were aligned with the Clustal X 2.1 software (Larkin et al., 2007). Then, the neighbor-joining (NJ) tree was constructed using MEGA 5 software (Tamura et al., 2011). To assess the confidence of phylogenetic relationships, the bootstrap tests were conducted with 1000 resamplings.

KNOCKOUT OF *erg5* HOMOLOG GENE IN *FUSARIUM VERTICILLIOIDES*

In order to knockout *erg5* homologous genes (*erg5A*: FVEG_07284; *erg5B*: FVEG_08786) in *F. verticillioides*, the upstream and downstream flanking sequences of the FVEG_07284 and FVEG_08786 were amplified. The primer pairs were shown in **Table 2**.

The resulting PCR products were cloned into the plasmid pCX62 (Zhao et al., 2004) and results in knockout construct pCX62-ΔFv07284 and pCX62-ΔFv08786, in which the PCR products were ligated with the hygromycin phosphotransferase (*hph*) gene. Then, the deletion cassette was transformed into *F. verticillioides* 7600 and resulted in deletion mutants. Fungal transformation followed the protocol reported by Miller et al. (1985), with minor modification as described by Li et al. (2006).

RESULTS

TRANSCRIPTIONAL RESPONSES TO KETOCONAZOLE STRESS BY GENES INVOLVED IN ERGOSTEROL BIOSYNTHESIS

Genome-wide transcriptional responses to ketoconazole treatment from two independent experiments were analyzed by DGE method. For genes involved in ergosterol biosynthesis, seven were

Table 2 | Primer pairs used for knockout of *erg5* homolog gene in *F. verticillioides*.

Primers	Sequence (5′ → 3′)	Product size (bp)	Amplified region
Fv07284(p)F-*Xho*I	CCGCTCGAGCACCCGATGAACTCGCCAATA	1859	FVEG_07284 5′ flanking region
Fv07284(p)R-*Eco*RI	CGGAATTCATCATACGCAACGCAAAGAGC		
Fv07284(3)F-*Bam*HI	CGGGATCCATGATGGGAAAGCGAGTTGA	1717	FVEG_07284 3′ flanking region
Fv07284(3)R-*Xba*I	GCTCTAGAGCTGACAGCGACCAGTAGGA		
Fv08786(p)F-*Kpn*I	GCGGTACCCGAGGATGATTGCTTGGTGAG	1455	FVEG_08786 5′ flanking region
Fv08786(p)R-*Eco*RI	CGGAATTCCATGCTGGGTCTAGTTGAGGG		
Fv08786(3)F-*Xba*I	GCTCTAGAGGGGCAAGGTGTTTGTGAATA	1797	FVEG_08786 3′ flanking region
Fv08786(3)R-*Xba*I	GCTCTAGAAATGCCACTGAGTTCGGATG		

Table 3 | Transcriptional response to ketoconazole stress by genes involved in ergosterol biosynthesis in *N. crassa* wild type.

Locus	Gene	Function	TPM-wt1	TPM-wt(k)1	Fold [wt(k)1/wt1]	TPM-wt2	TPM-wt(k)2	Fold [wt(k)2/wt2]
NCU08280	*erg1*	Squalene epoxidase	78.1	34.6	0.44	16.1	27.4	1.70
NCU04156	*erg2*	Sterol biosynthesis	107.1	327.7	3.06	26.8	296.4	11.07
NCU06207	*erg3*	C-5 sterol desaturase	1000.3	767.0	0.77	639.5	999.0	1.56
NCU01333	*erg4*	C-24 reductase	1.4	0.6	0.42	1.4	2.0	1.36
NCU05278	*erg5*	C-22 sterol desaturase	67.4	308.9	4.58	83.5	174.6	2.09
NCU03006	*erg6*	C-24 sterol methyltransferase	23.8	791.5	33.20	19.3	174.6	9.06
NCU01119	*erg7*	Oxidosqualene:lanosterol cyclase/lanosterol synthase	34.0	11.3	0.33	23.0	4.5	0.19
NCU08671	*erg8*	Phosphomevalonate kinase	24.7	8.7	0.35	26.5	10.6	0.40
NCU06054	*erg9*	Squalene synthetase	21.1	14.7	0.70	10.1	14.6	1.44
NCU02571	*erg10*	Acetoacetyl-CoA thiolase	181.6	178.8	0.98	323.8	103.8	0.32
NCU02624	*erg11*	Cytochrome P450 lanosterol 14α-Demethylase	110.1	812.0	7.37	69.1	676.7	9.80
NCU03633	*erg12*	Mevalonate kinase	70.1	90.6	1.29	21.6	77.5	3.59
NCU03922	*erg13*	Hydroxymethylglutaryl-coenzyme A synthase	117.0	47.3	0.40	227.4	35.0	0.15
NCU11381	*erg19*	Mevalonate pyrophosphate decarboxylase	38.6	21.1	0.55	49.5	18.2	0.37
NCU01175	*erg20*	Polyprenyl synthetase	188.5	81.1	0.43	116.0	50.9	0.44
NCU08762	*erg24*	Sterol C-14 reductase	7.1	11.8	1.66	9.2	34.7	3.77
NCU06402	*erg25*	C-4 methyl sterol oxidase	2512.8	5111.7	2.03	2894.3	5348.9	1.85
NCU02693	*erg26*	C-3 sterol dehydrogenase	57.0	63.2	1.11	81.5	48.4	0.59
NCU05991	*erg27*	3-Keto sterol reductase	3.0	4.6	1.53	0.6	1.7	2.90
NCU04461	*erg28*	Endoplasmic reticulum membrane protein	67.7	28.9	0.43	30.2	33.3	1.10
NCU04144	*are2*	Acyl-CoA:sterol acyltransferase	1.4	1.4	1.05	0.6	0.1	0.17
NCU00712	*hmg1/hmg2*	Hydroxymethylglutaryl-coenzyme A reductase	14.8	8.1	0.55	9.8	10.1	1.03
NCU07719	*idi1*	Isopentenyl diphosphate isomerase	59.2	15.6	0.26	39.4	37.5	0.95
NCU08139	*mvd1*	Diphosphomevalonate decarboxylase	0	0	–	0	0	–

wt1 and wt(k)1 represent the first batch samples, wt2 and wt(k)2 represent the second batch samples; wt: wild type; wt(k): wild type treated with ketoconazole.

consistently upregulated while four consistently downregulated upon ketoconazole treatment in two independent experiments (**Table 3**). The most dramatically upregulated genes were *erg11* (NCU2624), *erg6* (NCU03006), *erg2* (NCU04156), and *erg5* (NCU05278), which were consistently increased upon ketoconazole treatment by at least three folds in two independent experiments. The consistently downregulated genes include *erg7* (NCU01119), *erg8* (NCU08671), *erg13* (NCU03922), and *erg20* (NCU11381). However, the transcriptional changes in these downregulated genes were less dramatic than those in *erg11*, *erg6*, *erg2*, and *erg5*. None of them had a transcriptional change higher than three folds.

DELETION OF *erg5* INCREASES AZOLE SUSCEPTIBILITY IN *N. CRASSA*

Six ketoconazole-responsive genes involved in ergosterol synthesis have the knockout mutants available in Fungal Genetics Stock Center. All these genes are ketoconazole-upregulated genes, including *erg2*, *erg3*, *erg5*, *erg6*, *erg24*, and *erg25*. On the solid medium without antifungal drugs, all deletion mutants had the growth rates similar to the wild-type strain and no obvious defect was observed in their asexual and sexual reproduction (the data not shown)[2]. Drug sensitivity test showed that the deletion of only

[2]http://www.broadinstitute.org/annotation/genome/neurospora/Phenotypes.html

erg5 altered the sensitivity to azoles. On agar plates, all three tested azoles, including ketoconazole, itraconazole, and fluconazole, had greater inhibition to the *erg5* mutants than the wild-type strain (**Figure 1**). MIC analysis in the liquid medium showed that MIC values of the *ERG5* mutants for ketoconazole, itraconazole, and fluconazole were only 60, 50, and 40% of that of the wild-type strain, respectively (**Table 4**).

DELETION OF *erg5* DOES NOT CAUSE ACCUMULATION OF 14α-METHYLATED STEROLS DURING KETOCONAZOLE STRESS IN *N. CRASSA*

ERG5 catalyzes the biosynthesis of ergosta 5,7,22,24(28)-trienol, a direct precursor for ergosterol biosynthesis. ERG5 mutations caused accumulation of ergosta 5,7,24(28)-trienol and ergosta 5,7-dienol in *S. cerevisiae ERG5* deletion mutant (Barton et al., 1974; Skaggs et al., 1996). Thus, it is possible that the *erg5* mutant might accumulate more toxic 14α-methylated sterols than the wild-type strain. To test this possibility, a comparative analysis of sterol profiles between the *erg5* mutant and the wild type was performed by HPLC-MS. Our results showed that, in the normal liquid medium without ketoconazole, the *erg5* mutant produced more ergosta 5,7-dienol than the wild-type strain. As expected, ergosterol in the *erg5* mutant was almost undetectable (**Figure 2**). When treated with ketoconazole, 4,4-dimethyl-ergosta 8,14,24(28)-trienol, the direct product catalyzed by ERG11 (lanosterol 14α-methylase), was reduced in the wild-type strain and the *erg5* mutant at the similar level. Ketoconazole treatment greatly increased levels of 14α-methylated sterols, such as eburicol and 14α-methyl-ergosta-8,24(28)-dien-3β, 6α-diol, in both the wild-type strain and the *erg5* mutant (**Figure 2**). However, the levels of these compounds were similar between the wild-type strain and the *erg5* mutant, suggesting that the azole-hypersensitive phenotype in the *erg5* mutant is not due to over-accumulation of 14α-methylated sterols.

ERG5 HOMOLOGS ARE HIGHLY CONSERVED IN FUNGAL KINGDOM

To gain an insight into the sequence conservation and phylogenic relationship of ERG5 homologs in fungi, Blast and phylogenetic analysis were conducted. Blast analysis revealed that ERG5 homologs are high conserved in amino acid sequences. Phylogenetic analysis with amino acid sequences of 27 ERG5 homologs (*E* value = 0; Alignment coverage >90%; sequence similarity >76%) from 3 yeast fungal species (*C. albicans*, *S. cerevisiae*, *Schizosaccharomyces pombe*) and 16 filamentous fungal species showed that all ERG5 homologs were clustered into 2 big clades (**Figure 3**). The ERG5 homologs from yeast fungal species were distributed in the first clade (clade I), which represents the most ancestral group, while those from filamentous fungal species were located in the second clade (clade II).

As shown in **Figure 3**, ERG5 in filamentous fungi can be further categorized into two classes based on their phylogenic relationship, namely Type IIA and Type IIB, respectively. *N. crassa*, *N. fischeri*, *A. niger*, *A. nidulans*, *T. reesei*, *M. grisea*, and *V. albo-atrum* have only one *erg5* gene, all in Type IIA. *A. fumigatus*, *A. oryzae*, and *A. clavatus* and all examined *Fusarium* species have two *erg5* genes, one in Type IIA and the other in Type IIB. *A. terreus* is the only species that has three *erg5* genes, two in Type IIA and one in Type IIB. Similar to ERG5, multiple ERG3 and ERG11 homologs were also found in *Aspergillus* and *Fusarium* species (Ferreira et al., 2005; Liu et al., 2010; Becher et al., 2011).

DELETION OF *erg5* INCREASES AZOLE SUSCEPTIBILITY IN *F. VERTICILLIOIDES*

To see if ERG5 in other filamentous fungi also mediate azole sensitivity, gene knockout mutants for Type IIA *erg5* gene *erg5A* (FVEG_07284) and for Type IIB *erg5* gene *erg5B* (FVEG_08786), in the pathogenic fungus *F. verticillioides* were generated. As shown in **Figure 4**, on the medium without drug, the growth rates of the *erg5* deletion mutants were similar to that of the wild-type strain and no defect in conidiation was observed in the mutants. When inoculated on the medium with 0.5 μg/ml ketoconazole, both *erg5A* and *erg5B* mutant displayed greater growth inhibition than the wild-type strain, indicating the ERG5 homologs have similar roles in azole resistance among different fungal species.

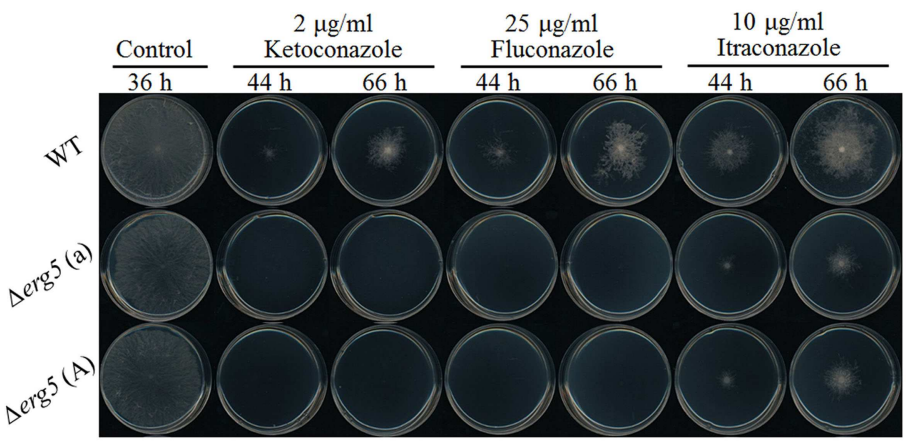

FIGURE 1 | Drug susceptibility analysis of the *erg5* knockout mutant of *N. crassa*. Two microliters of conidial suspension (1 × 104) conidia/ml were inoculated on the center of plates (Φ = 9 cm) with or without antifungal drugs, then incubated at 28°C for 66 h. Each test had three replicates and the experiment was independently repeated twice.

DISCUSSION

Although transcriptional responses by genes of sterol biosynthesis to azole stress have been observed in many fungal species (Bammert and Fostel, 2000; De Backer et al., 2001; Agarwal et al., 2003; Liu et al., 2005, 2010; da Silva Ferreira et al., 2006; Yu et al., 2007; Becher et al., 2011; Florio et al., 2011), it is still mysterious about the biological significance of transcriptional activation for most of these genes. Taking advantage of the availability of knockout mutants of *N. crassa*, we analyzed the contributions of six ketoconazole-responsive genes in ergosterol biosynthesis, including *erg2*, *erg3*, *erg5*, *erg6*, *erg24*, and *erg25*, to azole resistance, and demonstrated that deletion of *erg5*, but not other ergosterol genes, could make *N. crassa* more susceptible to antifungal azoles. Since the deletion of *erg5* should result in an effect opposite to its overexpression, transcriptional increase of *erg5* upon azole stress might positively contribute to the azole resistance. As *erg5* deletion consistently resulted in azole hypersensitivity in both *N. crassa* and *F. verticillioides*, strategies that either silencing the expression of *erg5* or disrupting C-22 sterol desaturase (ERG5) activity can make fungal pathogens more susceptible to antifungal azoles. ERG5, thus, can be a promising target for antifungal drug design or fungal disease management in crops. Although no alteration in azole sensitivity was observed in the mutants for the rest of ergosterol biosynthesis genes, their transcriptional increases during azole treatment suggest that these genes are involved in the adaptation to azole stress. Their contributions to azole resistance might be able to be detected by combined mutation of multiple genes. Nevertheless, *erg5* is the most important contributor to azole resistance among these azole-responsive genes.

Table 4 | MIC of the *erg5* mutant and wild type for azoles.

	MIC for ketoconazole (μg/ml)	MIC for fluconazole (μg/ml)	MIC for itraconazole (μg/ml)
WT	2.5 ± 0	31.25 ± 0	5.0 ± 0
Δ*erg5 (a)*	1.5 ± 0	12.5 ± 0	2.5 ± 0
Δ*erg5 (A)*	1.5 ± 0	12.5 ± 0	2.5 ± 0

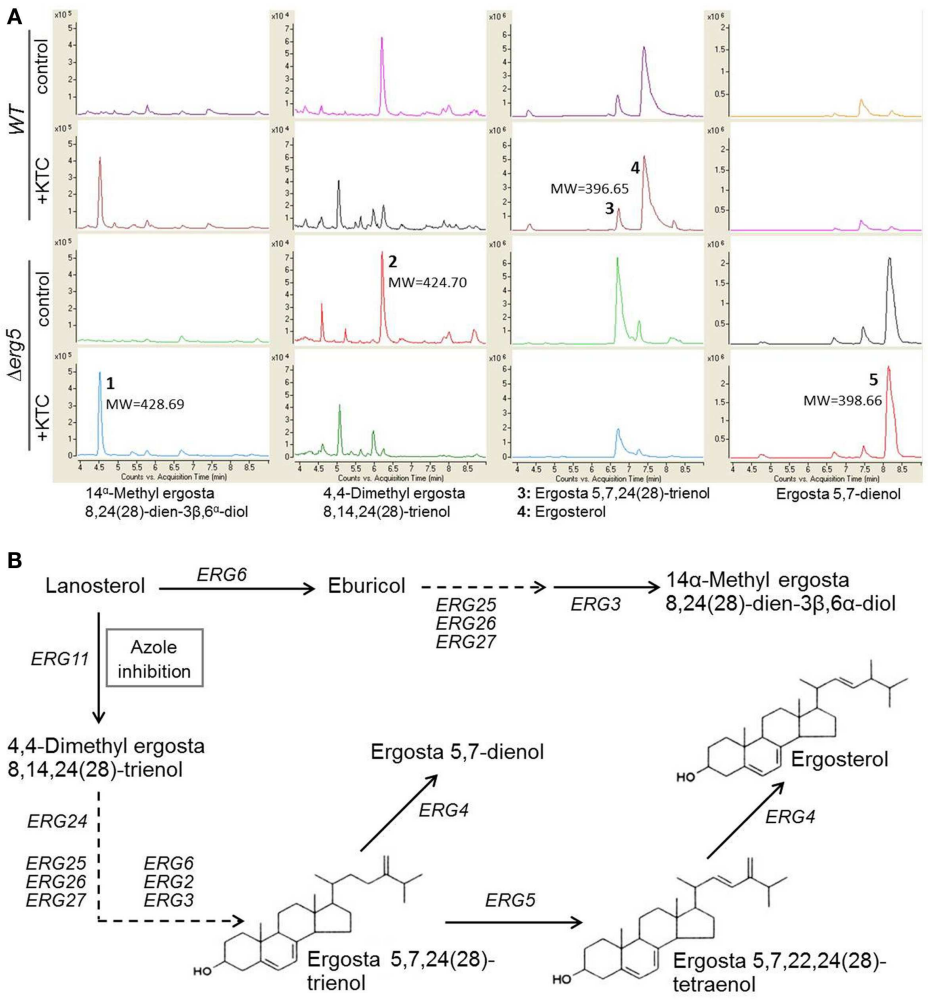

FIGURE 2 | HPLC-MS chromatogram of sterol extracts (A) and schematic representation of the ergosterol biosynthetic pathway (B) in *N. crassa*.

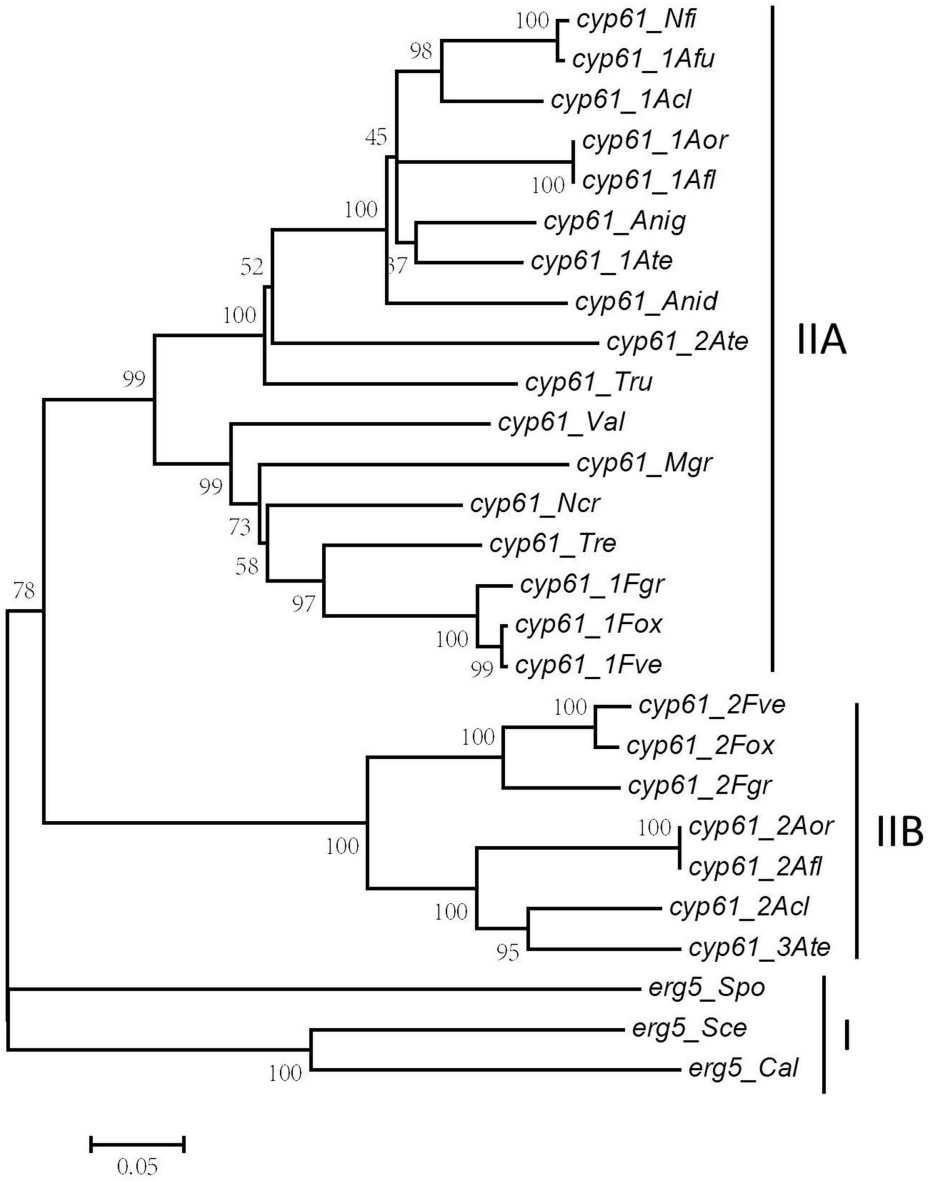

FIGURE 3 | Phylogenic analysis of fungal ERG5 homologs. Predicted REG5 proteins from 19 fungal species were aligned and a condensed neighbor-joining (NJ) tree was constructed with cut-off bootstrap values of 50% obtained from 1000 replicates using MEGA 5 software (Tamura et al., 2011). (*Cal, C. albicans*; *Sce, S. cerevisiae*; *Spo, S. pombe*; *Ncr, N. crassa*; *Afu, A. fumigatus*; *Afl, A. flavus*; *Anid, A. nidulans*; *Anig, A. niger*; *Acl, A. clavatus*; *Ate, A. terreus*; *Aor, A. oryzae*; *Nfi, N. fischeri*; *Fve, F. verticillioides*; *Fox, F. oxysporum*; *Fgr, F. graminearum*; *Tru, T. rubrum*; *Val, V. albo-atrum*; *Tre, T. reesei*; *Mag, M. grisea*).

Membrane sterols regulate membrane fluidity, permeability, the activity of membrane-bound enzymes and growth rate (Skaggs et al., 1996). In *S. cerevisiae*, genes involved in early steps of ergosterol biosynthesis, such as *ERG9* (squalene synthase), *ERG1* (squalene epoxidase), *ERG7* (lanosterol synthase), *ERG11* (lanosterol 14α demethylase), and *ERG24* (C-14 reductase), are essential, while genes participating the late steps of ergosterol biosynthesis, such as *ERG2*, *ERG3*, *ERG6*, *ERG5*, *ERG25*, and *ERG4*, are not essential (Fryberg et al., 1973; Lees et al., 1995). In *N. crassa*, viable homokaryotic mutants for only these non-essential genes were generated. Thus, the end-product ergosterol is not necessary for fungal survival. When ergosterol is absent, other sterol intermediates might function as substitutes of ergosterol. Similar to previous observation in *S. cerevisiae* (Kelly et al., 1995), our data showed that disruption of lanosterol 14α-demethylase by ketoconazole reduced 4,4-dimethyl-ergosta 8,14,24(28)-trienol biosynthesis and accumulated 14α methylated sterols such as 14α-methyl-ergosta-8,24(28)-dien-3,6-diol. ERG5 catalyzes the chemical reaction downstream to 4,4-dimethyl-ergosta 8,14,24(28)-trienol biosynthesis. As shown in **Figure 2**, deletion of *erg5*

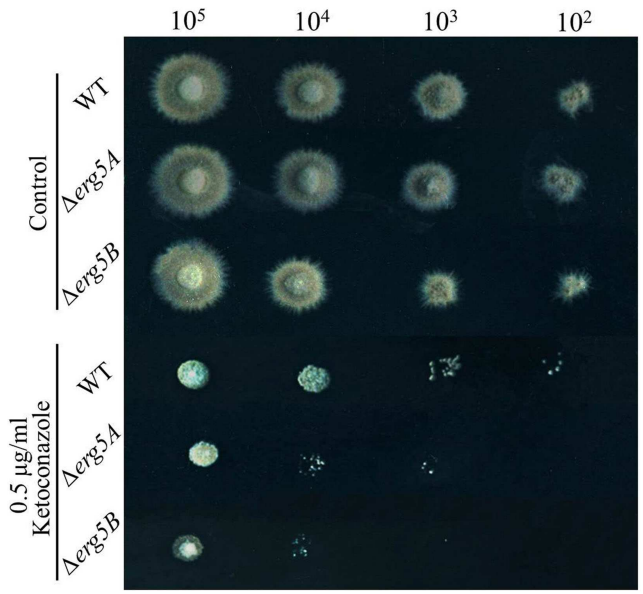

FIGURE 4 | Drug susceptibility analysis of the *erg5A* (FVEG_07284) and *erg5B* (FVEG_08786) knockout mutants of *F. verticillioides*. Two microliters of conidial suspensions with different concentration (1 × 105, 1 × 104, 1 × 103, or 1 × 102 conidia/ml, respectively) were inoculated on the plates (Φ = 9 cm) with or without antifungal drugs, and incubated at 28°C for 72 h. Each test had three replicates and the experiment was independently repeated twice.

is not likely to reduce the biosynthesis of 4,4-dimethyl-ergosta 8,14,24(28)-trienol under ketoconazole stress. Therefore, the azole-hypersensitive phenotype in *erg5* mutants is not related to the biosynthesis of 4,4-dimethyl-ergosta 8,14,24(28)-trienol. In addition, deletion of *erg5* did not lead an increase in accumulation of 14α-methylated sterols upon ketoconazole stress. Thus, over-accumulation of 14α-methylated sterols is also not a possible cause to the azole-hypersensitive phenotype in *erg5* mutants. An *in vitro* experiment has shown that ketoconazole can bind to ERG5 (CYP61) of *S. cerevisiae* with an affinity similar to that of ERG11 (Kelly et al., 1997). Binding of azole to ERG5 might reduce the concentration of azoles attacking ERG11, providing a protective buffer to ERG11. If it is true, removal of *erg5* will increase azole sensitivity. In addition, when ergosterol is absent, localization of some membrane proteins, such as azole pumps, might be affected and make cells accumulate more azoles. The real mechanism requires further investigation.

In summary, this study reported azole sensitivities of knockout mutants for 6 azole-responsive genes involved in ergosterol biosynthesis and demonstrated that deletion of *erg5* increase azole sensitivity in both *N. crassa* and *F. verticillioides*. Our findings provide a new insight into in the mechanism of azole resistance.

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Who possesses drug resistance genes in the aquatic environment?: sulfamethoxazole (SMX) resistance genes among the bacterial community in water environment of Metro-Manila, Philippines

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Recent evidence has shown that antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are ubiquitous in natural environments, including sites considered pristine. To understand the origin of ARGs and their dynamics, we must first define their actual presence in the natural bacterial assemblage. Here we found varying distribution profiles of *sul* genes in "colony forming bacterial assemblages" and "natural bacterial assemblages." Our monitoring for antibiotic contamination revealed that sulfamethoxazole (SMX) is a major contaminant in aquatic environments of Metro-Manila, which would have been derived from human and animal use, and subsequently decreased through the process of outflow from source to the sea. The SMX-resistant bacterial rate evaluated by the colony forming unit showed 10 to 86% of the total colony numbers showed higher rates from freshwater sites compared to marine sites. When *sul* genes were quantified by qPCR, colony-forming bacteria conveyed *sul1* and *sul2* genes in freshwater and seawater (10^{-5}–10^{-2} copy/16S) but not *sul3*. Among the natural bacterial assemblage, all *sul1*, *sul2*, and *sul3* were detected (10^{-5}–10^{-3} copy/16S), whereas all *sul* genes were at an almost non-detectable level in the freshwater assemblage. This study suggests that *sul1* and *sul2* are main *sul* genes in culturable bacteria, whereas *sul3* is conveyed by non-culturable bacteria in the sea. As a result marine bacteria possess *sul1*, *sul2* and *sul3* genes in the marine environment.

Keywords: sulfonamide resistance, sul gene, non-culturable, marine, aquatic environment

INTRODUCTION

Antibiotic resistant bacteria (ARB) are selected under low concentrations of antibiotics (Gullberg et al., 2011), in which the mutant selection window (MSW) comprising a range of concentrations where resistant bacteria can be selectable is designated (Drlica, 2001). Low concentrations of antibiotics selects for low-level ARB due to an adaptive mutation, which can sometimes result in high-level resistance (Baquero, 2001). Such a situation can be found in natural aquatic environments. Thus, the bacterial response to very low concentrations of antibiotics in the environment has been of particular focus (Gullberg et al., 2011), and the response behavior of environmental bacteria under MSW has been reviewed (Andersson and Hughes, 2011). However, a majority of environmental bacteria, especially in the ocean, are non-culturable (Colwell and Grimes, 2000), and hence it is necessary to study ARB including the non-culturable community while simultaneously monitoring antibiotic concentrations.

Antibiotics used in human and animal medicine are released through manure, wastewater and subsequently to rivers, lakes, and oceans, the culmination of which is a major concern in the development of ARB. Although the released antibiotics are diluted and degraded in the ocean, trace level concentrations can potentially select for and preserve ARB, even in areas considered to be pristine. Our previous studies have shown the potential for ARB and antibiotic resistance genes (ARGs) to be reserved in natural aquatic environments and even in non-contaminated areas (Rahman et al., 2008; Tamminen et al., 2011). It is generally understood that clinically derived ARGs are a risk to patients; however, whether the ARGs found in the environment are a risk to humans is not understood. To clarify the origin, movement and preservation of ARGs in natural environments and to assess risk, it is necessary to quantitatively track ARGs from terrestrial water ways to the ocean. Moreover, concentrations of antibiotics in various water ways should be quantified as a baseline to see correlation between antibiotic contamination and the occurrence of ARGs.

Among antibiotics used throughout tropical Asia, sulfonamides have been widely applied in human and animal medicine, with previous studies showing their bacterial resistance rate to be higher (2–90%; Hoa et al., 2011) than tetracycline (0.07–0.18%; Kobayashi et al., 2007) and quinolone (0.1–15%; Takasu et al., 2011). Sulfonamides have a low chelating ability, low

binding constants, high water solubility, and stability (Sukul and Spiteller, 2006). Therefore, once sulfonamides are released into the aquatic environment, they remain active against bacteria due to their chemical characteristics, and can furthermore accelerate the development of ARB in natural microbial communities.

Although contamination by antibiotics and ARB in Vietnam, Thailand and other countries of Indochina has recently been summarized (Suzuki and Hoa, 2012), the extent to which this occurs in other tropical Asian areas is not known. As for aquatic environments in the Philippines, the occurrence of ARB has previously been reported (Tendencia and de la Peña, 2001; Kim et al., 2003), however, despite the frequent use of sulfonamides in humans and animals, sulfonamide resistance has not been addressed.

As mentioned above, to clarify the role of the environmental bacterial assemblage on the dynamics of ARGs, the full community including non-culturable bacteria should be targeted. Here we monitored antibiotic concentrations and sulfonamide resistance genes through a lake, river and bay system within Metro-Manila. Furthermore, comparison of culturable to the total bacterial assemblage provided new evidence that the possession profile of ARGs (*sul*) in bacteria varied between freshwater and marine bacteria.

MATERIALS AND METHODS

SAMPLING

Water samples were taken at four sites in Laguna Lake (st. MNL-1 ~4), two sites in the Pasig River (st. MNR-1 and 2) and four sites in Manila Bay (st. MNB-1 ~4), in November 3–4, 2009, in Metro-Manila, the Philippines (**Figure 1**). Among the lake sites, MNL-4 was closes to land relative to the other MNL sites.

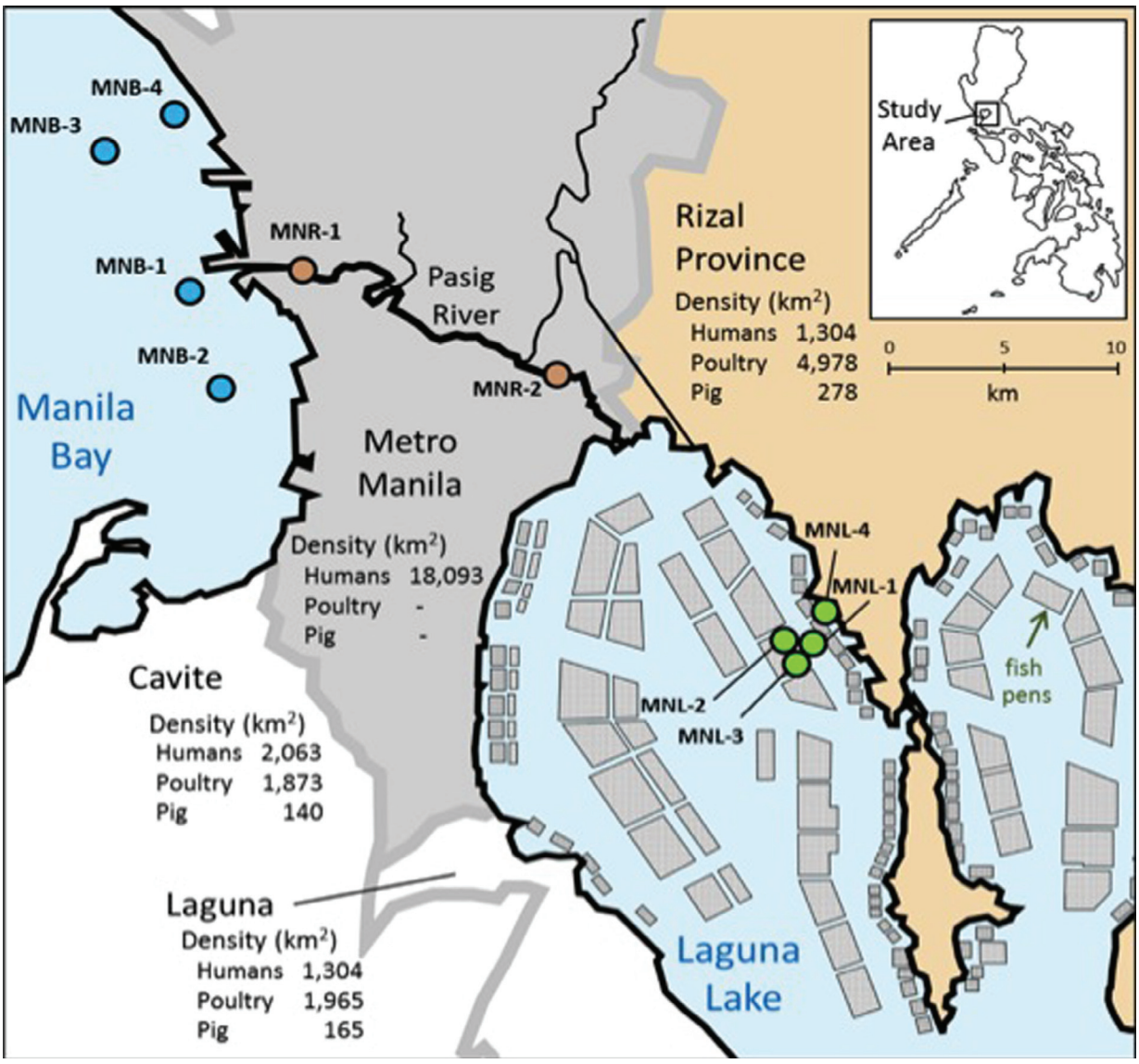

FIGURE 1 | Map of sampling sites in Metro-Manila. Surface water was taken from four sites (MNL-1~4) in Laguna Lake, two sites (MNR-1, 2) in the Pasig River, and four sites (MNB-1~4) in Manila Bay. Conditions in surrounding are inserted on map. Squares in Laguna Lake denote areas of aquaculture net pens.

From the river sites, MNR-2 was in the upper river location closest to Laguna Lake and –1 was the lower site closer to Manila Bay. The marine Manila Bay site MNB-1 was near the mouth of the Pasig River whereas MNB-4 was closer to land with a very high density of people with low capacity for wastewater control. For the entire study area, information on human population densities and agriculture activities are shown in **Figure 1**. Sampling occurred one month after Typhoon Ondoy, and there was still substantial high-flood levels in Laguna Lake that flooded well within the surrounding towns. For all sites, surface water was taken by alcohol-sterilized bucket, and stored in a sterilized polypropylene bottle for the bacterial experiment and a glass bottle for antibiotic analysis. All samples were transported on ice and taken to the lab within several hours for the experiment. At each site environment measurements of salinity, pH and temperature were taken by a pH/conductivity meter (Horiba D-54, Horiba, Kyoto, Japan). Total suspended solids (TSS) were measured by filtering 50 ml of subsurface water samples through a pre-weighed 47 mm glass fiber filter (GF/F, retention $>0.7\,\mu$m), then drying in a drying oven at 60°C for 24 h. The filter was then allowed to cool and be reweighed in a temperature/humidity controlled room. The TSS was calculated as the added weight in mg to the filter divided by the total volume of water filtered (mg/ml).

ANTIBIOTIC CONCENTRATIONS

Target antibiotics of oxytetracycline (OTC), sulfamethoxazole (SMX), sulfamethazine, trimethoprim, and lincomycin, were all analyzed according to Ye et al. (2006). Briefly, the antibiotics were solid-phase extracted by using Oasis HLB (200 mg; Waers) and the extracts were analyzed by liquid chromatograph (Agilent series 1100, Tokyo, Japan) equipped with a tandem mass spectrometer (LC-MS/MS; TSQ Quantum 7000, Thermo Finnigan, Japan). The antibiotics quantified by LC-MS/MS were separated in a Xterra MS C18 (2.1 mm i.d. × 50 mm; particle size: 2.5 μm; Waters) with a guard column (Xterra MS C18; 2.1 mm i.d. 20 mm; particle size: 3.5 μm; Waters) by using a binary gradient system (solvent A: 1% formic acid in H_2O; solvent B: acetonitrile) at a flow rate of 0.2 ml/min. The run started at 5% B for 5 min, followed by a 11-min linear gradient to 95% B, then the initial conditions were reestablished and the column was equilibrated for 17 min. Analytes were quantified in selected reaction monitoring mode on positive electrospray, ESI positive mode. This procedure was used for other cases reported in Managaki et al. (2007) and Hoa et al. (2011).

BACTERIAL COUNTS

The colony-forming bacteria were counted on nutrient agar (NA) plates. Organic nutrients may affect the sensitivity on SMX. We have confirmed that nutrient concentration in NA did not have an effect on susceptibility of bacteria using a sensitive strain (*E. coli* AG1). Each water sample (0.5 ml) was 10-fold serially diluted with 4.5 ml of phosphate-buffered saline (PBS, pH 7.4). A 100-μl of aliquot was spread on Nutrient Broth (Difco Laboratories, Detroit, MI) plus 1.5% agar and 0.5% NaCl plate, and incubated at 30°C for 7 days in duplicates. NA plates containing 60 μg/ml of SMX or oxytetracycine (OTC) were used to enumerate SMX-resistant (SMX^r) and OTC-resistant bacteria. For total cell count, glutaraldehyde was added to 1 ml of sample to a final concentration of 2% to fix the cells. After filtration with a black polycarbonate filter (0.2 μm pore size, Millipore, Billerica, MA, USA), bacterial cells were stained with 5 μg/ml ethidium bromide. The bacteria were enumerated by epifluorescence microscopy (BX60, Olympus Co., Tokyo, Japan). More than 300 cells were enumerated, and a minimum of 20 fields were randomly selected. However, appropriate enumeration could not be done from lake and river (upper reach) samples due to interference by small clay particles.

DNA PREPARATION

Extraction of DNA was performed from filters and from mixtures of colonies on plates. To obtain DNA from the total bacterial assemblage, an appropriate amount of water samples (30–200 ml) was filtered through 47-mm polycarbonate filters (0.2 μm pore size, Millipore), which were kept at −20°C and transported to the laboratory. Triplicate filters were used for DNA extraction. All colonies that appeared on agar plates with SMX were suspended in PBS and cells were harvested by centrifugation. The cells were kept at −80°C until DNA extraction. The DNA of culturable SMX^r bacteria was obtained from this sample. The extraction of DNA from filters and mixtures of colonies was carried out according to the cethyltrimethylammonium bromide (CTAB)-method (Wilson, 1987) with some modification. Briefly, thawed filters were dipped in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing sodium dodecyl sulfate (SDS, 0.5%), Proteinase K (0.1 mg/ml, TaKaRa, Otsu, Japan) and RNase A (0.05 mg/ml, SIGMA-ALDRICH, St. Louis, MO, USA). The filter was incubated at 37°C for 1 h. To remove polysaccharides, a CTAB/NaCl solution (10% CTAB and 0.7 M NaCl) was added, and the samples were incubated at 65°C for 10 min. The freeze-thawing was repeated with three cycles of freezing at −80°C and thawing at 65°C to increase the recovery of DNA from bacterial cells. Subsequently, an equal volume of phenol-chloroform-iso amyl alcohol (25:24:1) was added, and the tubes were inverted and centrifuged at 2100 ×g at 4°C for 10 min. The supernatant was divided between two 1.5-ml tubes, and an equal volume of chloroform-iso amyl alcohol (24:1) was added. The tubes were inverted and centrifuged at 21,600 ×g for 10 min at 4°C, and supernatant was collected in another 1.5-ml tube. The samples were precipitated with an addition of 0.1 volume of 3 M sodium acetate and then a 0.6 volume of iso-propanole. The precipitated pellets were dried under vacuum and dissolved in 50 μl of sterilized Milli-Q water. The recovered DNA was quantified by ultraviolet absorption meter (DU640, BECKMAN COULTER, Orange County, CA, USA), and the quality of the DNA was checked by electrophoresis on 1.0% agarose gel with ethidium bromide staining.

DENATURED GRADIENT GEL ELECTROPHORESIS (DGGE)

Community structure was estimated by DGGE targeting 16S rRNA gene, and the dominant microbial diversity was compared with banding profile (Boon et al., 2002; Takasu et al., 2011). Total DNA was purified from a filter (as environmental DNA) and pooled-colony on NA plate (as culturable bacterial DNA). PCR and electrophoresis conditions were the same as Muyzer et al. (1993). The representative bands, common ones and specific

ones, on the DGGE gel were cut out from the gel and were sequenced.

QUANTITATIVE PCR

Quantitative PCR was performed using a CFX 96 Real-Time system (BioRad Laboratories, Hercules, CA, USA) to detect an increase of double-stranded DNA with an increase in fluorescence. PCR amplifications were performed in a 20 μl reaction volume containing 1× Sso Fast EvaGreen Supermix (BioRad), 500 nM of each primer and 1 μl of sample DNA. Quantitative PCR was performed using previously designed primers; bacterial 16S rRNA genes (Suzuki et al., 2000), *sul*1 (Heuer and Smalla, 2007), *sul*2 (Heuer et al., 2008), and *sul*3 (Pei et al., 2006). Serial 1:10 dilutions of plasmids constructed from the pGEM-T Easy vector (Promega, Madison, WI, USA) and 16S rRNA gene from *E. coli* K12, *sul*1 from plasmid R388, *sul*2 from plasmid RSF1010 and *sul*3 from plasmid pUVP4401 fragments were used as standards for quantification (Heuer and Smalla, 2007). The qPCR program consisted of an initial denaturation of 30 s at 95°C and 40 cycles of 5 s at 95°C (denaturation) and 10 s at 50°C for 16S rRNA gene and 10 s at 51°C for *sul*1 and *sul*2 and 20 s at 60°C (extension) for *sul*3 respectively. Melting curves for the amplicons were measured by raising the temperature slowly from 60°C and 65°C to 95°C for 16S rRNA gene, *sul1, sul2,* and *sul3*, respectively, while monitoring fluorescence (**Figure A1**). Each sample was measured in triplicate. The copy numbers of *sul1*, *sul2,* and *sul3* were normalized by dividing by the 16S rRNA gene copy number at the respective time points to take into account any temporal variation in bacterial cell numbers. Unit of the copy number is described as copies/16S through text. The results were analyzed using a Big Dye terminator kit on a 3130 ABI Prism sequencer (Applied Biosystems, Foster City, CA, USA). PCR products were sequenced and phylogenetic relationship among *sul1, sul2,* and *sul3* was analyzed.

DNA SEQUENCING

PCR product of *sul* genes and 16S rRNA genes on DGGE were sequenced to show phylogenicity of these genes. Purified PCR products were sequenced on an ABI Genetic Analyzer 3130 (Applied Biosystems) with BigDye Terminator, version 3.1. The sequencing primers for *sul* gene were the same as above and 341f was for 16S rRNA gene. Sequences were aligned with known sequences in the DDBJ database using BLAST. Phylogenetic relationships were inferred by pairwise comparison and the neighbor joining method using Clustal X (Thompson et al., 1997). Phylogenetic trees were edited using Treebiew (Page, 1996), and out groups were *E. coli* dihydropteroate synthase (DHPS) (accession number: CP001637) for *sul* genes and *Ketogulonigenium vulgrum* WSH-001 (accession number: NC-17384) for 16S rRNA gene. The accession numbers of the newly sequenced *sul* genes were shown in **Figure 3**.

RESULTS AND DISCUSSION

ENVIRONMENTAL CONDITION

We systematically sampled water from Laguna Lake to Manila Bay (**Figure 1**). Laguna Lake is the largest freshwater body in the Philippines (911 km^2) and the extensive area is used for fish pen-aquaculture as shown in **Figure 1**. The north side of Laguna Lake is Rizal Province, where the density of animal farms is high. Water from Laguna Lake flows out through the Pasig River to Manila Bay, with the Pasig River running through and receiving wastewater from Metro-Manila, the highest populated area of the Philippines. Physico-chemical conditions of sampling sites are summarized in **Table 1**. In Manila Bay, we collected surface water from four sites. The MNB-1 site is located near the mouth of the Pasig River, where salinity was lower than the other bay sites (**Table 1**). Since the sampling period was only one month after Typhoon Ondoy, surface seawater salinity indicated freshwater flow into the bay. The lake and river water samples contained clay particles, which interfered with cell counting under the microscope, whereas this was extensively lower in the bay samples due to dilution, aggregation and sedimentation.

ANTIBIOTICS CONTAMINATION

It has been reported that more developed countries frequently use macrolides as major antibiotics for humans and animals, whereas

Table 1 | Environmental and microbial data at sampling sites.

Site	Water temp (°C)	pH	Salinity	Suspended solid (mg/l)	EtBr* count (cells/ml)	Viable count (cells/ml)	SMX^r count (cells/ml) (%)***
MNL-1	27.0	8.10	0	86.5	CI**	1.6×10^4	2.7×10^3 (16.9)
MNL-2	27.4	8.16	0	94.6	CI	2.1×10^4	3.8×10^3 (18.1)
MNL-3	27.4	8.09	0	92.5	CI	1.5×10^4	2.8×10^3 (18.7)
MNL-4	27.2	7.49	0	72.3	CI	4.7×10^4	1.2×10^4 (25.5)
MNR-2	27.0	7.81	0	66.8	CI	5.5×10^4	2.2×10^4 (40.0)
MNR-1	26.0	7.80	0	114.2	3.37×10^5	3.4×10^5	7.1×10^4 (20.9)
MNB-1	25.0	8.09	16	54.7	1.04×10^6	1.4×10^5	4.9×10^4 (35.0)
MNB-2	25.4	8.11	25	16.3	9.98×10^5	1.3×10^4	2.6×10^3 (13.0)
MNB-3	25.3	7.85	27	24.3	1.66×10^6	1.8×10^4	1.9×10^3 (10.6)
MNB-4	25.4	7.74	27	13.4	1.61×10^6	3.0×10^4	2.6×10^4 (86.7)

Ethidium bromide.

**Count impossible due to clay particles having self-fluorescence.*

***% was calculated by SMX^r count/viable count × 100.*

Asian developing counties use more inexpensive drugs such as sulfonamides (Managaki et al., 2007; Luo et al., 2011). We monitored OTC, SMX, sulfamethazine, trimethoprim and lincomycin in this study. As shown in **Table 2**, OTC was not detected from all of the sites, whereas SMX was a major antibiotic in all sites followed by sulfamethazine. Trimethoprim, which is usually used as a combination drug with SMX, and lincomycin were lower in concentration than sulfonamides. This profile is the same as that observed from Vietnam (Managaki et al., 2007) and China (Luo et al., 2011). The concentration gradient was the same for all antibiotics, i.e., concentration was highest in the river mouth (MNB-1) and the lower reach of the river (MNR-1), Laguna Lake (MNL-1 ∼4) showed similar concentrations among sites (27.8–41.6 ng/l for SMX), and the open Manila Bay marine sites MNB-2 to MNB-4 showed similar concentrations (7.8–17.7 ng/l for SMX). The SMX concentrations detected at the ng/l level in Laguna Lake and Pasig River sites were much lower than the inhibition concentration for susceptible bacteria. For sulfonamide antibiotics, concentrations of 10–400 mg/l could inhibit microbial activity, which is found in activated sludge by more than 20% (Ingerslev and Halling-Sørensen, 2000).

MICROBIAL NUMBER AND DIVERSITY

The total cell number was enumerated by ethidium bromide staining, showing 10^5–10^6/ml from the Pasig River and Manila Bay sites (**Table 1**). As mentioned above, microscope cell counts were impossible for the Laguna Lake and the upper reach of the Pasig River due to interference from clay particles. Viable cell numbers by colony counting showed that lake and bay sites were 10^4-levels/ml, and one order of magnitude larger in river-related sites (MNR-2, -1 and MNB-1) (**Table 1**). Microbial diversity was estimated in the DGGE method. The DGGE profiles of all colonies pooled from NA agar plate (**Figure 2A**) and of the water assemblage from filters (**Figure 2B**) was obtained. Representative common and specific bands were sequenced (**Figure 3**). Among the cultured colony, common species of γ-Proteobacteria closed to *Pseudomonas* (band number 1, 2, 3, 7, 11, 12, and 13) and *Alteromonas* (band number 5, 6, 8, 10, and 15) were detected through the lake, river, and bay system. These genera could be detected as an abundant group in the water column by the culture method (Fuhrman and Hagström, 2008). Environmental assemblage samples showed different profiles. Cyanobacteria related bands were commonly detected through Laguna Lake, Pasig River and Manila Bay (band number 16, 18, 21, 22, 24, 28, and 31). Abundant common species from the DGGE profiles varied between culturable bacteria and the assemblage. This suggests that culturable bacteria are not a major component of the natural assemblage, which are selected by the culture method. Thus, the detection of ARB by culture further suggests selection of ARGs from the total community. Enteric bacteria can be commonly detected from freshwater systems (Hoa et al., 2008, 2011; Hu et al., 2008), suggesting freshwater ARB most likely occur from aquatic, terrestrial and enteric species. Although the present study did not show enteric species among the sequenced band of DGGE, we still cannot ignore the possibility of horizontal gene transfer (HGT) across the bacterial community including enteric/terrestrial bacteria. In-part this is because of the high survival of enteric bacteria in water over time (Vital et al., 2008). We have previously suggested that species diversity may relate to a high ARG reserving potential (Suzuki et al., 2008). If physical turbulent mixing is occurring, the community may be resilient to the event and may recover over a short period, such as on a scale of days (Shade et al., 2012). Turbulence may also have an effect on ARG transfer among the community by quick HGT, which can occur within as short of a period as 60 min (Andrup and Anderson, 1999).

SMX^r BACTERIA

SMX^r rate is shown in **Table 1**. Laguna Lake sites (MNL-1∼4) showed an SMX^r rate of 16.0–25.5%, which increased to 20.9–40.0% in the Pasig River sites (MNR-2, -1, and MNB-1). Manila Bay sites (MNB-2 and -3) showed rates of 10.6–13.0% with the exception at MNB-4 of 86.7%. The notably higher percentage at MNB-4 may have been due to the fact that this site was very close to a land area with high-density housing and poor wastewater control, where unknown substances and/or human derived bacteria may have contaminated the sample water. From SMX^r rate (**Table 1**) and drug concentration (**Table 2**), it can be seen that SMX was likely urban wastewater runoff (point and non-point sources) to the river and subsequently diluted in the sea. The SMX^r bacterial rate was highest from lake and river sites,

Table 2 | Antibiotic concentrations at sampling sites.

Site	OTC (ng/l)	SMX (ng/l)	Sulfamethazine (ng/l)	Trimethoprim (ng/l)	Lincomycin (ng/l)
MNL-1	<LOQ*	37.8	8.6	1.7	2.4
MNL-2	<LOQ	28.8	8.3	2.3	2.6
MNL-3	<LOQ	27.8	9.2	2.1	2.2
MNL-4	<LOQ	41.6	16.2	2.5	2.6
MNR-2	n.d.	46.6	12.0	2.3	5.5
MNR-1	<LOQ	79.8	73.1	9.2	7.4
MNB-1	<LOQ	93.8	61.1	18.3	6.7
MNB-2	n.d.	11.7	2.4	2.3	0.83
MNB-3	n.d.	7.8	3.9	1.6	0.80
MNB-4	n.d.	17.7	4.6	5.8	6.5

**LOQ, limit of quantitation, where compound was detected as a peak but not quantitative concentration; n.d., not detectable.*

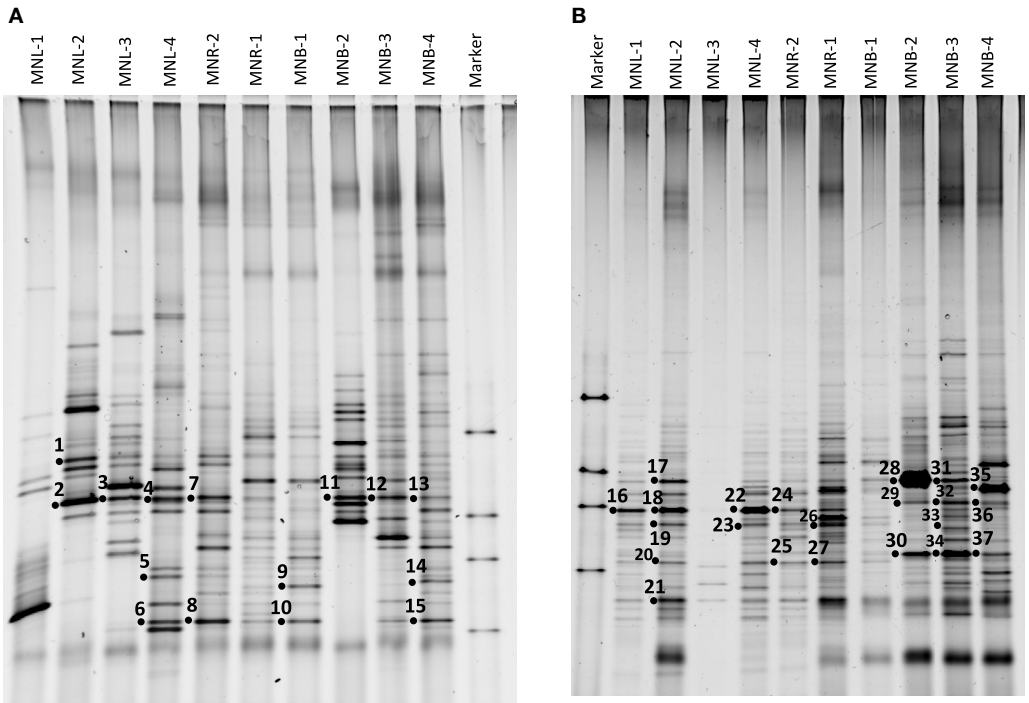

FIGURE 2 | DGGE analysis of each site using a primer set for 16S rRNA gene. (A) Cultured bacterial community, and **(B)** Natural environmental assemblage. Numbers are sequenced bands.

suggesting that SMX^r-culturable bacteria were minor in seawater. Correlation between concentrations of antibiotics and ARG (*sul1*) has been reported for wastewater treatment plants, WWTP (Gao et al., 2012b), suggesting that higher concentrations select ARB in the case of sulfonamides. In the WWTP reported by Gao et al. (2012b), 191 ng/l SMX was detected even in effluent, which is possibly an effective concentration for bacteria. Bacteria continuously exposed to higher concentrations of antibiotics should contain higher copies of *sul* genes, which spread among the community. The *sul* genes were reported from enteric and environmental bacteria, being mostly culturable species. However, particular attention should be given to non-culturable bacteria as an AGR reservoir. The present study examined the distribution of *sul* genes with qPCR among waters as well as colony forming assemblages as shown below.

QUANTIFICATION OF *sul* GENES

Total DNA was recovered from "SMX^r-colony forming bacterial assemblages" and "natural bacterial assemblages" which were purified from 0.2 μm Nuclepore filters. Detection of *sul* genes is shown in **Figure 4**. Colony forming bacteria possessed *sul1* as the major *sul* gene from Laguna Lake samples, and *sul2* was detected at a similar level from Pasig River and Manila Bay samples (**Figure 4A**). The *sul3* was not detected from all sites with the exception of MNR-1. Exceptionally high copies found in MNR-1 may have been due to colony formation of *sul3* possessing bacteria. The present study and other studies indicated that *sul3* is a minor sulfonamide resistance determinant in bacterial isolates in the aquatic environment (Hoa et al., 2008; Su et al., 2011). The origin of *sul3* is suspected to be human (Grape et al., 2003), however, Su et al. (2012) recently reported a high incidence (<40%) of *sul3* in environmental SMX-trimethoprim^r *E. coli*, suggesting that the distribution of *sul3* varies among environments. On the other hand, *sul* detection profiles from natural assemblages were quite different. Both *sul1* and *sul2* were mostly under the detection limit from lake and river sites (**Figure 4B**). This suggests that although *sul* genes are a minor gene in lake and river bacterial communities, the cultivation procedure effectively selected the *sul* possessing bacteria from the total assemblage. Gao et al. (2012a) recently reported that a natural assemblage from an aquaculture site and measured for several isolates, showed that SMX^r bacteria were found in a relatively wide group whereas *sul1* and *sul2* were detected in restricted species. In contrast to freshwater lake and river sites, bay site samples showed 10^{-5} to 10^{-3} copies/16S for all *sul1, sul2,* and *sul3*. The quantitative detection of *sul* genes in the marine environment has not been reported, and the present results are the first to indicate that the total bacterial assemblage possesses not only *sul1* and *sul2* but also *sul3*. It is thus clear that *sul* genes including *sul3* are relatively abundant in the marine bacterial assemblage, the majority of which are the non-culturable bacteria.

We sequenced *sul* genes from culturable bacteria and an environmental assemblage. Results showed no difference among each *sul1* and *sul2* gene (**Figure 5**). The *sul3* from culturable bacteria in MNR-1 and the seawater sample in MNB-4 were slightly different. This suggests that each *sul* group is possibly homogenous around this area.

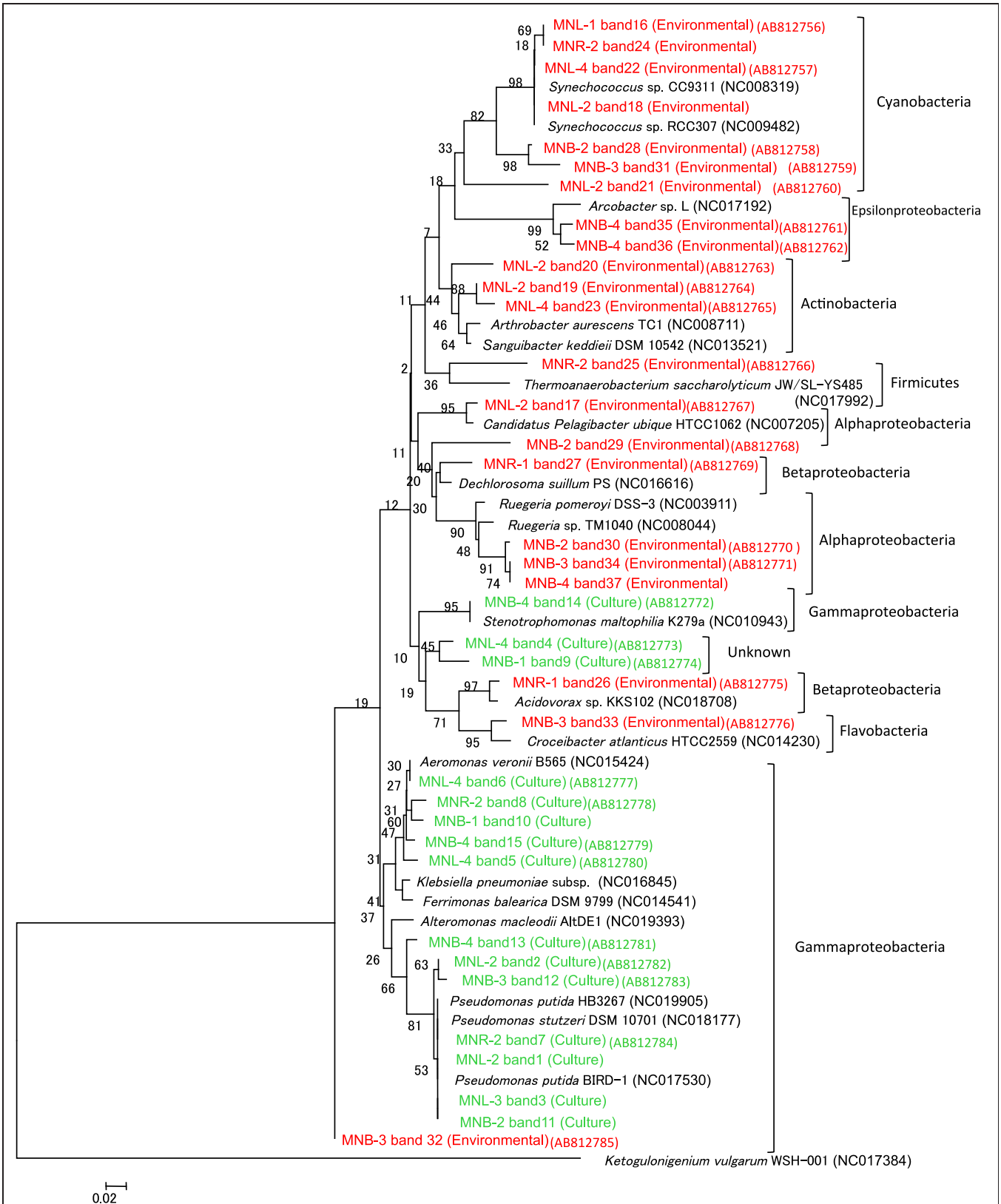

FIGURE 3 | Phylogenetic tree of 16S rRNA gene in DGGE (Figure 2). Representative common and specific 37 bands were sequenced. Green color is DNA of the pooled colony (Culture) and red color is DNA of the environmental water assemblage (Environmental).

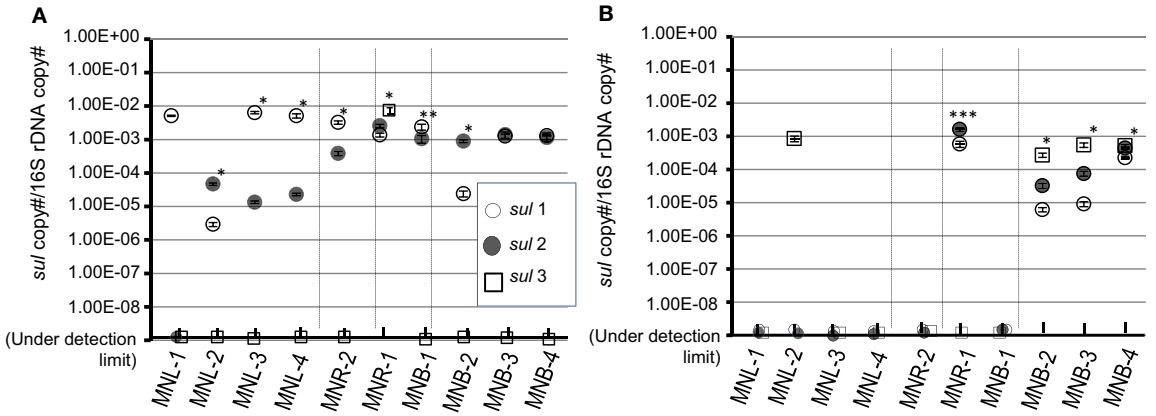

FIGURE 4 | Copy number of *sul* genes in a pooled-colony of SMX^r bacteria (A) and that of a natural total assemblage (B). Copy number was normalized by 16S rRNA gene (16S). Symbols are denoted as: open circle is *sul1*, closed circle is *sul2* and open square is *sul3*. SD from triplicate experiments is shown with vertical bar with symbol. *, significantly different three genes (ANOVA, $p < 0.001$) or two genes (*t*-test, $p < 0.001$); **, significantly different two genes (*t*-test, $p = 0.013$); ***, significantly different two genes (*t*-test, $p = 0.002$); no asterisk, not significantly different or only single gene was detected.

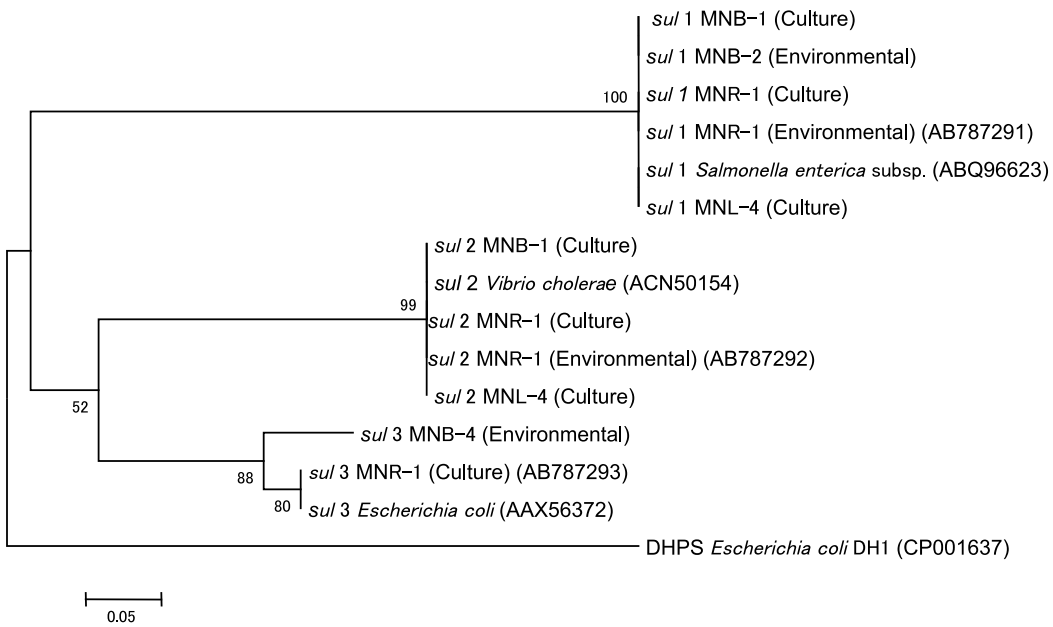

FIGURE 5 | Phylogenetic tree of *sul1, sul2,* and *sul3* from culturable bacteria (Culture) and the environmental water assemblage (Environmental).

The *sul* genes are frequently found on plasmids with integrons, suggesting these are transferable among bacteria (Sholz et al., 1989; Hall and Collis, 1998), although SMX^r based on chromosomal mutation is also known (Gibreel and Sköld, 1999). The present study used known PCR-primers to detect *sul* genes, suggesting the *sul* genes in seawater are the same as those genes prevalent among enteric and soil bacteria (Pei et al., 2006; Heuer and Smalla, 2007; Heuer et al., 2008). Thus, the origin(s) of the genes are suspected to be human and terrestrial bacteria, and the genes have a pathway and potential to be transferred to marine bacteria. The opposite HGT can be occurred from marine *Vibrio* to *E. coli* in OTC resistance gene (Neela et al., 2008). Generally ARGs are thought to originate within antibiotic producing bacteria, which are horizontally transferred to environmental microbes; known as the "producer hypothesis" based on Benveniste and Davies (1973). However, since sulfonamides are synthetic small molecules and there are no producers in the environment, the sulfonamide case does not fit the "producer hypothesis." Sulfonamide resistance genes have been suggested to be a fixed-mutation, which have been proposed to be a reservoir of ARGs (Sköld, 2010), although the *in situ* HGT between environmental bacteria and human pathogenic/enteric bacteria is unclear.

Recent evidence shows that enteric bacteria can survive and grow in aquatic environments by biofilm formation (Soreira et al., 2012) and also in soil (Byappanahalli et al., 2012). The bacteria conveying the *sul* genes with class 1 and 2 integrons predominantly originate from the discharge of wastewater (Su et al., 2012). Therefore, the contaminated bacteria having *sul* genes are mainly of human, animal and terrestrial origin, which transfer the genes to marine bacteria including non-culturable ones. The *sul1, sul2,* and *sul3* found in the seawater assemblage thus should flow into the sea and eventually accumulate in the marine bacteria community.

It is known that ARGs are circulated among animals and humans (Wooldridge, 2012). Once ARGs have become fixed in a bacterium, they are difficult to eliminate (Andersson and Hughes, 2011). We have reported that the OTC resistance gene *tet*(M) is distributed even in pristine ocean sediments (Rahman et al., 2008). This evidence also supports that ARGs in the marine environment would be of human and terrestrial origin, and ultimately fixed into the marine bacterial assemblage. Our study focusing on non-culturable bacteria in relation to ARGs is a useful approach to reveal potential reservoirs of ARGs in natural environments, with the potential of including different hosts in different environments.

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APPENDIX

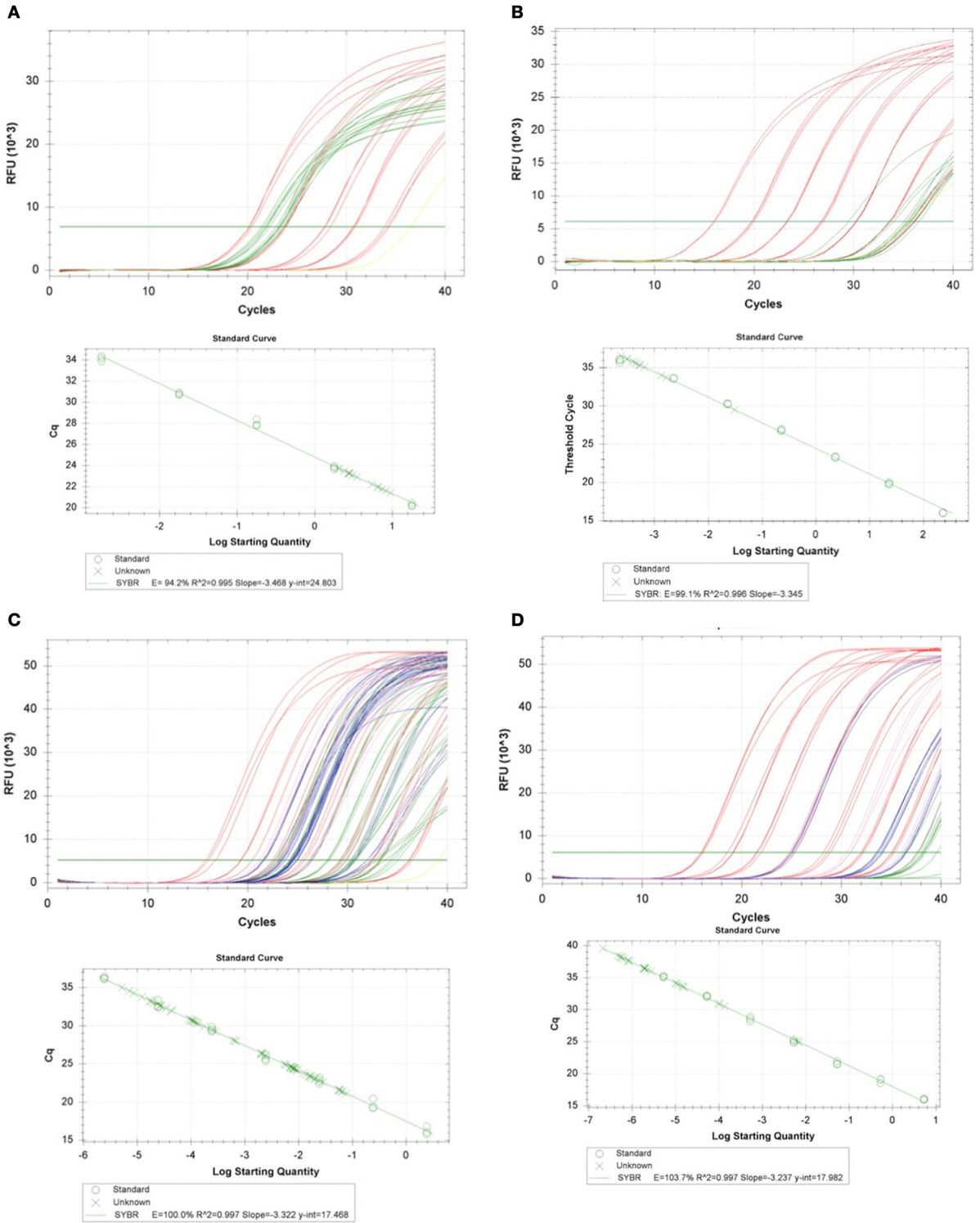

FIGURE A1 | (A) qPCR standard curve, 16S rRNA gene (Colony DNA) Amplification efficiency = 94.2%, Linearity (R^2) = 0.995. **(B)** qPCR standard curve, 16S rRNA gene (Filter DNA) Amplification efficiency = 99.1%, linearity (R^2) = 0.996. **(C)** qPCR standard curve, sul1 gene (Colony DNA) Amplification efficiency = 100.0%, linearity (R^2) = 0.997. **(D)** qPCR standard curve, sul1 gene (Filter DNA) Amplification efficiency = 103.7%, linearity (R^2) = 0.997.

(Continued)

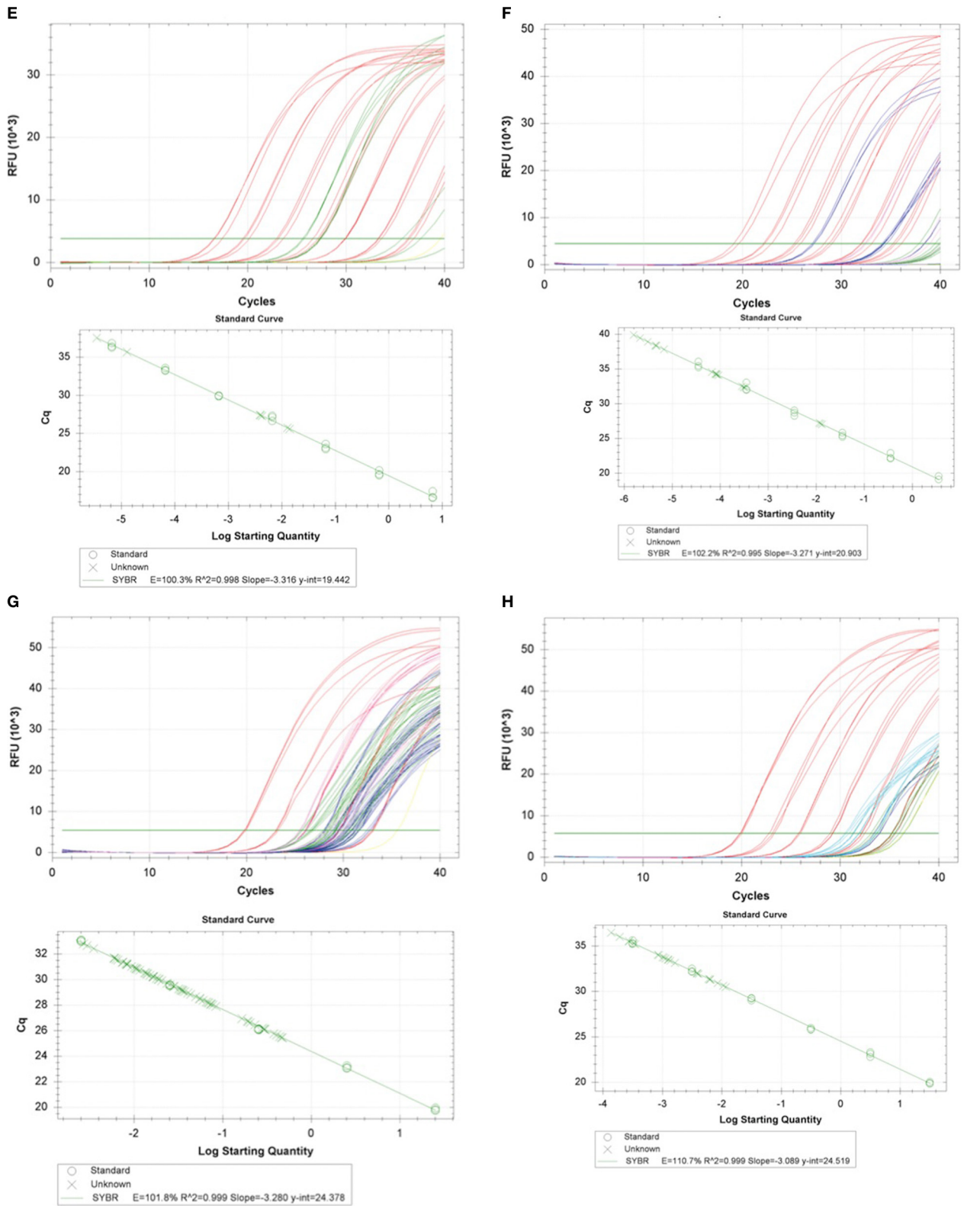

FIGURE A1 | Continued
(E) qPCR standard curve, sul2 gene (Colony DNA) Amplification efficiency = 100.3%, linearity (R^2) = 0.998. **(F)** qPCR standard curve, sul2 gene (Filter DNA) Amplification efficiency = 102.2%, linearity (R^2) = 0.995. **(G)** qPCR standard curve, sul3 gene (Colony DNA) Amplification efficiency = 101.8%, linearity (R^2) = 0.999. **(H)** qPCR standard curve, sul3 gene (Filter DNA) Amplification efficiency = 110.7%, linearity (R^2) = 0.999.

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Effect of antimicrobial exposure on AcrAB expression in *Salmonella enterica* subspecies *enterica* serovar Choleraesuis

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Understanding the impact of antimicrobial use on the emergence of resistant bacteria is imperative to prevent its emergence. For instance, activation of the AcrAB efflux pumps is responsible for the emergence of antimicrobial-resistant *Salmonella* strains. Here, we examined the expression levels of *acrB* and its multiple regulator genes (RamA, SoxS, MarA, and Rob) in 17 field isolates of *S.* Choleraesuis by using quantitative PCR methods. The expression of *acrB* increased in eight of the field isolates ($P < 0.05$). The expression of *acrB* was associated with that of *ramA* in one isolate, *soxS* in one isolate, and both these genes in six isolates. Thereafter, to examine the effect of selected antimicrobials (enrofloxacin, ampicillin, oxytetracycline, kanamycin, and spectinomycin) on the expression of *acrB* and its regulator genes, mutants derived from five isolates of *S.* Choleraesuis were selected by culture on antimicrobial-containing plates. The expression of *acrB* and *ramA* was higher in the mutants selected using enrofloxacin (3.3–6.3- and 24.5–37.7-fold, respectively), ampicillin (1.8–7.7- and 16.1–55.9-fold, respectively), oxytetracycline (1.7–3.3- and 3.2–31.1-fold, respectively), and kanamycin (1.6–2.2- and 5.6–26.4-fold, respectively), which are AcrAB substrates, than in each of the parental strains ($P < 0.05$). In contrast, in AcrAB substrate-selected mutants, the expression of *soxS*, *marA*, and *rob* remained similar to that in parental strains. Of the four antimicrobials, the level of *ramA* expression was significantly higher in the enrofloxacin- and ampicillin-selected mutants than in the oxytetracycline- and kanamycin-selected mutants ($P < 0.05$), whereas the expression levels of *acrB* and multiple regulator genes in spectinomycin-selected mutants were similar to those in each parental strain. These data suggest that exposure to antimicrobials that are AcrAB substrates enhance the activation of the AcrAB efflux pump *via* RamA, but not *via* SoxS, MarA, or Rob in *S.* Choleraesuis.

Keywords: AcrAB efflux pump, antimicrobial resistance, RamA, *Salmonella* Choleraesuis, SoxS

INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Choleraesuis is a bacterial pathogen that causes severe diarrhea, pneumonia, and septicemia in pigs and in elderly and immunocompromised humans (Chiu et al., 2004). These bacteria sometimes cause severe infections, necessitating antimicrobial treatment of affected patients. A high rate of multidrug resistance among *S.* Choleraesuis has been reported in several countries (Lee et al., 2009; Asai et al., 2010), raising a concern for public and animal health.

Activation of an efflux system that removes the drug from the cell is one antimicrobial resistance mechanism in bacteria (Chen et al., 2007; Li and Nikaido, 2009). The AcrAB-TolC system is the main multidrug efflux system in Gram-negative bacteria (Giraud et al., 2000). The expression level of *acrAB* mRNAs correlates with efflux activities and is regulated by global regulatory proteins and local repressors (Rosenberg et al., 2003; Li and Nikaido, 2004; Olliver et al., 2004; Abouzeed et al., 2008). In particular, the expression of *acrAB* is regulated by the global regulators, RamA, SoxS, MarA, and Rob (Rosenberg et al., 2003; Abouzeed et al., 2008). Moreover, mutations within the local repressor AcrR contribute to the overexpression of *acrAB* (Olliver et al., 2004). In *S.* Typhimurium and *S.* Haardt, RamA mainly regulates the expression of *acrAB* (**Figure 1**; Nikaido et al., 2008; Zheng et al., 2009; Kim and Woo, 2011). Other regulators, SoxS and MarA, are also known to regulate *acrAB* expression, but the contributions of SoxS and MarA to antimicrobial susceptibilities are lower than that of RamA (Abouzeed et al., 2008). Little is known about Rob and its contribution to the enhancement of AcrAB in *Salmonella* (Rosenberg et al., 2003). In *S.* Pullorum, the expression of *acrB*, which shifts from sensitivity to resistance against bile salt (deoxycholate, an AcrAB substrate), was independent of *ramA*, *soxS*, *marA*, and *rob* expression (Usui et al., 2011).

We have previously recorded the various efflux activity levels of *S.* Choleraesuis under field conditions, suggesting that elevated efflux activities are related to the emergence of fluoroquinolone resistance (Usui et al., 2009). In fluoroquinolone-selected *S.* Choleraesuis, efflux pumps including AcrAB were enhanced and resulted in decreasing susceptibilities to several

antimicrobials (Usui et al., 2010). However, little is known about roles of regulator genes for efflux pumps in *S.* Choleraesuis. In this study, we examined the expression levels of *acrAB* and multiple regulator genes in 17 clinical swine isolates of *S.* Choleraesuis and antimicrobial-selected mutants in order to understand the impact of antimicrobial exposure on the efflux activities in this organism.

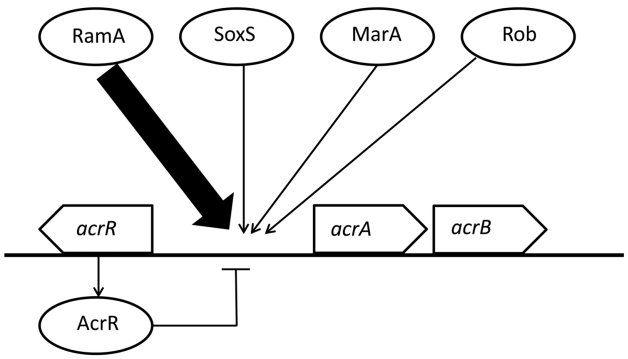

FIGURE 1 | Regulation of *acrAB* expression by multiple regulators in *Salmonella* Typhimurium and *S.* Haardt. This figure was produced using data from the literature (see main text). Functional interactions are represented as arrows for activation/induction and as "⊣" for repression. The size of arrows indicates the estimated impact.

MATERIALS AND METHODS

BACTERIAL STRAINS

Seventeen strains of *S.* Choleraesuis, obtained from different diseased pigs between 2001 and 2005 (Asai et al., 2010), were used in this study (**Table 1**). These strains comprised 6 nalidixic acid-resistant strains (low enrofloxacin-accumulation), and 11 nalidixic acid-susceptible strains (two strains: low enrofloxacin-accumulation; three strains: intermediate enrofloxacin-accumulation; six strains: high enrofloxacin-accumulation) as previously recorded (Usui et al., 2009). The intracellular concentration of enrofloxacin was significantly lower in nalidixic acid-resistant isolates, and the nalidixic acid-susceptible isolates ZSC-8 and 582 as compared to other susceptible isolates with the exception of isolates 143, ZSC-12, and 1002 ($P < 0.1$; Usui et al., 2009). The intracellular enrofloxacin concentrations of isolates 143, ZSC-12, and 1002 were at an intermediate level. Minimum inhibitory concentrations (MIC) of enrofloxacin, ampicillin, oxytetracycline, and kanamycin had been determined in our previous study (Asai et al., 2010). MIC of spectinomycin was

Table 1 | Characterization of *Salmonella* Choleraesuis used in this study.

Strain no.	Susceptibility to nalidixic acid[a]		Enrofloxacin-accumulation		mRNA expression		Mutations	
	Category[b]	MIC (mg/L)[b]	Category[c]	(ng/10^5cfu)[c]	*acrA*[d]	*acrB*[d]	AcrR[e]	Binding site
13-PLS-6	Resistance	256	Low	8.2	1.6 ± 0.2*	1.8 ± 0.2*	WT	WT
14-PLS-21	Resistance	>512	Low	8.7	3.9 ± 0.3*	2.7 ± 0.2*	WT	WT
16-PLS-45	Resistance	>512	Low	6.5	1.5 ± 0.1*	1.9 ± 0.3*	Q78ter	WT
16-PLS-46	Resistance	>512	Low	6.6	2.4 ± 0.2*	2.4 ± 0.4*	Q78ter	WT
sal-1372	Resistance	>512	Low	6.3	1.8 ± 0.1*	2.2 ± 0.2*	WT	WT
16-PLS-33	Resistance	>512	Low	5.1	1.5 ± 0.1*	1.9 ± 0.2*	Q78ter	WT
ZSC-8	Susceptible	8	Low	8.1	1.7 ± 0.1*	1.8 ± 0.2*	Q78ter	WT
582	Susceptible	8	Low	7.2	1.4 ± 0.1*	1.4 ± 0.2*	Q78ter	WT
143	Susceptible	8	Intermediate	9.1	1.1 ± 0.1	1.3 ± 0.1	Q78ter	WT
ZSC-12	Susceptible	4	Intermediate	9.5	1.0 ± 0.1	1.0 ± 0.1	Q78ter	WT
1002	Susceptible	4	Intermediate	9.8	1.1 ± 0.2	1.2 ± 0.2	Q78ter	WT
916	Susceptible	4	High	10.7	1.1 ± 0.1	1.1 ± 0.1	Q78ter	WT
03-197-1	Susceptible	2	High	11.4	0.9 ± 0.1	0.8 ± 0.1	Q78ter	WT
03-228-1	Susceptible	2	High	10.7	1.0 ± 0.2	1.1 ± 0.1	Q78ter	WT
ZSC-14-1	Susceptible	4	High	11.0	0.9 ± 0.1	1.1 ± 0.1	Q78ter	WT
ZSC-40	Susceptible	4	High	10.5	0.9 ± 0.2	0.9 ± 0.2	WT	WT
419	Susceptible	4	High	12.7	1.0 ± 0.2	0.9 ± 0.1	Q78ter	WT

MIC, minimum inhibitory concentration; WT, wild-type sequence.

[a] *MIC break point of nalidixic acid is 32 mg/L.*

[b] *Data from a previous report (Asai et al., 2010).*

[c] *Data from a previous report (Usui et al., 2009).*

[d] *Relative expression level compared to the average for six high enrofloxacin-accumulating nalidixic acid-susceptible strains.*

[e] *Q78ter, stop codon mutation, glutamine-78-termination.*

**Indicates mRNA expression is significantly higher than mRNA expression of six high enrofloxacin-accumulating nalidixic acid-susceptible strains ($P < 0.05$).*

determined by the broth microdilution method according to CLSI guidelines (Clinical and Laboratory Standard Institute, 2008).

LABORATORY-SELECTED MUTANTS

Five strains (582, 143, 916, 1002, and 419), susceptible to all of the antimicrobial agents evaluated in this study, were selected as parent strains from which mutants were isolated. Cultures grown on agar containing various agents was employed, including three AcrAB substrates (ampicillin, oxytetracycline, and kanamycin) and one non-substrate (spectinomycin; Bailey et al., 2008; Ricci and Piddock, 2009a,b). For selection of mutants, 0.1 mL of each strain, adjusted to 10^8–10^9cfu/mL were exposed to 4 × MIC of the respective substance for each strain in the agar medium, as described previously (Ricci and Piddock, 2009b). A single strain was selected for each combination. In addition, as fluoroquinolone is an AcrAB substrates, enrofloxacin-selected mutants obtained in our previous report were used as control (Usui et al., 2010). MICs of antimicrobials for mutants were determined as described above.

QUANTITATIVE PCR ANALYSIS OF EXPRESSION OF 16S rRNA, *acrAB*, AND MULTIPLE GLOBAL REGULATORS

To evaluate the genes conferring efflux pump activity and their multiple global regulators, quantitative PCR was applied to the detection and quantification of mRNAs (Usui et al., 2010, 2011). The preparation of cDNA was performed as described by Zheng et al. (2009), with slight modifications. In brief, extraction of total RNA from 5 mL of bacterial suspensions was carried out with an ISOGEN kit (Nippon Gene, Tokyo, Japan). cDNA was synthesized from these RNA samples using the PrimeScript RT reagent kit (TaKaRa, Shiga, Japan). Quantitative PCR was performed with SYBR premix EX TaqII (TaKaRa) on a One Step real-time system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The oligonucleotide primers used for the detection of cDNA are listed in **Table 2**. The yields of amplicons from *acrA*, *acrB*, *ramA*, *soxS*, *marA*, and *rob* were normalized to those originating from 16S rRNA. Within bacterial cells, 16S rRNA was assumed to be transcribed at a constant rate throughout the growth conditions used in this study.

After normalization to the levels of 16S rRNA, gene expression was compared with the average values for the six high enrofloxacin-accumulating susceptible strains.

acrR DNA SEQUENCE ANALYSIS

Mutations in the local repressor gene, *acrR*, and the regulator-binding site were detected by direct DNA sequencing, using previously reported primer sets (Zheng et al., 2009). Amplification of the gene and purification of the resulting amplicon were performed as described before (Zheng et al., 2009). Nucleotide

Table 2 | Primers used in this study.

Primer name	Sequence (5′–3′)	Reference
acrA-F	AAAACGGCAAAGCGAAGGT	Usui et al. (2011)
acrA-R	GTACCGGACTGCGGGAATT	Usui et al. (2011)
acrB-rt1	GGCATTGGGTATGACTGGAC	Zheng et al. (2009)
acrB-rt2	GCATTACGGAGAACGGGATAG	Zheng et al. (2009)
ramA-rt1	TTTCCGCTCAGGTTATCGAC	Zheng et al. (2009)
ramA-rt2	CGGGCAATATCATCAATACG	Zheng et al. (2009)
soxS-rt1	AAATCGGGCTACTCCAAG	Zheng et al. (2009)
soxS-rt2	TACTCGCCTAATGTTTGATG	Zheng et al. (2009)
marA-rt1	ATTCTCTATCTGGCGGAAC	Zheng et al. (2009)
marA-rt2	CGGGTCAATGTTTGCTGTG	Zheng et al. (2009)
robA-rt1	TATTCCGCCAGTGCTTTATG	Zheng et al. (2009)
robA-rt2	CCTGCTCATCGTCTTTCTCC	Zheng et al. (2009)
16S rRNA-F	CCAGCAGCCGCGGTAAT	Usui et al. (2010)
16S rRNA-R	TTTACGCCCAGTAATTCCGATT	Usui et al. (2010)

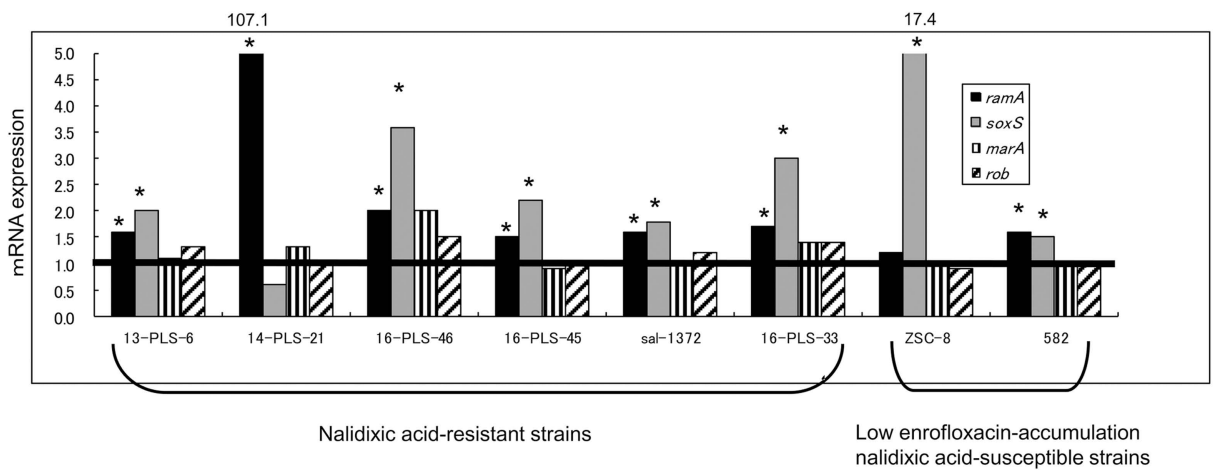

FIGURE 2 | Expression of multiple regulator genes in field isolates of *S.* Choleraesuis. The expression of multiple regulator genes in resistant strains and low enrofloxacin-accumulating nalidixic acid-susceptible strains was compared to the average expression of the corresponding genes in high enrofloxacin-accumulating nalidixic acid-susceptible strains ($n = 6$). Horizontal line indicates the average gene expression in the six susceptible strains, which expressions were set to be 1.0. *Indicates that mRNA expression is significantly higher in a strain compared to mRNA expression in the six susceptible strains ($P < 0.05$).

Table 3 | The MICs of several antimicrobials and gene expressions of *acrB* and multiple regulator genes in laboratory-selected mutants.

Strain no.	Selecting agent[a]	MIC (mg/L)					mRNA expression[c]				
		Enrofloxacin (2)[b]	Ampicillin (32)[b]	Oxytetracycline (16)[b]	Kanamycin (64)[b]	Spectinomycin (128)[b]	*acrB*	*ramA*	*soxS*	*marA*	*rob*
582		0.0625	1	2	2	32	1.4	1.6	1.5	1.1	1.0
582/E	Enrofloxacin	0.25	4	4	2	32	6.9	39.2	0.8	1.2	0.6
582/A	Ampicillin	0.25	4	8	2	32	7.7	89.5	0.3	1.2	0.8
582/O	Oxytetracycline	0.125	4	4	2	32	4.2	9.5	0.3	1.1	0.9
582/K	Kanamycin	0.125	2	4	4	32	3.1	9.0	0.5	1.3	0.6
582/S	Spectinomycin	0.0625	1	2	2	128	1.7	1.4	1.5	1.2	0.8
143		0.0625	1	2	2	32	1.3	1.2	1.2	1.1	1.0
143E	Enrofloxacin	0.25	4	4	2	32	4.3	45.2	0.6	0.9	0.7
143/A	Ampicillin	0.125	4	4	2	32	2.9	38.8	0.1	0.7	0.5
143/O	Oxytetracycline	0.0625	2	8	2	32	2.2	10.2	1.3	1.2	0.3
143/K	Kanamycin	0.0625	2	4	8	32	2.1	9.3	1.1	1.2	0.4
143/S	Spectinomycin	0.0625	1	2	2	128	1.6	0.9	0.9	0.7	0.7
1002		0.0625	1	2	2	32	1.2	1.0	0.8	1.0	1.0
1002/E	Enrofloxacin	0.25	4	4	2	32	8.0	29.8	0.1	1.2	1.0
1002/A	Ampicillin	0.125	4	2	2	32	2.4	16.1	0.1	0.3	0.5
1002/O	Oxytetracycline	0.125	2	8	2	32	2.2	14.7	0.2	1.2	0.9
1002/K	Kanamycin	0.125	2	4	4	32	2.4	14.2	0.3	1.0	0.9
1002/S	Spectinomycin	0.0625	1	2	2	128	1.1	1.4	0.4	1.1	0.9
916*		0.0625	1	2	2	32	1.1	1.1	0.8	0.9	0.9
916/E	Enrofloxacin	0.5	4	4	2	32	7.4	34.2	0.4	0.8	0.9
916/A	Ampicillin	0.25	4	4	2	32	8.5	58.2	0.1	0.9	0.8
916/O	Oxytetracycline	0.0625	1	4	2	32	2.2	3.5	0.2	1.0	0.9
916/K	Kanamycin	0.0625	1	4	8	32	2.2	6.5	0.1	1.0	0.6
916/S	Spectinomycin	0.0625	1	2	2	128	1.2	0.6	0.2	0.7	0.6
419*		0.0625	4	2	2	32	0.9	0.7	1.0	1.0	1.0
419/E	Enrofloxacin	0.25	16	4	2	32	5.7	24.2	0.2	1.7	0.8
419/A	Ampicillin	0.0625	8	2	2	32	1.6	32.5	0.3	0.7	0.9
419/O	Oxytetracycline	0.125	8	8	2	32	3.0	21.8	0.8	3.4	1.6
419/K	Kanamycin	0.0625	8	4	8	32	1.5	18.5	0.3	1.1	0.9
419/S	Spectinomycin	0.0625	4	2	2	128	0.9	1.1	0.4	2.1	1.2
03-197-1*		0.0625	>128	256	2	32	0.8	1.1	1.3	1.2	1.2
03-228-1*		0.0625	1	256	8	32	1.1	1.1	1.0	0.9	0.9
ZSC-14-1*		0.0625	1	256	2	32	1.1	1.0	1.0	1.0	0.8
ZSC-40*		0.0625	>128	256	2	32	0.9	0.8	0.8	1.1	0.9

MIC, minimum inhibitory concentration.

[a] *Mutants were selected by exposing to fourth the MIC of the antimicrobials for each susceptible strain.*

[b] *MIC data of antimicrobials except for spectinomycin in parental strains from a previous report (Asai et al., 2010). Parenthesis indicates break points.*

[c] *Relative expression level of acrB and multiple regulator genes compared to average of six high accumulation susceptible strains represented as asterisks.*

sequences were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with a 3130 Genetic Analyzer (Applied Biosystems).

STATISTICAL ANALYSIS

Student's *t*-test was used to compare results between and within experiments. *P*-values < 0.05 were considered significant.

RESULTS

RELATIONSHIP BETWEEN THE ENHANCEMENT OF EFFLUX PUMPS AND THE EXPRESSION OF *acrAB*

Expression of *acrA* and *acrB* in the six resistant strains and in the two low enrofloxacin-accumulation nalidixic acid-susceptible strains was significantly higher than that in the other susceptible strains ($P < 0.05$; **Table 1**). Expression levels of *acrB* correlated

significantly with those of *acrA* in this study (correlation coefficient: 0.89).

RELATIONSHIP BETWEEN INCREASED EXPRESSION OF *acrB* AND GLOBAL REGULATORS

Of the eight strains with a high *acrB* expression level, six strains showed higher levels of both *ramA* and *soxS* expression compared with the average values for the six high accumulating susceptible strains ($P < 0.05$; **Figure 2**). The remaining strains showed a higher level of *ramA* or *soxS* expression ($P < 0.05$): the 14-PLS-21 strain showed only a higher expression level of *ramA* (107.1-fold ± 42.0-fold), while the ZSC-8 strain showed only a higher expression level of *soxS* (17.4-fold ± 2.7-fold). Neither the expression of *marA* nor that of *rob* changed in any of the strains tested.

SEQUENCING OF THE LOCAL REPRESSOR GENE *acrR* AND OF THE REGULATOR-BINDING SITE

A stop codon mutation in AcrR (glutamine-78-to-terminal; Q78ter) was found in 13 strains (**Table 1**). No significant relationship was found between the Q78ter stop codon mutation in AcrR and the expression of *acrB*. None of the strains tested had a point mutation in the regulator-binding site, which affects the *acrB* expression, previously determined using the *S.* serovar Typhimurium strain BN18 (Olliver et al., 2004).

GENE EXPRESSION IN LABORATORY-SELECTED MUTANTS

Expression levels of both *acrB* and *ramA* increased significantly in AcrAB substrate-selected mutants compared to these levels in parental strains ($P < 0.05$; **Table 3**). Among four AcrAB substrates, a higher level of *ramA* expression was observed in the enrofloxacin- and ampicillin-selected mutants than in the oxytetracycline- and kanamycin-selected mutants ($P < 0.05$). In contrast, the expression of *soxS* decreased significantly in AcrAB substrate-selected mutants compared to that in parental strains ($P < 0.05$; **Table 3**). MICs of AcrAB substrates were increased in AcrAB substrate-selected mutants as compared with those in the parental strains (**Table 3**).

Expression of *acrB* and multiple regulator genes remained stable in spectinomycin-selected mutants. In spectinomycin-selected mutants, MICs of AcrAB substrates were of the same level as compared with those in the parental strains (**Table 3**).

DISCUSSION

The current study showed that the expression of *ramA* and/or *soxS* was associated with *acrAB* overexpression in field isolates of *S.* Choleraesuis. In this study, expression of *ramA*, but not *soxS*, *marA*, or *rob*, was enhanced in laboratory-derived *S.* Choleraesuis mutants selected using antimicrobials that are AcrAB substrates. Several previous studies have reported that *ramA*, but not *soxS*, is involved in activation of the AcrAB-TolC system in *S.* Typhimurium (Ricci et al., 2006; Bailey et al., 2008; Zheng et al., 2009). Interestingly, in this study, *soxS* expression was downregulated in AcrAB substrate-selected mutants of *S.* Choleraesuis. In addition, enrofloxacin- and ampicillin-selected mutants derived from the ZSC-8 strain with a high level of *soxS* expression, showed increased expression of *ramA*, but not of *soxS* (data not shown). Several studies have suggested that increased expression of *ramA* downregulates *soxS* expression (Nikaido et al., 2008; O'Regan et al., 2009). These results suggest that the *acrA* and *acrB* expression in antimicrobial-selected mutants of *S.* Choleraesuis were dependent on *ramA* expression.

The present study showed that increased levels of both *ramA* and *soxS* expression were demonstrated by some field isolates of *S.* Choleraesuis upon activation of the AcrAB-TolC system. There was a marked difference in *soxS* expression between the field isolates and the laboratory-derived strains. As all of the strains used in this study were isolated from diseased pigs, these isolates would have been exposed to antimicrobials and disinfectants used in disease treatment and hygiene management. This suggests that the resistance in some field isolates was mediated through a different mechanism, and therefore could have arisen from a different selective pressure.

Activation of the AcrAB efflux pump is responsible for the emergence of fluoroquinolone-resistant *Salmonella* strains (Ricci et al., 2006; Usui et al., 2009). In Japan, the description of veterinary fluoroquinolone drugs includes an explanation that the drug is considered as a second-line drug. In the AcrAB substrate-selected mutants, the level of *acrB* expression generally increased, depending on *ramA* expression. It is possible that the use of an AcrAB substrate as a first-line drug crucially selects strains of *S.* Choleraesuis with activated AcrAB efflux systems. Among AcrAB substrates used in this study, the degree of *acrB* expression may be different due to exposure to the various antimicrobial agents. Therefore, the antimicrobial class used as first-line drugs may be associated with the frequency of fluoroquinolone resistance in bacterial strains. Further studies are needed in order to clarify the effects of each antimicrobial on *acrB* expression in these bacteria.

In conclusion, the AcrAB-TolC efflux system in field isolates of *S.* Choleraesuis is likely regulated by external factors in the environment, such as antimicrobials and drug residues *via ramA* and *soxS*. In particular, exposure of AcrAB substrates enhances activation of AcrAB *via* RamA in *S.* Choleraesuis.

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Novel β-lactamase inhibitors: a therapeutic hope against the scourge of multidrug resistance

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The increasing incidence and prevalence of multi-drug resistance (MDR) among contemporary Gram-negative bacteria represents a significant threat to human health. Since their discovery, β-lactam antibiotics have been a major component of the armamentarium against these serious pathogens. Unfortunately, a wide range of β-lactamase enzymes have emerged that are capable of inactivating these powerful drugs. In the past 30 years, a major advancement in the battle against microbes has been the development of β-lactamase inhibitors, which restore the efficacy of β-lactam antibiotics (e.g., ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin/clavulanate, and piperacillin/tazobactam). Unfortunately, many newly discovered β-lactamases are not inactivated by currently available inhibitors. Is there hope? For the first time in many years, we can anticipate the development and introduction into clinical practice of novel inhibitors. Although these inhibitors may still not be effective for all β-lactamases, their introduction is still welcome. This review focuses on the novel β-lactamase inhibitors that are closest to being introduced in the clinic.

Keywords: antibiotic resistance, β-lactamase inhibitors

INTRODUCTION

The ongoing dissemination of multi-drug resistant (MDR) bacteria is a serious threat to global health. Microbiological and epidemiological surveys commissioned by public and private institutions paint a frightening portrait of the emergence of β-lactam resistance in both the community and the hospital setting. A major mechanism for antibiotic resistance among Gram-negative bacteria is the production of β-lactamases. β-lactamases are enzymes that inactivate β-lactam antibiotics by hydrolyzing the amide bond of the β-lactam ring. β-lactamases are bacterial resistance determinants that have been known for more than seventy years, yet the details of their evolution, dissemination and hydrolytic capacity still remains a great scientific challenge.

Two classification systems are presently used to categorize β-lactamases. Introduced more than thirty years ago, the Ambler classification system divides β-lactamases into four classes (A, B, C, and D) based on their amino acid sequences (Ambler, 1980). The Bush-Medeiros-Jacoby classification system groups β-lactamases according to functional properties; this classification system uses substrate and inhibitor profiles in an attempt to organize the enzymes in ways that can be correlated with their phenotype in clinical isolates (Bush and Jacoby, 2010). For purposes of simplicity in this review, we will refer to the Ambler classification system.

Class A enzymes include both plasmid-mediated and chromosomally-encoded β-lactamases that demonstrate broad-spectra (e.g., TEM-1 and SHV-1), extended-spectra (e.g., CTX-M-15), and carbapenemase activity (e.g., KPC-2). Class B enzymes are metallo-β-lactamases (MBLs) which can hydrolyze penicillins, cephalosporins, and carbapenems such as the recently described New Delhi metallo-β-lactamase (NDM-1) found in *Klebsiella pneumoniae* and *Escherichia coli* (Kumarasamy et al., 2010). Class C enzymes are cephalosporinases that are chromosomally-encoded for example the inducible *Pseudomonas aeruginosa* AmpC and P99 β-lactamase of *Enterobacter* spp., or plasmid-mediated such as CMY-2, first found in *Escherichia coli*. Class D enzymes have a substrate preference for oxacillin and are referred to as oxacillinases (e.g., OXA-1). Recent surveys have shown that class D enzymes are a rapidly expanding class of β-lactamases and have enzymes that can hydrolyze extended-spectrum cephalosporins (e.g., OXA-10) and carbapenems (e.g., OXA-23). Several class D enzymes are often found in non-fermenting bacteria such as *P. aeruginosa* and *Acinetobacter baumannii* and occasionally in *E. coli* and *K. pneumoniae*.

At present, there are three commercially available β-lactamase inhibitors: clavulanic acid, sulbactam and tazobactam (**Figure 1**). These are mechanism-based inhibitors that share a common β-lactam structure. As a group, they are best active against most

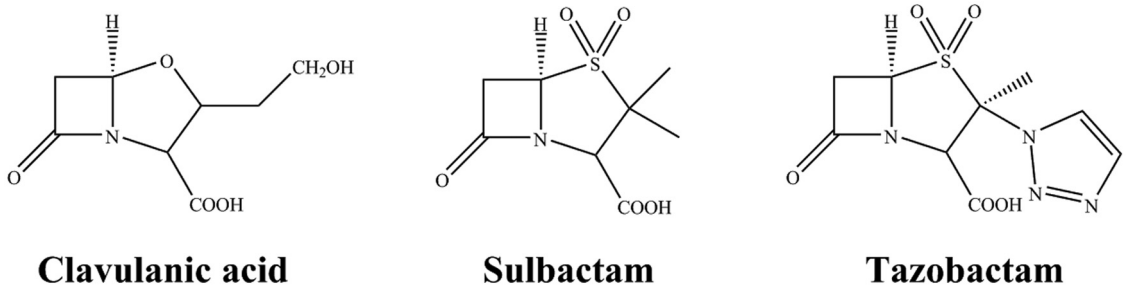

FIGURE 1 | Chemical structures of the clinically available β-lactamase inhibitors.

class A β-lactamases, exceptions include KPC-2 carbapenemase and inhibitor resistant TEMs (IRTs) and SHVs. Clavulanate, sulbactam, and tazobactam have less effect on class C enzymes, and are essentially inactive against class B and most class D enzymes (Bush and Jacoby, 2010).

Encouragingly, pharmaceutical companies are aggressively developing and bringing to market new combinations of β-lactam antibiotics with β-lactamase inhibitors. Several of these are now close to clinical availability. A promising new design for β-lactamase inhibitors has been to focus on scaffolds that can rapidly acylate a wide range of β-lactamases while minimizing hydrolysis. This review will focus on recent data regarding the mechanisms of inhibition of these novel agents, their antimicrobial activity, and the progress in their clinical trials. Specifically, avibactam and MK-7655 are members of a new class of non-β-lactam-β-lactamase inhibitors called diazabicyclooctanes (DBOs) with a broader spectrum of activity than other inhibitors. Recent modifications to boronic acid (BA) compounds have led to very potent *E. coli* AmpC inhibitors that are eagerly awaited. Finally, the discovery of a "universal" β-lactamase inhibitor has been an important goal of both academia and the pharmaceutical industry but has proven to be quite challenging. Emerging data show this ideal might not be feasible and researchers investigating mechanisms of β-lactamase inhibition will likely need to develop alternative strategies.

DIAZABICYCLOOCTANES

AVIBACTAM

Avibactam (AVI) is a non-β-lactam compound in the class of DBOs (**Figure 2**). As a β-lactamase inhibitor, AVI inactivates β-lactams by a reversible fast acylation and relatively slow deacylation reaction. Against most class A and class C β-lactamases this results in a low turnover ratio (Ehmann et al., 2012). The β-lactamase inhibition by AVI is mostly reversible and AVI demonstrates a half-life of 16 min for TEM-1 which closely approaches one generation time of *E. coli* (Ehmann et al., 2012). Thus despite reversibly of AVI, AVI is predicted to remain bound to TEM-1 during most of an entire generation cycle of *E. coli*; thus keeping the enzyme inactive. Unlike clavulanic acid and like sulbactam, AVI does not induce β-lactamase production (Coleman, 2011). In addition to TEM-1 and SHV-1, clinically important β-lactamases that are readily inhibited by AVI include the serine carbapenemase KPC-2, the ESBL CTX-M-15, class C β-lactamases such as the AmpC and some class D enzymes (OXA-48).

An interesting development is the combination of this DBO inhibitor with a number of β-lactam antibiotics that have traditionally been used to treat Gram-negative bacteria. Despite the tendency of this class of antibiotics to select ESBLs, expanded-spectrum cephalosporins are seen as potential partners because they have a broader spectrum of activity. As a result, the combination of ceftazidime-AVI has potent activity against *K. pneumoniae*

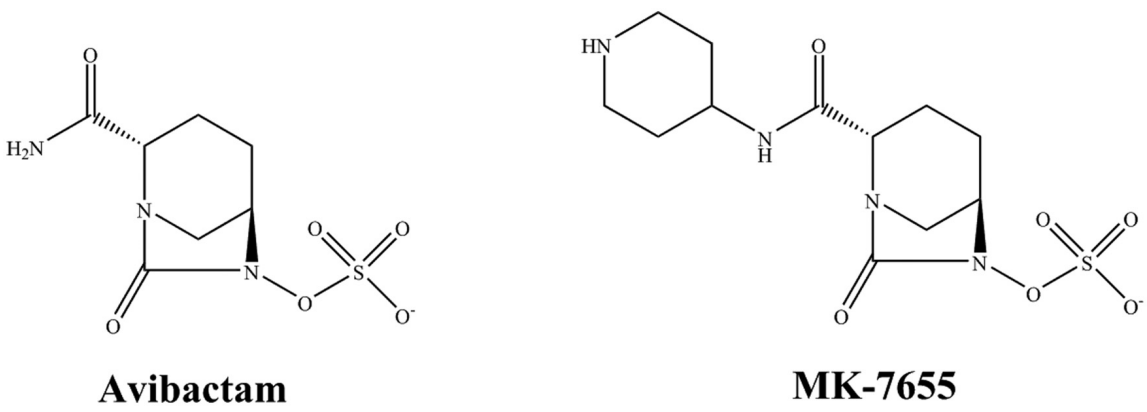

FIGURE 2 | Chemical structures of diazabicyclooctanes.

carrying ESBLs such as SHV-5, other ESBLs and AmpC enzymes and also against most *Klebsiella* spp. harboring the KPC enzyme (Livermore et al., 2011). Against *P. aeruginosa*, AVI reverses AmpC-mediated ceftazidime resistance, reducing MICs for fully derepressed mutants and isolates to ≤8 mg/L (Mushtaq et al., 2010). Unfortunately, ceftazidime-AVI lacks activity against *A. baumannii* and most species of anaerobic bacteria (Citron et al., 2011; Zhanel et al., 2013).

Emerging data from clinical trials that are registered show that ceftazidime-AVI is as effective as carbapenem therapy for complicated urinary tract infections (UTIs) and complicated intra-abdominal infections (cIAI), including those caused by expanded-spectrum cephalosporin-resistant Gram-negative organisms (Zhanel et al., 2013). Furthermore, a recent trial of ceftazidime-AVI plus metronidazole in the treatment of cIAIs found a favorable clinical response rate when compared to meropenem (Lucasti et al., 2013).

Ceftaroline is a novel semisynthetic anti-methicillin-resistant *Staphylococcus aureus* (MRSA) cephalosporin with broad-spectrum activity. The combination of ceftaroline-AVI is active against *Enterobacteriaceae* that produce KPC, various ESBLs (CTX-M types), and AmpC (chromosomally derepressed or plasmid-mediated enzymes), as well as against those producing more than one of these β-lactamase types (Castanheira et al., 2012b). However, ceftaroline's activity against *Acinetobacter* spp. and *P. aeruginosa* is limited. In a clinical study of diabetic foot infections (which are often polymicrobial), ceftaroline-AVI reduced ceftaroline MICs for strains of resistant *Enterobacter* spp. and one strain of *Morganella*, as well as for the anaerobes *Bacteroides fragilis* and *Prevotella* spp. (Goldstein et al., 2013a). A Phase 2 clinical trial comparing ceftaroline-AVI to doripenem in adults with complicated UTIs is in progress[1].

Monobactams resist hydrolysis by MBLs, thus another promising partner for AVI is aztreonam. For example, if any *Enterbacteriaceae* and *P. aeruginosa* strains carrying MBLs and co-produce ESBLs or AmpC, the aztreonam would target the MBLs, while the avibactam would inhibit the ESBLs and AmpC (Livermore et al., 2011; Crandon et al., 2012). As such, this combination will be a very welcome addition to the antibiotic formulary as the safety and efficacy of aztreonam are already established in clinical practice.

MK-7655

MK-7655, a novel DBO that is structurally similar to AVI except for an additional piperidine ring, exhibits synergy in combination with imipenem against KPC-producing *K. pneumoniae* and *P. aeruginosa* expressing AmpC (**Figure 2**; Mangion et al., 2011; Hirsch et al., 2012). Studies show that at a concentration of 4 mg/L, MK-7655 lowers imipenem MICs for *Enterobacteriaceae* with KPC carbapenemases from 16–64 mg/L to 0.12–1 mg/L (Livermore et al., 2013). Interestingly, synergy is also seen for *Enterobacteriaceae* with carbapenem resistance mediated by porin loss. Among strains of *P. aeruginosa*, 4 mg/L of MK-7655 reduces the MIC of imipenem for all isolates, except those with MBLs.

Two separate Phase 2 clinical trials of two doses (125 mg or 250 mg) of MK-7655 plus imipenem-cilastatin vs. imipenem-cilastatin alone for treatment of complicated UTIs or cIAIs began in early 2012[1]. Results from these trials are eagerly awaited.

BAs

The inhibitory effects of BAs on β-lactamases have been known for several decades. Boron forms a reversible bond with β-lactamases. Recent studies have shown that different BAs are high affinity inhibitors of the AmpC β-lactamase of *E. coli*, class A β-lactamases TEM-1, CTX-M, and SHV-1, and class C β-lactamase, ADC-7 from *Acinetobacter* spp. and *P. aeruginosa* (Drawz et al., 2010a; Winkler et al., 2013). Many BAs are in early developmental stages, however the progress of these compounds is rapidly advancing.

Despite the large number of BAs in development, only one so far is approaching clinical trials. First introduced at the 2012 Interscience Conference on Antimicrobial Agents and Chemotherapy, RPX7009 is a new boron-based inhibitor being developed in combination with biapenem (RPX2003; **Figure 3**; Castanheira et al., 2012a; Hecker et al., 2012; Sabet et al., 2012). RPX7009 lacks direct antibacterial activity but it does enhance the activity of biapenem against class A carbapenemase-producing *Enterobacteriaceae* (e.g., KPC, SME, or IMI/NMC-A; Livermore and Mushtaq, 2013). Moreover, RPX7009 lowers the MICs of biapenem against *Enterobacteriaceae* with complex β-lactamase backgrounds (AmpC or ESBL activity) and porin losses. Unfortunately, RPX7009 does not inhibit class B MBLs and class D carbapenemases. Against *Bacteroides* and other select anaerobes, biapenem and RPX7009 demonstrates comparable activity to meropenem alone (Goldstein et al., 2013b). Regarding other anaerobes (*Fusobacterium* spp and *Prevotella*) biapenem and RPX7009 are reasonable active. *Clostridia* are a notable exception with the range extending up to 8 mg/L. As expected against MBL-producing *Bacteroides*, activity is poor.

BAL30072 AND BAL30376

BAL30072 (**Figure 4**) is a novel siderophore monosulfactam similar to aztreonam. BAL30072 demonstrates activity against a broad range of Gram-negative bacilli including *Acinetobacter* spp., *P. aeruginosa*, *Burkholderia cepacia*, and some MDR *Enterobacteriaceae* (Page et al., 2010; Russo et al., 2011; Higgins et al., 2012). BAL30072 shows potency against carbapenem-resistant *Enterobacteriaceae* including those with AmpC, ESBL, and KPC enzymes, *P. aeruginosa* including most strains with MBLs and most isolates of *A. baumannii* except those producing OXA-58 (Mushtaq et al., 2013). However, resistance is still observed with the *K. pneumoniae* ST258 isolates carrying KPC. The addition of meropenem to BAL30072 increases activity against certain individual isolates of *A. baumannii*. BAL30072 is currently in a Phase 1 study and will likely be combined with meropenem in future clinical development[1].

In addition to BAL30072, researchers have also developed another compound with broad activity against β-lactamases called BAL30376, which combines three β-lactams: the siderophore monobactam BAL19764, the bridged monobactam class C β-lactamase inhibitor BAL29880 for class C cephalosporinases, and clavulanic acid to inhibit class A enzymes (Bush and Macielag,

[1] www.clinicaltrials.gov

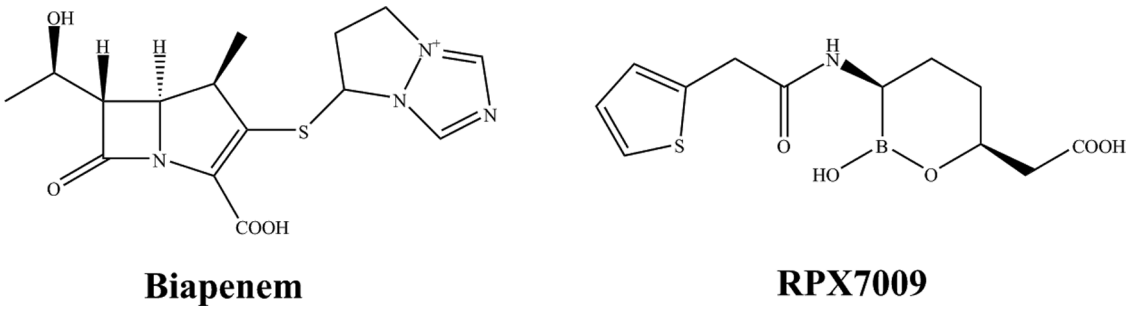

FIGURE 3 | Chemical structures of a novel combination: a carbapenem (left) with a new boron-based β-lactamase inhibitor (right).

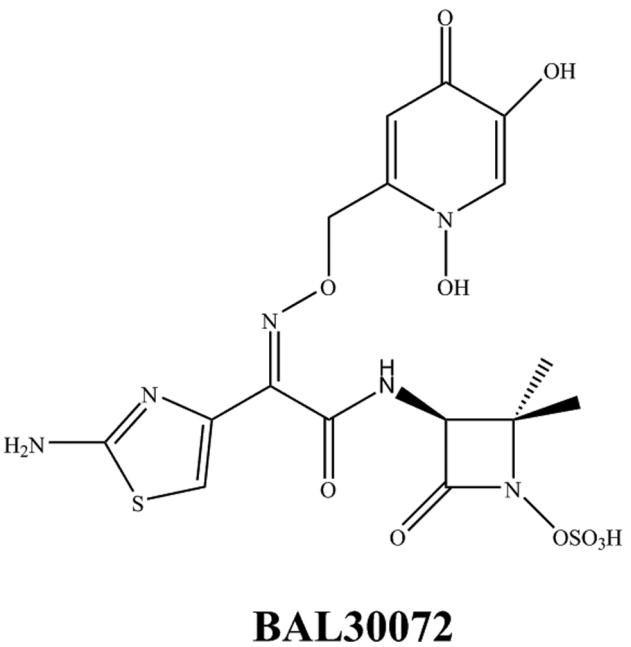

FIGURE 4 | Chemical structure of a novel siderophore monosulfactam.

2010). Further *in vitro* analysis and animal studies of BAL30376 will be necessary before its developmental pathway is clear.

NEW CARBAPENEMS AND BEYOND

Originally developed in the 1970s, carbapenems are among the most broad-spectrum antibiotics in clinical use. One major advantage of this class of agents is their stability against hydrolysis by many ESBLs and class C cephalosporinases. The unique property of carbapenems that merits their inclusion in this review is their ability to inhibit both class A and class C β-lactamases (Drawz and Bonomo, 2010; Papp-Wallace et al., 2011) and their high affinity for the bacterial transpeptidases and carboxypeptidases that synthesize the peptidoglycan-based cell wall. The carbapenem class of β-lactams act as a "slow substrates." Crystallographic analyses show how these compounds inactivate the serine-based class A and C enzymes by adopting unique conformations in the active site that disfavor hydrolysis (carbonyl oxygen outside of the oxyanion hole). The remaining parts of this section will examine the promise of some of these carbapenems that are apart from imipenem, meropenem, ertapenem, and doripenem.

Biapenem has been available in Japan since 2002 and is currently in Phase 2 clinical study in the USA. Biapenem achieves high concentration in respiratory tissue making it an attractive choice for pulmonary infections (Bassetti et al., 2011). Biapenem is hydrolyzed by MBLs and its bicyclic derivative has significant affinity for these enzymes (Garau et al., 2005). Recent experimental evidence shows it might be possible to obtain new competitive inhibitors of B2 MBLs by modification of this bicyclic compound (Gatti, 2012).

Razupenem (SMP-601; **Figure 5**) is a β-methyl carbapenem with activity against MRSA, enterococci including *Enterococcus faecium* and many species of Enterobacteriaceae. The activity of razupenem is not abrogated by ESBLs but AmpC and class A carbapenemases seem to affect it more than ertapenem or imipenem (Livermore et al., 2009). Pharmacodynamic data suggest razupenem can be dosed the same for *E. coli*, *Proteus mirabilis* and *Klebsiella* spp. as for MRSA (MacGowan et al., 2011). However, the development of razupenem has been discontinued.

LK-157 is a novel tricyclic carbapenem with potent inhibitory activity against serine β-lactamases (**Figure 5**; Plantan et al., 2007). LK-157 is a close structural analog to sanfetrinem, an oral, broad-spectrum antibiotic whose development was stopped after Phase 2 clinical trials (Babini et al., 1998). LK-157 restores the diminished activity of β-lactam antibiotics against a number of bacterial strains producing class A ESBLs (excluding CTX-M and KPC) as well as class C β-lactamases (Paukner et al., 2009). Of note, data from a rat jejunum model suggest the compound has good bioavailability, raising the exciting possibility of an oral broad-spectrum agent active against class A and C enzymes (Iglicar et al., 2009).

S-649266 is a novel cephem antibiotic that promises to be stable against MBLs[2]. Details are still forthcoming about this compound, but early reports indicate S-649266 is stable against MBL producing strains and is effective against *A. baumannii*, *Stenotrophomonas maltophilia*, and *Burkholderia* spp. This is not a new β-lactamase inhibitor, but the activity against MBLs merits close attention.

[2]www.shionogi.com/pdf/RDmeeting2012.pdf

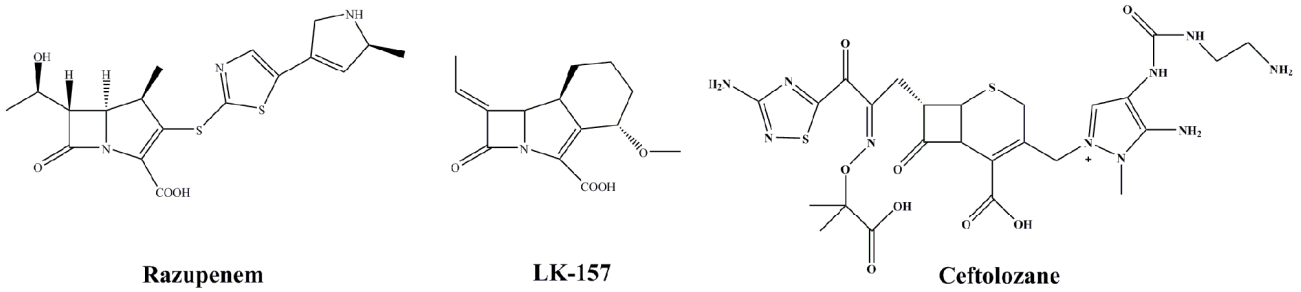

FIGURE 5 | Chemical structures of novel β-lactams with β-lactamase inhibitor activity.

CXA-202 is the combination of ceftolozane (CXA-201) with tazobactam (**Figure 5**). This formulation is targeted for *P. aeruginosa* and other MDR strains and has advanced into Phase 3 trials. *Per se*, this is not a novel β-lactamase inhibitor but is a new partner. The enhanced activity of the cephalosporin partner will be examined closely as this combination represents a novel testing paradigm in this area.

INHIBITORS OF CLASS B ENZYMES

Except for aztreonam-AVI, BAL30072, and S-649266, none of the other aforementioned experimental β-lactams and β-lactamase inhibitors have significant activity against isolates expressing MBLs. This is problematic because MBLs can spread rapidly through mobile genetic elements, as seen with the global emergence of NDM-1 (Liu et al., 2013).

The hydrolytic mechanisms of MBLs are significantly different from other classes of β-lactamases, requiring one or two zinc atoms depending on the subclass. Our understanding of MBLs is emerging as compared to the better studied class A and C enzymes (Dubus et al., 1995; Powers and Shoichet, 2002; Chen et al., 2006, 2009; Fisher and Mobashery, 2009). One class of agents that appear promising against MBLs is the thiol derivatives. Thiols, including the anti-hypertensive medication captopril, effectively inhibit several MBLs including NDM-1 and subclass B1, B2, and B3 enzymes (Heinz et al., 2003; King et al., 2012). Thiol compounds utilize the same mechanisms of zinc chelation and hydrolytic displacement. Additional clinical studies using these compounds in combination with antibiotics seem warranted.

CHALLENGES OF INHIBITING CLASS D ENZYMES

Similar to MBLs in their diversity, class D β-lactamases are designated OXA-type because of their ability to hydrolyze oxacillin. Their substrate profiles range from narrow to broad-spectrum, including carbapenems (Nazik et al., 2012). At present, β-lactamase inhibitors effective against class D enzymes are not available but promising data are emerging.

Several class D enzyme inhibitors are in development. For instance, substituted penicillin sulfones demonstrate efficacy against a number of OXA enzymes including OXA-24/40, a clinically relevant enzyme found in *A. baumannii* (Bou et al., 2010; Drawz et al., 2010b). A compound in development, 4,7-dichloro-1-benzothien-2-yl sulfonylaminomethyl BA (DSABA), is the first BA-based class D enzyme inhibitor. DSABA inhibits class A and C enzymes as well and demonstrates synergy with imipenem against *A. baumannii* (Tan et al., 2010). A series of thiophenyl oxime phosphonate β-lactamase inhibitors with potency against OXA-24/40 have also been discovered (Tan et al., 2011). Of interest, one compound reduces the MIC of imipenem against a highly imipenem-resistant strain of OXA-24/40 producing *A. baumannii*.

CHOOSING THE RIGHT PARTNER ANTIBIOTIC AND THE CHALLENGES AHEAD

Determining the ideal β-lactam for a given β-lactamase inhibitor and defining the ratio of the inhibitor to that β-lactam is a complex process. Indeed, it has been suggested that several considerations should be taken into account: (1) the ability of the inhibitor to protect the β-lactam ring from hydrolysis by key target enzymes; (2) the quantity of inhibitor needed to protect the β-lactam ring; (3) the feasibility and stability of the formulation; (4) pharmacokinetic and dosing parameters; and (5) cost (Shlaes, 2013). However, it is difficult to use standard pharmacokinetic and pharmacodynamic indices with inhibitors because they have weak to no intrinsic antimicrobial activity and they are usually partnered with an active antimicrobial agent. Mathematical modeling is one approach to these challenges. Using mathematical systems in pharmacodynamic models may help define regimens for inhibitors to prevent false labeling of a drug as ineffective because of dosing failures (Bush, 2012).

The report of a single isolate of *K. pneumoniae* producing a serine carbapenemase, a MBL, an ESBL and a plasmid-encoded AmpC carbapenemase underscores the challenge of using β-lactam antibiotics in the clinical setting (Pournaras et al., 2010). Treating this kind of pathogen with a β-lactam will likely require one with high stability to many common β-lactamases (e.g., aztreonam), together with two or more β-lactamase inhibitors that inhibit MBLs and serine β-lactamases. An example is the triple compound BAL30376 (Bush and Macielag, 2010; Livermore et al., 2010; Page et al., 2011). In addition to exerting a bactericidal effect against a wide range of β-lactamase-producing organisms including strains that were resistant to other β-lactams (except for KPC carbapenemases); BAL30376 is also relatively refractory toward selection of resistant mutants (Page et al., 2011).

FUTURE PERSPECTIVE

The majority of the compounds reviewed in this paper are in preclinical stages and (with the exception of AVI and MK-7655) are years away from availability. Thus, the pace of drug development must increase in order to meet the Infectious Diseases Society of America's goal of 10 new systemic drugs to treat infections caused by resistant bacteria by 2020 (Infectious Diseases Society of America, 2010; Boucher et al., 2013). The lack of drug candidates potentially active against MBLs is a great concern. For infections caused by bacteria harboring MBLs, treatment options are limited to polymyxins, tigecycline, and fosfomycin. Moreover, new β-lactamases are reported worldwide with alarming frequency, which continues to put strain on our existing antibiotic armamentarium (Lamoureaux et al., 2013). While novel β-lactamase inhibitors with new mechanisms of action provide substantial advances compared to currently available agents, incremental advances to existing classes are also valuable and should be encouraged (Page and Heim, 2009). The long quest for a universal β-lactamase inhibitor is becoming increasing quixotic with more pragmatic approaches, such as drug combinations, now a leading paradigm.

A plethora of strategies to invigorate drug development have been recently proposed (Infectious Diseases Society of America, 2012; Spellberg et al., 2013). These include conducting superiority and organism-specific clinical trials, transparency through public reporting of antibiotic usage tied to reimbursement, using molecular techniques for diagnostic confirmation of antibiotic indications, and investigating agents that modify host immune responses to pathogens to circumvent resistance selection. We also suggest that attention be given to alternative agents with activity against β-lactamases. Additional research studies are warranted especially since MBLs are important drivers of pan-resistant phenotypes. We remain positive in our outlook as the progress to date merits confidence that new drugs will be available very soon.

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Effect of subtherapeutic vs. therapeutic administration of macrolides on antimicrobial resistance in *Mannheimia haemolytica* and enterococci isolated from beef cattle

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Macrolides are the first-line treatment against bovine respiratory disease (BRD), and are also used to treat infections in humans. The macrolide, tylosin phosphate, is often included in the diet of cattle as a preventative for liver abscesses in many regions of the world outside of Europe. This study investigated the effects of administering macrolides to beef cattle either systemically through a single subcutaneous injection (therapeutic) or continuously in-feed (subtherapeutic), on the prevalence and antimicrobial resistance of *Mannheimia haemolytica* and *Enterococcus* spp. isolated from the nasopharynx and faeces, respectively. Nasopharyngeal and faecal samples were collected weekly over 28 days from untreated beef steers and from steers injected once with tilmicosin or tulathromycin or continuously fed tylosin phosphate at dosages recommended by manufacturers. Tilmicosin and tulathromycin were effective in lowering ($P < 0.05$) the prevalence of *M. haemolytica*, whereas subtherapeutic tylosin had no effect. *M. haemolytica* isolated from control- and macrolide-treated animals were susceptible to macrolides as well as to other antibiotics. Major bacteria co-isolated with *M. haemolytica* from the nasopharynx included *Pasteurella multocida*, *Staphylococcus* spp., *Acinetobacter* spp., *Escherichia coli* and *Bacillus* spp. With the exception of *M. haemolytica* and *P. multocida*, erythromycin resistance was frequently found in other isolated species. Both methods of macrolide administration increased ($P < 0.05$) the proportion of erythromycin resistant enterococci within the population, which was comprised almost exclusively of *Enterococcus hirae*. Injectable macrolides impacted both respiratory and enteric microbes, whereas orally administered macrolides only influenced enteric bacteria.

Keywords: macrolides, antimicrobial resistance, *Mannheimia haemolytica*, enterococci, beef cattle

INTRODUCTION

Bovine respiratory disease (BRD), commonly known as shipping fever continues to be one of the most economically significant health issues in feedlot cattle. The pathogenesis of BRD is multifactorial, being influenced by stress, immune status as well as viral/bacterial interactions within the respiratory tract. Regardless of what initiates the disease, *Mannheimia haemolytica* is considered to be the predominant bacterial pathogen associated with BRD (Confer, 2009).

To reduce or treat BRD, antibiotics are commonly administered to cattle upon arrival in North American feedlots. The use of antimicrobial therapy to control BRD increases in high-density feedlots where conditions are favorable for the introduction and transmission of infectious microbes. The macrolides, tilmicosin and tulathromycin are frequently administered subcutaneously to high-risk cattle, either prophylactically, metaphylactically, or therapeutically to cattle suffering from the disease. In North America, the macrolide tylosin phosphate is also included in beef cattle diets as a growth promoter and to prevent liver abscesses, a practice banned in Europe. After ionophores and tetracycline, macrolides are the most frequently used antimicrobials in cattle production in Canada (CIPARS, 2013). In the United States, a survey of 84% of the US feedlots revealed that about 42% of cattle received tylosin in feed for 138–145 days whereas over two-thirds of the cattle received injectable macrolides (USDA, 1999).

Macrolides belonging to the antimicrobial drug superfamily MLS_B (macrolide–lincosamide–streptogramin B) are classified as category II antimicrobials by the WHO and Health Canada (http://hc-sc.gc.ca/dhp-mps/vet/antimicrob/amr_ram_hum-med-rev-eng.php) emphasizing their importance in treating infections in humans. As reviewed by Gow (2005), as a proportion of total DDDs (Defined Daily Dose) for humans, after penicillins (27%), macrolides (20%) constitute the second most common systemic antibacterial class dispensed by retail pharmacies in Canada, followed by tetracyclines (14%), fluoroquinolones (12%), first- and second-generation cephalosporins (10%).

Although tilmicosin, tulathromycin and tylosin are exclusively used in food animals, they belong to the same category II MLS_B superfamily as erythromycin, which is used in both humans, food and companion animals. Despite having slight structural differences these drugs cross-select for resistance to all drugs of this superfamily, including several drugs used to treat infections in humans such as erythromycin and its derivatives azithromycin and clarithromycin (Roberts, 2008; Desmolaize et al., 2011). Consequently, use of macrolides in livestock could affect the efficacy of these antibiotics in controlling infections in humans through selection for resistance. Macrolide resistance can be conferred by discrete point mutations at nucleotide A2058 and its neighbours in the 23S rRNA, altering the main anchoring point for these antibiotics (Schlünzen et al., 2001) or by methylation of the A2058 at the N6 position as catalyzed by the Erm family of methyltransferases (Skinner et al., 1983). Drug efflux systems have also been shown to result in macrolide resistance (Roberts et al., 1999).

Enterococci are common members of the normal gut flora of both livestock and humans (Yost et al., 2011), but they can also be important human pathogens as *Enterococcus faecalis* and *Enterococcus faecium* are often implicated in nosocomial infections. Macrolide resistant enterococci have been isolated from cattle and depending on the species, could potentially colonize the intestinal tract of humans if they enter the food chain (Giraffa, 2002; Jensen et al., 2002).

The present study investigated and compared the response of respiratory and digestive tract bacteria in feedlot cattle to no antibiotic treatment or treatment with macrolide antibiotics at subtherapeutic (in-feed) or therapeutic (via injection) levels. Our specific objective was to evaluate the effects of administering macrolides to beef cattle either systemically through a single subcutaneous injection (tilmicosin and tulathromycin) or continuously in-feed (tylosin phosphate), on the prevalence and the antimicrobial resistance profiles of faecal *Enterococcus* spp., and *M. haemolytica* from the nasopharynx.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The study was conducted at the individual feeding barn facility at the Lethbridge Research Centre (Lethbridge, Alberta, Canada) using 40–eleven month old beef steers (394 ± 37 kg). All steers originated from the same ranch and had not received antibiotics during their lifetime prior to their arrival at the Lethbridge Research Centre. Steers were housed in individual pens with 10 replicate animals for each of the four treatments (1) control, no antibiotics; (2) tilmicosin (Micotil® Elanco Animal Health) single subcutaneous injection at 10 mg/kg bodyweight (BW) on day 1; (3) tulathromycin (Draxxin® Pfizer Animal Health, www.pfizer.ca) single subcutaneous injection at 2.5 mg/kg BW on day 1; (4) tylosin phosphate (Tylan®, Elanco Animal Health, www.elanco.ca) at 11 ppm in feed for the entire 28 day experimental period (**Figure 1**). Adjacent pens within the same treatment group shared a common water trough and cattle within each treatment were housed in separate, but otherwise identical wings of the barn. Throughout the study, care of the steers was in accordance with the guidelines set by the Canadian Council on Animal Care (http://www.ccac.ca/).

DIET AND FEEDING

Steers were housed in individual pens and fed a typical forage-based growing diet consisting of 70% barley silage, 25% barley grain, and 5% supplement (Addah et al., 2011) on a dry matter (DM) basis, for the entire experimental period (**Figure 1**). Steers were fed once daily in a manner that ensured that all feed that was allotted was consumed. To avoid cross contamination of feed, tylosin was mixed with 5 kg of supplement and manually spread over the surface of feed within each of the appropriate pens during the morning feeding. All cattle were provided feed at the same time each day with no feed remaining in the bunk prior to the next feeding. Cattle assigned to the control treatment had no access to medicated feed throughout the entire experiment and were not injected with any antibiotics.

SAMPLING AND SAMPLE PROCESSING

Rectal faecal and nasopharyngeal swab samples were taken from all 40 animals on arrival (day 0) at the facility, and then weekly thereafter for 4 weeks and processed as follows:

Faecal samples

Rectal grab faecal samples were taken and used for subsequent determination of antimicrobial resistant and total numbers of enterococci bacteria. For the isolation of *Enterococcus* species faecal samples (1 g) were diluted 1:5 in phosphate buffered saline, from which serial dilutions were made up to 10^{-5} and 100 µL of each dilution was spread-plated, in duplicate, onto Bile-Esculin-Azide (BEA) agar plates. Dilutions 10^{-1} and 10^{-2} were also spread-plated onto BEA agar containing erythromycin at a concentration of 8 µg/mL of media (BEA+Ery) to isolate macrolide-resistant enterococci. Plates were incubated at 37°C for 24 h and colonies from both BEA and BEA+Ery plates were enumerated. Three to five presumptive *Enterococcus* colonies per sample per media type were isolated and streak-purified onto BEA or BEA+Ery plates accordingly. Purified isolates were stored in glycerol stocks at −80°C until further characterized.

Nasopharyngeal swab samples

Nasopharyngeal swab samples were collected using a commercially available deep, double guarded culture swab (Jorgensen Laboratories, Inc., Loveland, CO, USA) from all 40 steers on arrival at the beef-barn facility prior to administration of antibiotics, and then weekly thereafter for 4 weeks following antibiotic treatment. Swab samples were transported to the lab on ice and immediately suspended in 0.7 mL of Brain Heart Infusion (BHI) broth. Aliquots (100 µL) were cultured at 37°C for 16 h onto BAC-agar plates (tryptic soy agar plates containing 5% sheep blood and 15 µg/mL of bacitracin; Dalynn Biologicals, Inc., Calgary, AB, Canada) with and without the addition of erythromycin (Ery) 8 µg/mL at 37°C for 16 h. Colonies (1–5) indicative of *Mannheimia*, were selected and tested for catalase and oxidase activity as described previously (Klima et al., 2011). Isolates that exhibited typical *M. haemolytica*

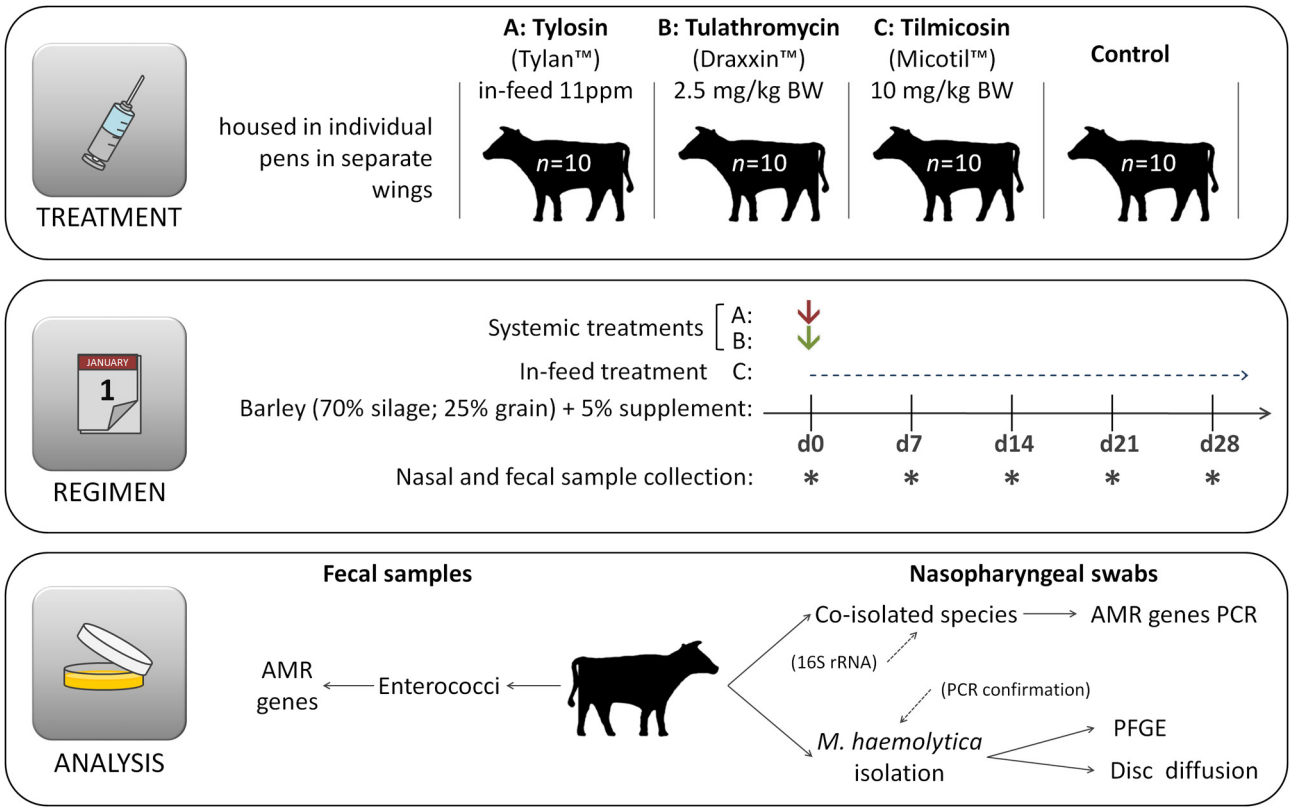


FIGURE 1 | Experimental design. Immediately after first sample collection on day 0 (d0) animals were administered appropriate treatments and hence the same day is also referred as day 1, denoting the beginning of the experimental period. Cattle had no prior direct exposure to antibiotics prior to initiation of the experiment.

colony morphology and were both catalase and oxidase positive were subsequently confirmed using a multiplex PCR assay (Alexander et al., 2008). Confirmed *M. haemolytica* isolates were stored at −80°C in BHI broth containing 20% glycerol for further characterization. Colonies that did not exhibit morphology indicative of *Mannheimia* on BAC or BAC+Ery agar plates were identified and 1–2 colonies representing each morphotype were selected and stored at −80°C in BHI broth containing 20% glycerol until further characterized. Bacitracin in BAC media inhibited the growth of the majority of gram positive bacteria, thereby improving the likelihood of isolating *M. haemolytica*. Although bacitracin resistant gram positive bacteria were co-isolated with *Mannheimia*, these isolates were subsequently identified using 16S rRNA profiling as described below.

CHARACTERIZATION OF *Mannheimia haemolytica* AND CO-ISOLATED NASOPHARYNGEAL BACTERIA

Confirmed *M. haemolytica* isolates were serotyped as previously described (Klima et al., 2011) with antisera prepared in rabbits against formalin-killed whole cells of *M. haemolytica* reference strains UGCC G1 (serotype 1), UGCC G2 (serotype 2), ATCC 29697 (serotype 6), ATCC 29698 (serotype 7), and ATCC 29700 (serotype 9). Isolates were subject to pulsed field gel electrophoresis (PFGE) profiling using *Sal*I restriction enzyme as previously described (Klima et al., 2011). The 16S rRNA genes were PCR amplified from bacteria co-isolated with *M. haemolytica* using universal bacterial 16S rRNA gene primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) and subject to DNA sequencing (Eurofins MWG Operon, Huntsville, Alabama, USA) using one (27F) or both (27F and 1492R) primers.

ANTIBIOGRAMS

Disk susceptibility tests were conducted for *Mannheimia* in accordance with the Clinical and Laboratory Standards Institute documents M31-A3 and M45-A (CLSI, 2008a,b). The antimicrobials tested, suppliers and resistance breakpoints applied are listed in **Table 1**. Reference strains *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Streptococcus pneumoniae* ATCC 49619, and *M. haemolytica* ATCC 33396 were used as quality controls. Briefly, cultures grown on Muller–Hinton agar supplemented with 5% defibrinated sheep blood (MHB; 16–18 h at 37°C) were suspended into Muller–Hinton broth to an absorbance reading between 0.125 and 0.145 at 625 nm. Using sterile swabs, the prepared inocula were swabbed onto MHB followed by the dispensation of the antibiotic containing disks onto the plate surface. The plates were incubated at 37°C in ambient air, with the exception of *S. pneumoniae* ATCC 49619 which required cultivation in a 5% CO_2 atmosphere for 24 h.

Table 1 | Antimicrobial agents, suppliers, disk contents, and interpretative criteria used for disk susceptibility testing.

Antimicrobial	Supplier	Supplier code	Disk content (μg)	Zone diameter (mm) breakpoints[c]		
				S	I	R
Amoxicillin/clavulanic Acid[a]	BD	AMC-30	20/10	≥27	n/a	≤26
Ampicillin[a]	BD	AM-10	10	≥27	n/a	n/a
Ceftiofur[b]	BD	XNL-30	30	≥21	18–20	≤17
Danofloxacin[b]	Pfizer	DNO	5	≥22	n/a	n/a
Erythromycin[a]	BD	E-15	15	≥27	25–26	≤24
Florfenicol[b]	BD	FF-30	30	≥19	15–18	≤14
Gentamicin[b]	BD	GM-10	10	≥15	13–14	≤12
Oxytetracycline[a]	BD	T-30	30	≥23	n/a	n/a
Spectinomycin[b]	BD	SPT-100	100	≥14	11–13	≤10
Sulfamethoxazole[a]/trimethoprim	BD	SXT	23.75/1.25	≤24	n/a	n/a
Tilmicosin[b]	BD	TIL-15	15	≥14	11–13	≤10
Tulathromycin[b]	Pfizer	TUL	30	≥18	15–17	≤14

[a] *M45-A: Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline (CLSI, 2008b). Due to the unavailability MIC breakpoints for Mannheimia spp., guidelines for Pasteurella spp. were followed.*

[b] *M31-A3: Performance standard for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard—third edition (CLSI, 2008a).*

[c] *Zone diameter value used to indicate susceptible (S), intermediate (I) and resistant (R), n/a, not available.*

The resulting zones of inhibition were read using the BioMic V3 imaging system (Giles Scientific, Inc., Santa Barbara, CA, USA).

CHARACTERIZATION OF ENTEROCOCCI

The enterococci isolated from faecal samples were confirmed to be *Enterococcus* spp. by PCR using primers Ent-ES-211-233-F (5′-GHACAGAAGTRAAATAYGAAGG-3′) and Ent-EL-74-95-R (5′-GGNCCTAABGTHACTTTNACTG-3′) and 130 select isolates representing both erythromycin susceptible and resistant categories were further analyzed for species identification by pyrosequencing as described by Zaheer et al. (2012). Thirty six select isolates were subject to PFGE profiling using *Sma*I restriction enzyme using an adaptation of the procedure of Turabelidze et al. (2000). Briefly, bacteria from overnight brain-heart infusion-agar (BHI-agar) cultures were harvested using sterile swabs and suspended in cell suspension buffer [100 mM Tris-HCl (pH 8.0) and 100 mM EDTA], to an optical density (OD) of 1.2–1.3 at 610 nm (1-cm light path). Aliquots (1 ml each) of the suspensions were centrifuged (10,000 × g) for 2 min. in a microcentrifuge and 2/3rd of the supernatant was removed from the tube. The bacterial pellet was resuspended in the remaining supernatant, concentrating the cell suspension to an OD_{610} of 3.6–4.0 (ca. 2.5×10^9 CFU/ml). An aliquot (100 μL) of cell suspension was added to an equal volume of lysis buffer (50 mM Tris-HCl (pH 8), 50 mM EDTA, 625 U/ml mutanolysin, 2.5 mg/ml lysozyme, 1.5 mg/ml proteinase K, 20 μg/ml RNase), mixed gently and incubated at 37°C for 10 min. An equal volume of 1.2% molten SeaKem Gold agarose (FMC BioProducts, Rockland, Maine) containing 1% sodium dodecyl sulfate was added, the mixtures were poured in duplicate into 2-cm by 1-cm by 1.5-mm reusable plug molds (Bio-Rad Laboratories, Hercules, CA) and allowed to solidify at room temperature for 10 min. The duplicate plugs were added to a tube containing 1.8 mL of proteolysis solution [0.44 M EDTA (pH 8.0), 1% sarcosyl, 400 μg/ml of proteinase K] and incubated with constant agitation at 300 rpm for 2 h at 55°C. Plugs were washed 3 times for 10 min each in H_2O (1.8 mL), followed by 3 times for 10 min in TE (1.8 mL) in a thermomixer set at 50°C and 300 rpm. One plug was cut in three equal slices latitudinally and two of the gel slices were pre-incubated in 200 μL of 1X restriction enzyme buffer for 15 min at 30°C. DNA in the plugs was restricted with 50 units of *Sma*I in a 200 μL reaction mixture for 3 h at 25°C. As a reference standard *Xba*I digested *Salmonella* serotype Braenderup (H9812) plugs were prepared as previously described (Klima et al., 2011).

The digested plugs were embedded in 1% SeaKem Gold low-melting temperature agarose (Lonza Canada, Inc., Shawinigan, QC) that was dissolved in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Prior to incorporation into the gel, digested plugs were incubated with 200 μl of 0.5 × TBE at room temperature for 20 min. The digested DNA were separated by PFGE using a CHEF DRII device (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), at 12°C. The voltage was maintained at 6 V/cm for a total of 21 h with switch times of 4–40 s for initial 12.5 h followed by switch times of 1.5–6 for 8.5 h. The gels were run in 0.5 × TBE buffer containing 0.45 mM thiourea. After electrophoresis, gels were stained with ethidium bromide (1 μg/ml) in distilled water for 20 min followed by three 20 min washes with distilled water. Gels were photographed with an AlphaImager gel documentation system (Alpha Innotech Corp., St. Leandro, CA). Fragment analysis was performed with BioNumerics V5.1 software (Applied Maths Inc., Austin, TX).

IDENTIFICATION OF MACROLIDE RESISTANCE DETERMINANTS

Erythromycin-resistant isolates were evaluated for the presence of the commonly found macrolide resistance determinants *erm*(A),

erm(B), *erm*(C), *erm*(F), *erm*(T), *erm*(X), *mef*(A) (http://faculty.washington.edu/marilynr/) by PCR analyses. For generating PCR template, a single bacterial colony was suspended in 50 μL of TE (10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0) and incubated at 95°C for 5 min followed by centrifugation at 10,000 × g for 5 min. Supernatant (2 μL) was used as template in a 20 μL volume PCR reaction mixture using PCR primers and reaction conditions as described elsewhere (Chen et al., 2007; Szczepanowski et al., 2009). The commercially available HotStarTaq Plus Master Mix Kit (Qiagen Canada, Inc., Mississauga, ON, Canada) was used according to manufacturer's instructions. Plasmids containing corresponding gene fragments previously cloned in our laboratory were used as positive controls. Select PCR fragments amplified from erythromycin resistant isolates originating from the present study were verified by DNA sequencing.

STATISTICAL ANALYSIS

Data were analyzed using commercially available statistical analysis software (SAS System for Windows, release 9.1.3, SAS Institute, Cary, NC). Prevalence of *M. haemolytica* and erythromycin resistance in enterococci were analyzed using logistic methodology within the GLIMMIX procedure of SAS, with treatment in the model and day of sampling treated as a repeated measure. Model adjusted means (LS means back-transformed to original scale) and standard errors were reported and used to estimate the efficacy of the antibiotic treatments for controlling *M. haemolytica*. For all tests, the level of significance was set at $P < 0.05$.

ETHICS STATEMENT

Experiments with beef steers were conducted according to the Canadian Council on Animal Care (CCAC) guidelines. The studies were approved by the institutional Animal Care Committee (ACC), Lethbridge Research Centre, Agriculture and Agri-Food Canada, under protocol number 1111. Antibiotics were administered or fed at levels approved by the Canadian Bureau of Veterinary Drugs and recommended by the manufacturer and used in accordance with industry practices.

RESULTS

Mannheimia haemolytica CHARACTERIZATION

A total of 274 suspect *M. haemolytica* isolates were obtained over the duration of the study of which 260 were confirmed by multiplex PCR assay (Alexander et al., 2008). Isolates were obtained from 29 out of the 40 steers used in the experiment. All of the confirmed *M. haemolytica* isolates were serotyped and 160 (1–3 isolates per *Mannheimia* positive animal per sampling event) were tested for antimicrobial susceptibility using the disk diffusion assay, and 65 (one isolate belonging to each of the *M. haemolytica* positive animals for each sampling event) were subjected to PFGE.

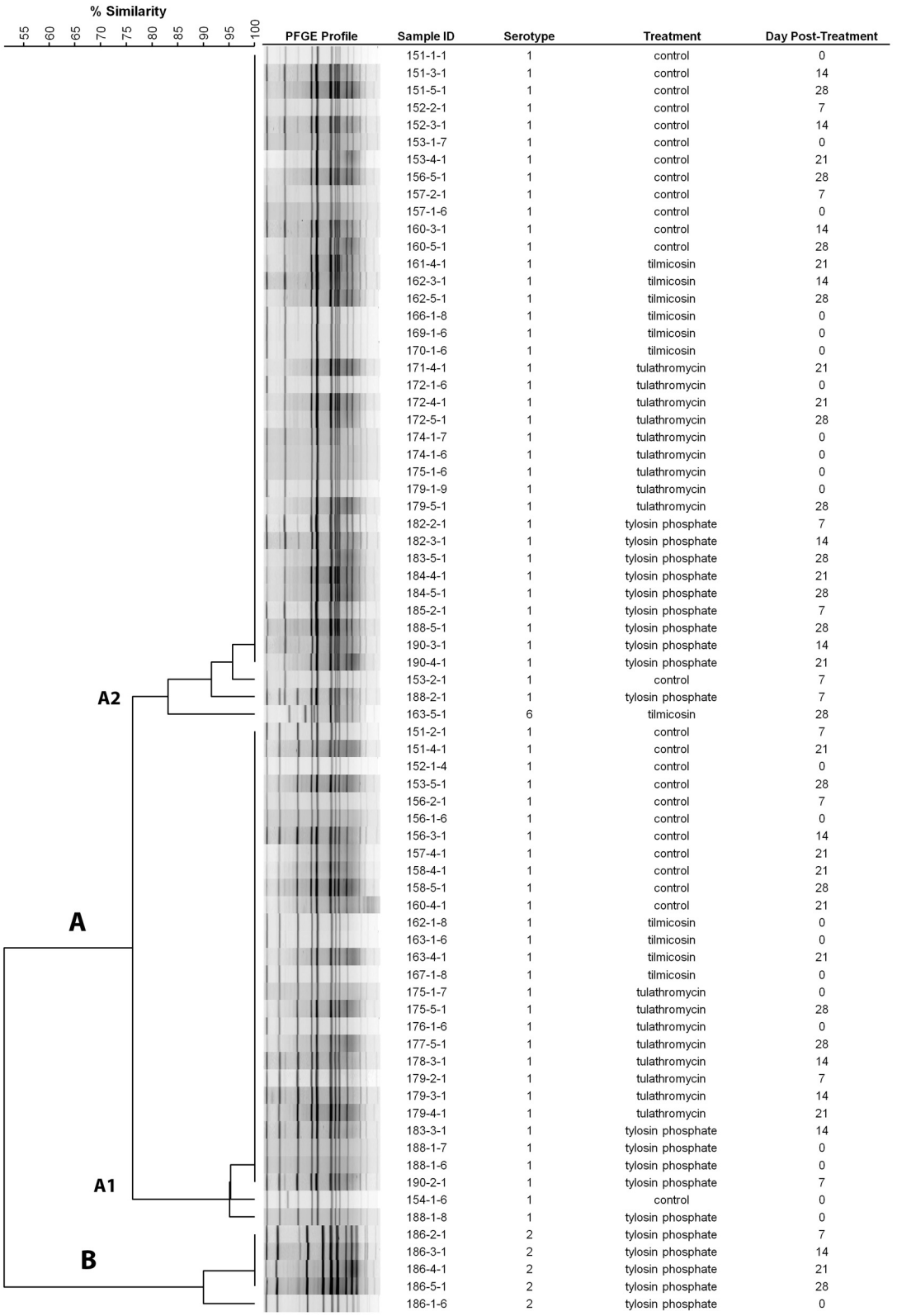
Serotyping revealed that 89% (232/260) of the *M. haemolytica* isolates that originated from 93% (27/29) of the positive animals were serotype 1, whereas 8.4% (22/260) of the isolates, all of which originated from a single steer were serotype 2. Two percent (5/260) of the isolates were identified as serotype 6, all of which were obtained from a single steer on the 28th day post treatment. Two main clusters were identified by PFGE analysis, cluster A consisted primarily of serotype 1 isolates with only a single serotype 6 isolate, whereas cluster B was comprised of serotype 2 isolates (**Figure 2**). Two sub-clusters, A1 and A2 were observed within cluster A; A1 solely comprising serotype 1 isolates and A2 consisting of a mixture of serotype 1 and 6 (**Figure 2**).

The prevalence of *M. haemolytica* dropped substantially from steers that were injected with tilmicosin or tulathromycin as compared to levels prior to treatment (**Figure 3A**). Compared to the control, the number of steers harboring *M. haemolytica* was reduced ($P < 0.05$) by systemic treatment with either tilmicosin or tulathromycin over the post treatment sampling period (days 7–28). Compared to the in-feed tylosin, tilmicosin and tulathromycin also resulted in a reduction ($P < 0.05$) in number of cattle positive for *M. haemolytica* (64 and 42%, respectively) (**Figure 3B**). In disk diffusion assays, *M. haemolytica* isolates cultured without erythromycin on primary isolation were all sensitive to amoxicillin/clavulanic acid, ampicillin, ceftiofur, danofloxacin, erythromycin, florfenicol, gentamicin, oxytetracycline, spectinomycin, trimethoprim/sulfamethoxazole, tilmicosin, and tulathromycin.

CHARACTERIZATION OF BACTERIA CO-ISOLATED WITH *M. haemolytica*

The non-*Mannheimia* bacterial colonies originating from nasopharyngeal samples cultured on BAC or BAC+Ery agar plates were divided into 11 morphological groups (**Table 2**). The 16S rRNA gene sequences from 165 select isolates with 5–15 isolates representing each morphology group were subsequently analyzed for genus/species identification through alignments using "seqmatch" (http://rdp.cme.msu.edu/index.jsp) or BLAST (http://blast.ncbi.nlm.nih.gov/). The morphotypes were found to be very consistent with 16S rDNA sequence based bacterial identification and therefore were used to define the identity of collected isolates. One representative of each morphotype per sampling event was used to determine bacterial prevalence. Along with *M. haemolytica* (13%), other bacteria found in abundance included *Pasteurella multocida* (25%), *Staphylococcus* spp. (25%), *Acinetobacter* spp. (9%), *E. coli*/*Shigella* group (8%), and *Bacillus licheniformis* (7%) (**Figure 4**). Among the *Staphylococcus* spp., *S. epidermidis*, *S. pasteuri*, and *S. cohnii* were abundant and collectively constituted 20% of isolated nasopharyngeal bacterial species, with *S. sciuri* only occasionally isolated. These *Staphylococcus* species had indistinguishable colony morphologies and thus collectively constituted one morphological group (**Table 2**). *Staphylococcus chromogenes* was placed in a separate morphological group due to its distinct yellow color and it constituted 5% of isolated nasopharyngeal bacterial species. Other species such as streptococci, *Macrococcus casseolyticus* and *Bacillus* spp. including *B. clausii* and *B. pumilus* were less abundant (**Figure 4**). However, collectively and irrespective of morphotypes, *Bacillus* spp. constituted ∼14% of isolated bacteria.

Erythromycin resistance was found in all isolated bacterial species except *M. haemolytica*, *P. multocida* and other less frequently isolated bacteria (**Table 2**). Of the seven macrolide resistance genes tested by PCR, the *erm*(C) was predominantly found in *Staphylococcus* spp. (**Table 2**). With regard to



PFGE Profile	Sample ID	Serotype	Treatment	Day Post-Treatment
	151-1-1	1	control	0
	151-3-1	1	control	14
	151-5-1	1	control	28
	152-2-1	1	control	7
	152-3-1	1	control	14
	153-1-7	1	control	0
	153-4-1	1	control	21
	156-5-1	1	control	28
	157-2-1	1	control	7
	157-1-6	1	control	0
	160-3-1	1	control	14
	160-5-1	1	control	28
	161-4-1	1	tilmicosin	21
	162-3-1	1	tilmicosin	14
	162-5-1	1	tilmicosin	28
	166-1-8	1	tilmicosin	0
	169-1-6	1	tilmicosin	0
	170-1-6	1	tilmicosin	0
	171-4-1	1	tulathromycin	21
	172-1-6	1	tulathromycin	0
	172-4-1	1	tulathromycin	21
	172-5-1	1	tulathromycin	28
	174-1-7	1	tulathromycin	0
	174-1-6	1	tulathromycin	0
	175-1-6	1	tulathromycin	0
	179-1-9	1	tulathromycin	0
	179-5-1	1	tulathromycin	28
	182-2-1	1	tylosin phosphate	7
	182-3-1	1	tylosin phosphate	14
	183-5-1	1	tylosin phosphate	28
	184-4-1	1	tylosin phosphate	21
	184-5-1	1	tylosin phosphate	28
	185-2-1	1	tylosin phosphate	7
	188-5-1	1	tylosin phosphate	28
	190-3-1	1	tylosin phosphate	14
	190-4-1	1	tylosin phosphate	21
	153-2-1	1	control	7
	188-2-1	1	tylosin phosphate	7
	163-5-1	6	tilmicosin	28
	151-2-1	1	control	7
	151-4-1	1	control	21
	152-1-4	1	control	0
	153-5-1	1	control	28
	156-2-1	1	control	7
	156-1-6	1	control	0
	156-3-1	1	control	14
	157-4-1	1	control	21
	158-4-1	1	control	21
	158-5-1	1	control	28
	160-4-1	1	control	21
	162-1-8	1	tilmicosin	0
	163-1-6	1	tilmicosin	0
	163-4-1	1	tilmicosin	21
	167-1-8	1	tilmicosin	0
	175-1-7	1	tulathromycin	0
	175-5-1	1	tulathromycin	28
	176-1-6	1	tulathromycin	0
	177-5-1	1	tulathromycin	28
	178-3-1	1	tulathromycin	14
	179-2-1	1	tulathromycin	7
	179-3-1	1	tulathromycin	14
	179-4-1	1	tulathromycin	21
	183-3-1	1	tylosin phosphate	14
	188-1-7	1	tylosin phosphate	0
	188-1-6	1	tylosin phosphate	0
	190-2-1	1	tylosin phosphate	7
	154-1-6	1	control	0
	188-1-8	1	tylosin phosphate	0
	186-2-1	2	tylosin phosphate	7
	186-3-1	2	tylosin phosphate	14
	186-4-1	2	tylosin phosphate	21
	186-5-1	2	tylosin phosphate	28
	186-1-6	2	tylosin phosphate	0

FIGURE 2 | Dendrogram of PFGE *Sal*I profiles from representative *M. haemolytica* isolates collected weekly. As an example, sample ID 151-3-1 represents isolate #1 from 3rd sampling event (day 14 post treatment) from animal #151. (Control: animal IDs 151–160; tilmicosin: animal IDs 161–170; tulathromycin: animal IDs 171–180; tylosin: animal IDs 181–190).

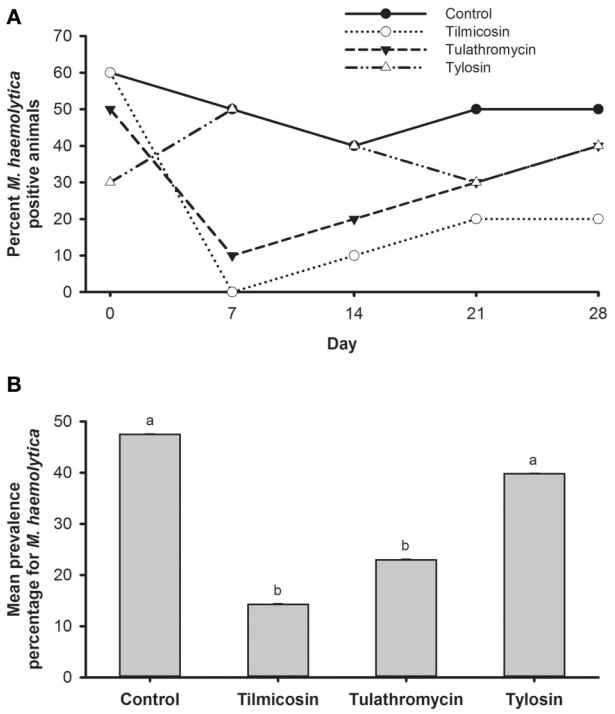


FIGURE 3 | (A) Percentage of animals positive for *M. haemolytica* for each of the five sampling events over 28 days study period. **(B)** Mean percentage of *M. haemolytica* prevalence in animals over the entire study period. Means with different superscripts differ ($P < 0.05$).

other erythromycin-resistant isolates, no resistance determinants matching any of the seven PCR primer pairs were amplified and therefore were considered as "not detected".

EVALUATION OF ENTEROCOCCI PRE- AND POST-MACROLIDE-TREATMENT

Enterococci were isolated from all 40 animals (control and treatment groups) on arrival (day 0), and weekly thereafter for 4 weeks (day 7, 14, 21, and 28). Enterococci colonies obtained on BEA and BEA+Ery plates were enumerated and the proportion of erythromycin resistant colonies was calculated for each sample (**Figure 5**). Compared to the control group, antibiotic treatment groups were 76 times more likely ($P < 0.02$) to have erythromycin resistant enterococci over the study period (days 7–28). Similarly, for all treatment groups, post-treatment samples from the entire study period were 66 times more likely to have erythromycin resistant enterococci ($P < 0.001$) when compared to pre-treatment (day 0) samples. No significant differences in the incidence of erythromycin resistance were observed between injectable (tilmicosin and tulathromycin) and in-feed (tylosin) macrolides.

Speciation of 130 of the enterococci isolates collected from day 0, 7, or 28 revealed that all were *Enterococcus hirae* with the exception of two which were *Enterococcus casseliflavus*. Fifty select isolates of erythromycin resistant enterococci from day 0 and day 7 sampling events were used for the identification of erythromycin resistance determinants. Of the seven macrolide resistance genes investigated via PCR only the *erm*(B) gene was identified in enterococci isolates.

Table 2 | Bacteria co-isolated with *M. haemolytica*.

Morphotypes	Morphology on BAC-agar plates	Species identification based on 16S rDNA sequencing	Ery resistance	Resistance determinant(s)
Mh	Small, glossy, grey, beta-haemolytic	*Mannheimia haemolytica*[a]	–	–
1	Small/small-medium, round, glossy, white	*S. epidermidis, S. cohnii, S. pasteuri, S. saprophyticus, S. sciuri*	+	*erm*(C), *erm*(C), *erm*(C), ND, ND
2	Large, mucoid, semi-transparent, grey-white	*Pasteurella multocida*	–	–
3	Very small, dense, brown/pale, alpha-haemolytic	*Streptococcus/Bacillus*	+	ND
4	Medium/large, wrinkly, crusty, fluid-filled, beta-haemolytic	*Bacillus licheniformis*	+	ND
5	Medium/large, glossy, grey-white, mostly beta-haemolytic	*Escherichia coli/Shigella*	+	–
6	Medium/large, rough edges, flat, granular	*Bacillus clausii/Bacillus spp.*	+	ND
7	Small, glossy, grey/cream	*Acinetobacter lwoffii, Acinetobacter spp.*	+	ND
8	Small/medium, glossy, pale-yellow	*Staphylococcus chromogenes*	NA	ND
9	Small/medium, pale-yellow, concentric with concave center	*Macrococcus caseolyticus*	NA	ND
10	Medium, yellowish, concentric circles, very haemolytic, greenish	*Bacillus pumilus*	NA	ND
U	Unique morphologies found occasionally	*Klebsiella*, *Neisseria* spp., *Paenibacillus* spp., uncultured bacteria	NA	ND

[a] *Also confirmed by multiplex PCR assay (Alexander et al., 2008); NA, not available, as those morphology groups were less commonly found on BAC plates and not found on BAC+Ery plates; ND, not determined (no positives detected in PCRs with any of the tested macrolide primer sets, see Materials and Methods).*

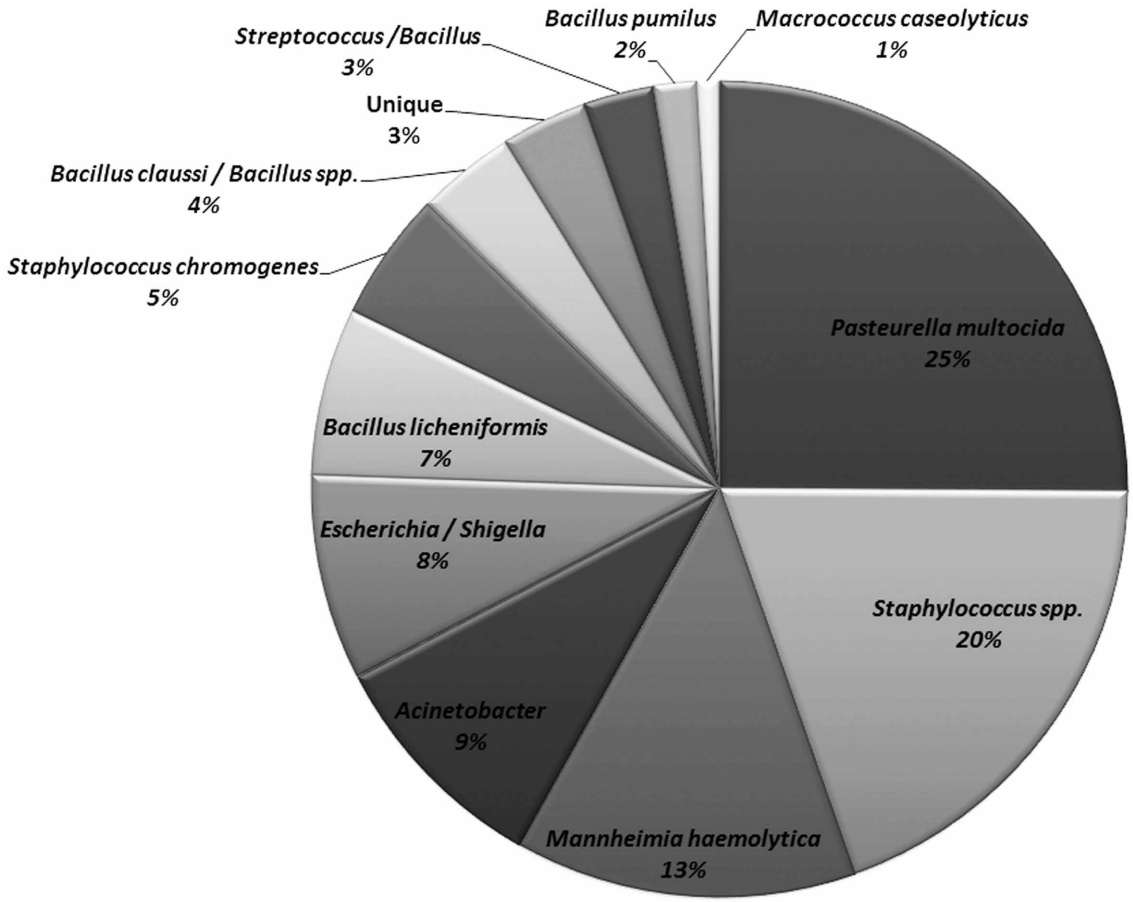


FIGURE 4 | Relative abundance of bacitracin resistant bacterial species isolated from nasopharynx over 28-days sampling period. One representative of each morphology per animal per sampling event were used to determine bacterial species prevalence ($n = 466$).

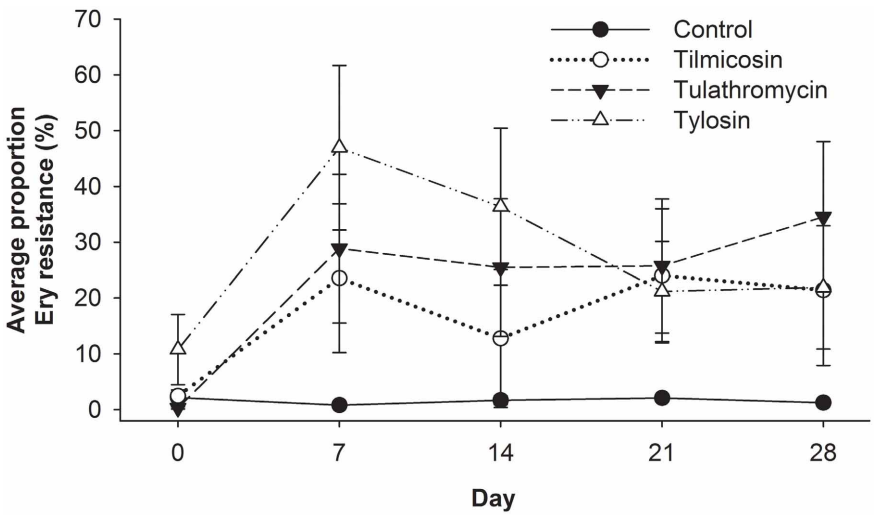


FIGURE 5 | Proportion of erythromycin-resistant faecal enterococci isolates for each of the five sampling events over 28 days study period, with day 0 samples collected prior to antibiotic treatment. From day 7 onward, Control isolates had less resistance detected ($P < 0.05$) than antibiotic treated groups, while resistance noted with injectable macrolides (tilimicosin and tulathormycin) did not differ from that cattle fed tylosin.

A representative 36 erythromycin resistant enterococci from 12 select animals from three sampling events (day 0, 7, and 28) were subject to PFGE and produced three closely related clusters (>85% similarity) (**Figure 6**). In eight of the twelve animals the PFGE profiles from the three sampling events had >90% similarity indicating that erythromycin resistant enterococci likely consisted of a persistent clonal population.

DISCUSSION

The objective of this study was to evaluate and compare the effect of in-feed subtherapeutic and injectable therapeutic administration of macrolides on antimicrobial susceptibility of indicator bacteria from the digestive and respiratory tract of feedlot cattle. For this purpose we selected faecal enterococci and *M. haemolytica* as our indicator bacteria for the digestive and respiratory tract, respectively. Injectable macrolide antimicrobials such as tilmicosin and tulathromycin are commonly used at therapeutic levels in beef cattle production to prevent and treat BRD. The macrolide, tylosin is frequently administered in-feed at subtherapeutic doses for improving feed efficiency and for the reduction of liver abscesses caused by *Fusobacterium necrophorum* and *Actinomyces pyogenes* (www.merckvetmanual.com). The subtherapeutic administration of antibiotics has been hypothesized to promote resistance development as bacteria are exposed to sublethal concentrations of antibiotic for prolonged periods of time. Consequently, the subtherapeutic administration of antibiotics in animal feeds for growth promotion has been proposed as a serious public health concern (Aarestrup and Wegener, 1999; Wegener et al., 1999; McEwen and Fedorka-Cray, 2002). Over the last 6–10 years the minimum inhibitory concentrations (MICs) of tilmicosin and tulathromycin towards *M. haemolytica* have markedly increased (Portis et al., 2012), questioning the continued effectiveness of these antibiotics against the etiological agents of BRD. Furthermore, *M. haemolytica* could serve as a reservoir of macrolide resistance genes, potentially disseminating them to other respiratory pathogens.

Deep nasopharyngeal swabs were taken to isolate *M. haemolytica* as this procedure is quick, simple and relatively non-invasive. In our study, a single systemic administration of therapeutic levels of either tilmicosin or tulathromycin was effective in lowering *M. haemolytica* in the nasopharynx of steers (**Figures 3A,B**). According to the manufacturer, (ELANCO Animal Health, Guelph, On, Canada, www.elanco.ca) injecting cattle with 10 mg of tilmicosin /kg of body weight results in lung concentrations exceeding the MIC(3.12 μg/mL) for *M. haemolytica* for at least 3 days, eliminating it from the respiratory tract for up to 6 days (Frank et al., 2000). Tulathromycin is believed to accumulate in neutrophils and alveolar macrophages (Siegel et al., 2004; Cox et al., 2010), with peak lung levels of 4.1 μg/mL occurring in cattle 24 h after a single injection and concentrations remaining above the MIC for *M. haemolytica* (2.0 μg/mL) for 10 days (www.pfizer.ca). Our data demonstrated that 7 days post-injection, *M. haemolytica* was detected in only one steer treated with tulathromycin and none of the steers treated with tilimicosin, whereas 60% of steers were positive for this bacterium upon arrival (day 0). This suggests that *M. haemolytica* in newly arrived cattle were not macrolide resistant, an observation supported by our inability to isolate erythromycin resistant *M. haemolytica* and likely a reflection of the fact that these cattle had no previous exposure to macrolides.

In contrast to injectable macrolides, tylosin had no effect on the number of *M. haemolytica* in steers administered this antibiotic in feed as compared to cattle that received no antibiotics (**Figures 3A,B**). This observation likely reflects differences in the method and concentration of the antibiotic administered and the sensitivity of *M. haemolytica* to tylosin. Tylosin is not effective at penetrating the outer membrane of Gram-negative bacteria and as a result its MIC (64 μg/mL) against *M. haemolytica* is much higher than either tilimicosin or tulathromycin (Andersen et al., 2012). Tylosin is known to be widely distributed in body fluids and tissues, but comparative pharmacokinetics of its distribution in the digestive tract relative to the respiratory tract when it is administered in feed are poorly characterized (Lewicki, 2006).

Mannheimia haemolytica isolated from animals belonging to control and all three macrolide treatment groups throughout the course of study were found to be susceptible to all tested macrolides indicating that both therapeutic and subtherapeutic administration did not contribute to macrolide resistance in *M. haemolytica* during the study. *Mannheimia haemolytica* isolated in the present study were also susceptible to all other antibiotics tested (**Table 2**). While tilmicosin and tulathromycin are generally effective against *M. haemolytica*, a few isolates originating from Germany, Japan and United States have shown resistance to these antibiotics (Katsuda et al., 2009; Watts and Sweeney, 2010; Michael et al., 2012). An integrative conjugative element (ICE) has been identified in *P. multocida* that exhibits high similarity to ICEs in *P. multocida* 36950, *Histophilus somni* 23364 and an ICE fragment within the incomplete *M. haemolytica* PHL23 genome (Michael et al., 2012). This is a matter of concern as *P. multocida*, *M. haemolytica*, and *H. somni* often share the same ecological niche in the bovine respiratory tract and exchange of this ICE element could lead to macrolide resistance in these BRD pathogens as transfer of this element among *P. multocida*, *M. haemolytica,* and *E. coli* has been demonstrated in the laboratory (Dabo et al., 2007; Watts and Sweeney, 2010).

The majority of *M. haemolytica* isolates collected from asymptomatic animals in this study were serotype 1 (**Figure 2**). The PFGE profiles of serotype 1 and 6 isolates clustered together with a ∼83% relatedness, a relationship observed previously for these serotypes (Klima et al., 2011). The predominance of a single serotype in the majority of steers is likely a reflection of sourcing them from the same isolated ranch in southern Alberta and transporting them directly to the Lethbridge Research Centre. This arrangement was necessary to ensure that steers had no exposure to antibiotics prior to arrival. Serotype 1 has frequently been linked to clinical disease and both serotypes 1 and 6 are often isolated from cattle with BRD (Zecchinon et al., 2005). However, none of the steers in the present study exhibited clinical BRD. The BRD complex consists of a bacteria (*M. haemolytica, Pasteurella multocida, Histophulus somni, Mycoplasma bovis*) and viruses (Bovine Viral Diarrhea, Infectious Bovine Rhinotracheitis, Bovine Respiratory Synctial Virus, Parainfluenza Type-3 Virus) which together supress innate immune responses and cause lung damage (Fulton, 2009; Pardon

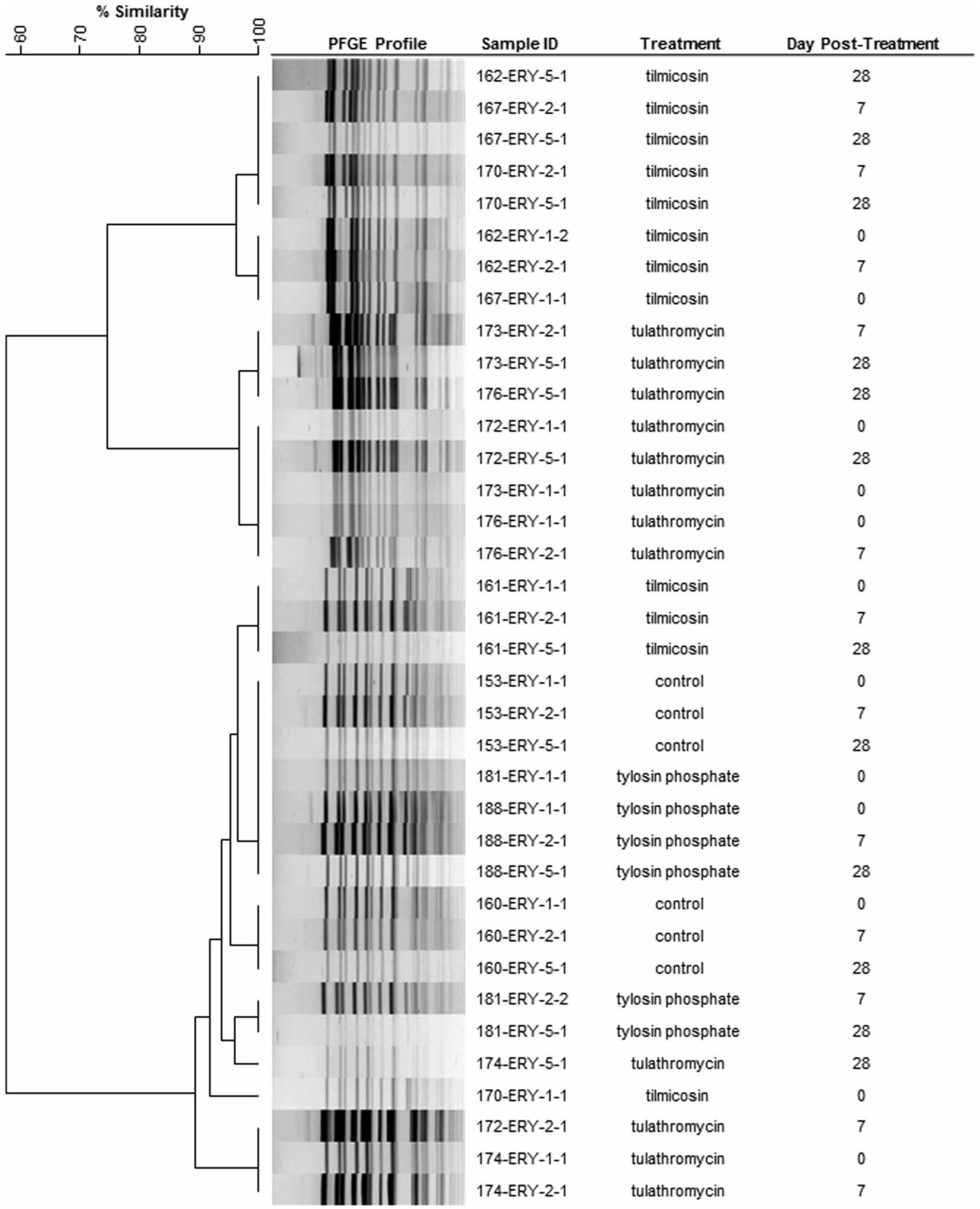


FIGURE 6 | Dendrogram of PFGE *Sma*I profiles from representative erythromycin resistant enterococci from day 0, 7, and 28. As an example, sample ID 153-ERY-5-1 represents isolate #1 collected from BEA+ERY plates on 5th sampling event (day 28) from animal #153. (Control: animal IDs 151–160; tilmicosin: animal IDs 161–170; tulathromycin: animal IDs 171–180; tylosin: animal IDs 181–190.

et al., 2011). It seems likely that in this study the steers lacked the infectious complex necessary for the development of BRD.

Pasturella multocida was the most ubiquitous bacterium isolated from the nasopharynx of steers and in contrast to *M. haemolytica*, prevalence of this bacterium was similar in pre- and post-treatment groups. However, none of the isolated *P. multocida* exhibited resistance to erythromycin. As *P. multocida* were not enumerated, it is possible that macrolides reduced the population of this bacterium. After *P. multocida*, *Staphylococcus* spp. was most abundant with a relative abundances of *S. epidermidis* > *S. pasteuri* > *S. cohnii* > *S. sciuri* > *S. saprophyticus*. Staphylococci are ubiquitous Gram-positive bacteria and are common in the microflora of skin and mucosal surfaces. Currently, there are 31 species recognized in the genus *Staphylococcus* and about half of these are indigenous to humans (Kloos and Bannerman, 1994). The role of some species, such as *S. epidermidis*, *S. saprophyticus*, *S. Pasteuri* and *S. cohni* in human disease has been well documented (Piette and Verschraegen, 2008; Savini et al., 2009). Consistent with previous studies (Aarestrup et al., 2000a; Simeoni et al., 2008), the majority of the erythromycin resistant *Staphylococcus* spp. harbored *erm*(C).

Thirteen percent of the isolated nasopharyngeal bacteria were *M. haemolytica* followed by *Acinetobacter*, Enterobacteriaceae (*Esherichia spp., Shigella spp.)* and *Bacillus* spp. including *B. licheniformis* and *B. clausii*. Most of these bacteria are recognized as part of normal flora of the skin, oropharynx and perineum of healthy individuals. While erythromycin resistance was found in the majority of isolated nasopharyngeal bacteria, macrolide determinants could only be detected in the *Staphylococcus* group. The primers we used to detect seven macrolide resistance genes would not be expected to capture all of the genes potentially conferring resistance. For example, primers for erythromycin resistance genes *erm*(D) and *erm*(34) previously characterized from *B. licheniformis* and *B. clausii*, respectively (Israeli-Reches et al., 1984; Bozdogan et al., 2004) were not included in our panel. Although we observed no increase in the diversity of erythromycin resistant nasopharyngeal bacteria in our study, determinants in those bacteria that possessed them could be disseminated into the broader environment.

Escherichia coli are commonly used as faecal indicator bacteria to assess AMR, but we chose enterococci as *E. coli* are intrinsically resistant to macrolides (Mao and Putterman, 1968). Enterococci are common inhabitants of the normal gut flora of both livestock and humans (Yost et al., 2011). Outside of their normal habitat, enterococci are viewed as pathogens and may present a public health concern as they can be transmitted to humans from other hosts or by ingestion of contaminated food or water (Heuer et al., 2006; Marshall and Levy, 2011). Enterococci, in particular *E. faecalis* and *E. faecium* are recognized as prevalent nosocomial pathogens (Fisher and Phillips, 2009; van Schaik and Willems, 2010) with many isolates being resistant to multiple antibiotics and capable of exchanging DNA with other bacteria (SchjØrring and Krogfelt, 2011). In the present study we did not isolate either *E. faecalis* or *E. faecium*, with *E. hirae* being the predominant species isolated from cattle, a species infrequently associated with hospital infections.

The present study revealed a significant increase in the proportion of erythromycin resistant enterococci following macrolide treatment regardless of the method of administration (**Figure 5**). Oral administration of tylosin was expected to have a direct impact on the enterococci population of the gut, but the occurrence of erythromycin resistant enterococci in cattle administered injectable macrolides was equally marked. Studies submitted to the Food and Drug Administration's Center for Veterinary Medicine (FDA/CVM) showed that with a single subcutaneous dose of tilmicosin to cattle, 24% was recovered in the urine and 68% in the feces, whereas with tulathromycin, 50% was recovered in the feces with 90% of this being in its original form. It has been proposed that tulathromycin losses activity at pH $\leq$ 7.0, (Food and Drug Administration's Center for Veterinary, 2013), but considering that the pH in intestinal digesta and in feces is usually neutral or acidic (Allison et al., 1979; Canh et al., 1997), our results would suggest that this antibiotic selected for resistant enterococci within the intestinal tract. It is possible that the forage rich diet used in our study resulted in intestinal contents having, a pH above 7.0, allowing the concentration of tulathromycin in digesta to exceed the MIC of enterococci. High forage diets in cattle are known to increase colonic pH to ranges between 7.4 and 8.0 (Scott et al., 2000; Loy et al., 2001), but results could be quite different on high grain diets where the pH of digesta is considerably lower.

Similar PFGE profiles were observed for erythromycin resistant enterococci from both pre- and post-treatment samples (**Figure 6**), suggesting that regardless of the method of administration, macrolides selected for erythromycin resistant enterococci that were already in the digestive tract. Selection for resistant enterococci combined with a reduction in susceptible enterococci significantly increased the presence of erythromycin resistant *Enterococcus* spp. within the digestive tract. This observation is of interest considering that the cattle used in this study originated from a very isolated ranch and never had prior direct exposure to macrolides. It would be interesting to examine the persistence of this resistant population for a prolonged period of time to understand population dynamics and to investigate if the metabolic burden/cost of antibiotic resistance genes in the absence of macrolides leads to a decline in resistance within the enterococci population over time. As is typical in industry, cattle in this study were treated with injectable macrolides early in the feeding period. Considering that they would have been fed for an additional 200 days prior to slaughter, loss of resistant enterococci from the intestinal tract at later points in the feeding period is a distinct possibility. However, resistant enterococci may persist in cattle fed tylosin as this antibiotic is often administered for a longer duration of the feeding period.

All of the isolated erythromycin resistant enterococci contained *erm*(B), a gene coding for rRNA adenine N-6-methyltransferase, which methylates the A2058 position of 23S rRNA. Macrolide resistance in enterococci isolates from humans and animal sources in Europe has been well documented (Jensen et al., 1999; Aarestrup et al., 2001). Occurrence of macrolide resistance in enterococci originating from swine is thought to stem from the subtherapeutic use of tylosin (Jackson et al., 2004). The co-existence of macrolide resistance genes with other

antibiotic resistance genes has also been observed, most notably a link between resistance to macrolides and vancomycin (Aarestrup et al., 2000b), attributable to *erm*(B) and the *vanA* gene occurring in close proximity on the same plasmid. The increased occurrence of both *erm* and *tet* (tetracycline resistance) genes in faecal microbial communities from beef cattle fed subtherapeutic levels of tylosin has also been identified (Chen et al., 2008). Linkage of determinants for MLS_B and chloramphenicol resistance has also been found on a single conjugative plasmid in *E. faecium* and dissemination of this cluster among streptogramin-resistant enterococci occurs (Werner et al., 2000). Resistance to MLS_B antibiotics in Gram-positive cocci colonizing humans is now recognized to be a serious problem, negatively affecting clinical outcomes (Lim et al., 2002; DiPersio and DiPersio, 2006).

Regardless of the hypothesized prospects of subtherapeutic administration of antimicrobials contributing towards AMR development, there is limited and conflicting data as to the extent that subtherapeutic vs. therapeutic drug administration contributes to livestock mediated antimicrobial resistance. Studies have shown that subtherapeutic administration of tylosin had no impact on the prevalence of erythromycin resistant *Campylobacter* in feedlot cattle (Inglis et al., 2005), whereas with broiler chickens the frequency of macrolide resistant *Campylobacter* in cecal contents was increased with subtherapeutic vs. therapeutic doses of tylosin (Ladely et al., 2007). These discrepancies may reflect species-specific (cattle vs. chicken) differences in gastrointestinal physiology and diet. Others have found that short-term therapeutic use of chlortetracycline in the diet was no less likely to select for resistant *Salmonella* populations than long-term subtherapeutic use (Kobland et al., 1987). In-feed and subcutaneous administration of oxytetracycline were also equally responsible for increasing the proportion of feedlot cattle excreting tetracycline resistant *E. coli* in faeces (Checkley et al., 2010).

The present study offers a comparison of subtherapeutic and therapeutic drug administration with regards to the prevalence of resistance among bacteria from two independent locations in cattle. In conclusion, the injectable macrolides had impact on both respiratory and enteric microbes whereas orally administered macrolides only influenced enteric bacteria. Therapeutic levels of tilmicosin and tulathromycin were effective in lowering nasopharyngeal *M. haemolytica*, whereas the in-feed levels of tylosin had no effect on the prevalence of this bacterium. *M. haemolytica* isolates from control and macrolide treated animals were found to be susceptible to macrolides as well as other antibiotics tested. The lack of AMR in *M. haemolytica* may be attributed to the possible absence of AMR determinants in *Mannheimia* as well as other closely related bacteria such as *P. multocida*. Erythromycin resistance was detected in nasopharyngeal bacteria co-isolated with *M. haemolytica*, regardless of the treatment group. All three macrolides increased the occurrence of erythromycin resistance *Enterococcus* spp. within the intestinal tract of cattle, but the species identified were not those most frequently linked to nosocomial infections in humans. To our knowledge this is the first report on increased occurrence of macrolide resistance in enterococci after systemic macrolide usage in cattle. It would be interesting to monitor the post-treatment AMR resistance over a period of weeks to months beyond treatment to determine if these macrolide-resistant enterococci continue to persist within the faecal bacterial populations of cattle.

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Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria

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Production of beta-lactamases, the enzymes that degrade beta-lactam antibiotics, is the most widespread and threatening mechanism of antibiotic resistance. In the past, extensive research has focused on the structure, function, and ecology of beta-lactamases while limited efforts were placed on the regulatory mechanisms of beta-lactamases. Recently, increasing evidence demonstrate a direct link between beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. Specifically, expression of beta-lactamase could be induced by the liberated murein fragments, such as muropeptides. This article summarizes current knowledge on cell wall metabolism, beta-lactam antibiotics, and beta-lactamases. In particular, we comprehensively reviewed recent studies on the beta-lactamase induction by muropeptides via two major molecular mechanisms (the AmpG–AmpR–AmpC pathway and BlrAB-like two-component regulatory system) in Gram-negative bacteria. The signaling pathways for beta-lactamase induction offer a broad array of promising targets for the discovery of new antibacterial drugs used for combination therapies. Therefore, to develop effective mitigation strategies against the widespread beta-lactam resistance, examination of the molecular basis of beta-lactamase induction by cell wall fragment is highly warranted.

Keywords: beta-lactamase, regulation, peptidoglycan

INTRODUCTION

Bacteria should continuously maintain and shape their envelopes to adapt enormous stresses they encounter in different niches and to meet physiological needs, such as growth and multiplication. Bacterial envelope is highly organized as a layer structure including cell wall, membrane(s), and the possible space between them. The structure of cell envelope varies in prokaryotes. In general, Gram-positive bacteria contain a thick layer of cell wall as well as a layer of cytoplasmic membrane. However, Gram-negative bacteria (e.g., *Escherichia coli*) typically contain an outer membrane, an intervening periplasmic space where a thin layer of cell wall resides, and a layer of cytoplasmic membrane.

The bacterial cell wall is unique to bacteria and plays a critical role in maintaining cell integrity. In addition, the conserved cell wall components, such as monomeric disaccharide tetrapeptide, could serve as a signal to trigger host immunologic or pathologic responses (Goldman et al., 1982; Melly et al., 1984; Viala et al., 2004; Watanabe et al., 2004; Dziarski and Gupta, 2005; Cloud-Hansen et al., 2006; Strober et al., 2006). Thus, given its significant role in bacterial pathophysiology, cell wall has been an effective target for developing various antimicrobials with different mode of actions, such as beta-lactam and glycopeptide antibiotics. Of these, beta-lactam antibiotics are the most commercially available antibiotics in the market. Until 2010, beta-lactam antibiotics account for sales of approximately 53% of the total antibiotic market worldwide (42 billion US dollars; Hamad, 2010). Beta-lactam antibiotics inhibit bacterial cell wall biosynthesis, consequently leading to cell lysis and death. Specifically, beta-lactam antibiotics bind and acylate active site of penicillin-binding protein (PBP), the enzyme essential for the biosynthesis of bacteria cell wall.

To counteract bactericidal effect of beta-lactams, bacteria have quickly evolved defense systems in which production of beta-lactamase is a major beta-lactam resistance mechanism. Bacterial resistance to beta-lactam antibiotics has become a worldwide health care problem, as exemplified by the recent emergence of broad-range beta-lactam resistant NDM-1 (New Delhi metallo-beta-lactamase 1) strains (Kumarasamy et al., 2010). Beta-lactamase is an enzyme that could hydrolyze beta-lactam ring, consequently deactivating beta-lactam antibiotics. In Gram-negative bacteria, the beta-lactamase was usually produced at very high concentration constitutively or by induction via direct interaction of beta-lactam antibiotic with regulatory system (e.g., MecR1/MecI in *Staphylococcus aureus*; Kogut et al., 1956; Richmond, 1963, 1965; Pollock, 1965; Zhu et al., 1992; Fuda et al., 2005; Safo et al., 2005). In Gram-negative bacteria, the expression level of beta-lactamase is usually low; however, it has been observed that production of beta-lactamase was inducible but molecular basis for this phenomenon was not clear (Ambler, 1980; Jacobs et al., 1997).

In the past, extensive research has focused on the structure, function, and ecology of beta-lactamases while limited efforts were placed on the regulatory mechanisms of beta-lactamases. In 1990s, the induction of beta-lactamase AmpC was observed to be correlated to the recycling process of cell wall in Gram-negative

Abbreviations: GlcNAc, *N*-acetylglucosamine; LT, lytic transglycosylase; MurNAc, *N*-acetylmuramic acid; PBP, penicillin-binding protein; PG, peptidoglycan; TCRS, two-component regulatory system.

bacteria, which shed light on the molecular basis of beta-lactamase induction (Jacobs et al., 1994). In the past two decades, accumulating evidence have shown the relationship between muropeptide release and beta-lactamase induction in Gram-negative bacteria (Holtje et al., 1994; Jacobs et al., 1994, 1997; Korsak et al., 2005). However, in Gram-positive bacteria, there is little evidence showing the induction of beta-lactamases by liberated murein fragments. Recently, Amoroso et al. (2012) observed that a cell wall fragment could re-enter in the cytoplasm of *Bacillus licheniformis* and function as a signal to induce the expression of beta-lactamase. However, whether this cell wall fragment is the major signal for beta-lactamase induction in this Gram-positive bacterium still needs to be determined in the future. Given the lack of information on the relationship between beta-lactamase induction and cell wall metabolism in Gram-positive bacteria, in this review, we only summarize the relevant background information and recent research on the mechanisms of beta-lactamase induction by cell wall fragments in Gram-negative bacteria. In addition, we also discuss potential strategies to mitigate beta-lactam resistance by targeting beta-lactamase induction pathways.

PEPTIDOGLYCAN BIOSYNTHESIS AND RECYCLING

In Gram-negative bacteria, peptidoglycan (PG), also called murein, is a mesh structure with units of continuous biopolymer residing on the intervening space between the outer and inner (cytoplasmic) membrane. Specifically, PG is a polysaccharide composed of repeating β-(1,4)-GlcNAc-β-(1,4)-MurNAc disaccharide interconnected by oligopeptide stems via covalent bond (Glauner et al., 1988; **Figure 1**). The PG maintains cell integrity by sustaining internal osmotic pressure and keeps the regular bacterial shape. The glycan strand in *E. coli* is averagely composed of 29 disaccharide-peptide units (Glauner, 1988).

The PG biosynthesis involves multi-stage enzymatic activities. First, the PG monomer unit (disaccharide with oligopeptide stem) is attached to a lipid in the cytoplasmic leaf of inner membrane (van Heijenoort, 2001b; Barreteau et al., 2008; Bouhss et al., 2008). Second, the PG monomer-lipid intermediate is flipped into periplasm and catalyzed into the end of extending glycan chain by glycosyltransferases (Goffin and Ghuysen, 1998; van Heijenoort, 2001a; Sauvage et al., 2008). Finally, the stem oligopeptides [L-Ala-γ-D-Glu-meso-A2pm-(L)-D-Ala-D-Ala pentapeptide in *E. coli*, **Figure 1**] that is linked to MurNAc are cross-linked to the adjacent stem oligopeptides from other glycan chains by transpeptidases (Goffin and Ghuysen, 2002; Sauvage et al., 2008). These transpeptidases are the target of beta-lactam antibiotics and also called PBPs (including PBP1a, PBP1b, PBP1c, PBP2, and PBP3; Goffin and Ghuysen, 1998; Sauvage et al., 2008). Thus, PBPs are involved in the final stage of PG synthesis. Each bacterial cell may produce different PBPs, leading to various types of cross-linkage, such as D-Ala → (D)-meso-A2pm, (L)-meso-A2pm → (D)-meso-A2pm, and so on (van Heijenoort, 2011), for making a rigid mesh structure of PG.

Notably, PG is not a static biological structure. The structural units of PG changes dynamically during bacterial growth and doubling, with old units degraded and new materials added. Instead of starting over the complete *de novo* synthesis as described above, large quantities of the new materials added are recycled from the degraded PG units. It's estimated that up to 60% of the parental cell wall is made of the recycled PG units during active bacterial growth (de Pedro et al., 2001; Park and Uehara, 2008).

The PG recycling also involves multi-stage enzymatic activities. First, the lytic transglycosylase (LT) cleaves the glycan strand between the MurNAc and GlcNAc, and forms the 1,6-anhydro bond at the newly exposed MurNAc end in the mean time. With the aid of the endopeptidases (e.g., PBP4) that could break the cross-linkage between stem oligopeptides, anhydro muropeptide monomers (GlcNAc-anhydro-MurNAc-peptides) are liberated from PG. The main muropeptides are GlcNAc-anhMurNAc-L-Ala-γ-D-Glu-meso-A2pm-D-Ala (GlcNAc-anhydroMurNAc-tetrapeptide), with small amount of tri-, pentapeptides (Glauner, 1988). Second, these muropeptides are transported into cytoplasm through the inner membrane transporter AmpG (Park and Uehara, 2008). Subsequently, in cytoplasm, the GlcNAc sugar residue is removed by the glycoside hydrolase NagZ (Cheng et al., 2000; Votsch and Templin, 2000). The resulting population of 1,6-anhydroMurNAc-oligopeptides are further transformed to UDP-MurNAc-pentapeptide (Park and Uehara, 2008), a PG precursor that can be reincorporated into the PG biosynthesis pathway (Park and Uehara, 2008). The muropeptides also could serve as a signal to induce the production of beta-lactamase, which will be discussed below in Section "Mechanisms of Beta-lactamase Induction."

BETA-LACTAM ANTIBIOTICS AND BETA-LACTAMASE

In 1928, Alexander Fleming observed the bactericidal effect of *Penicillium notatum*, leading to the identification of the first beta-lactam antibiotic, penicillin (Fleming, 1929). Since then, a variety of beta-lactam antibiotics with different antimicrobial profiles have been discovered or synthesized, such as penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems. All beta-lactam antibiotics share a common core containing a four-member beta-lactam ring (**Figure 2**). This beta-lactam ring displays phenomenal structural mimicry with the backbone of the D-alanyl-D-alanine, the substrate of PBP (**Figure 2**). Therefore, penicillin has been proposed to act as a substrate analog and binds to the active site of transpeptidases for inhibition of synthesis of the cross-linked PG (Tipper and Strominger, 1965). This hypothesis was later supported by the evidence that transpeptidases could bind radioactive-labeled penicillin; thus, transpeptidases were also called as PBPs (Cooper et al., 1949; Maass and Johnson, 1949a,b; Cooper, 1955; Schepartz and Johnson, 1956; Markov et al., 1960; Spratt and Pardee, 1975).

Beta-lactam antibiotics have been a primary choice for physicians to treat bacterial infections due to their high specificity and potent killing effect. Clinical introduction of beta-lactam antibiotics has ever claimed to be a historical victory against bacterial infection; the mortality rate due to bacterial infections in the USA was drastically dropped from 797 to 36 per 100,000 individuals between 1900 and 1980 (Armstrong et al., 1999). The emergence of antibiotic resistant bacteria quickly becomes the ghost of modern medicine (Cohen, 2000). In fact, even during the ground-breaking discovery of penicillin, Alexander Fleming has already isolated the *E. coli*, *Salmonella enterica* serovar Typhi, and *Haemophilus influenza* strains that were resistant to penicillin (Fleming, 1929).

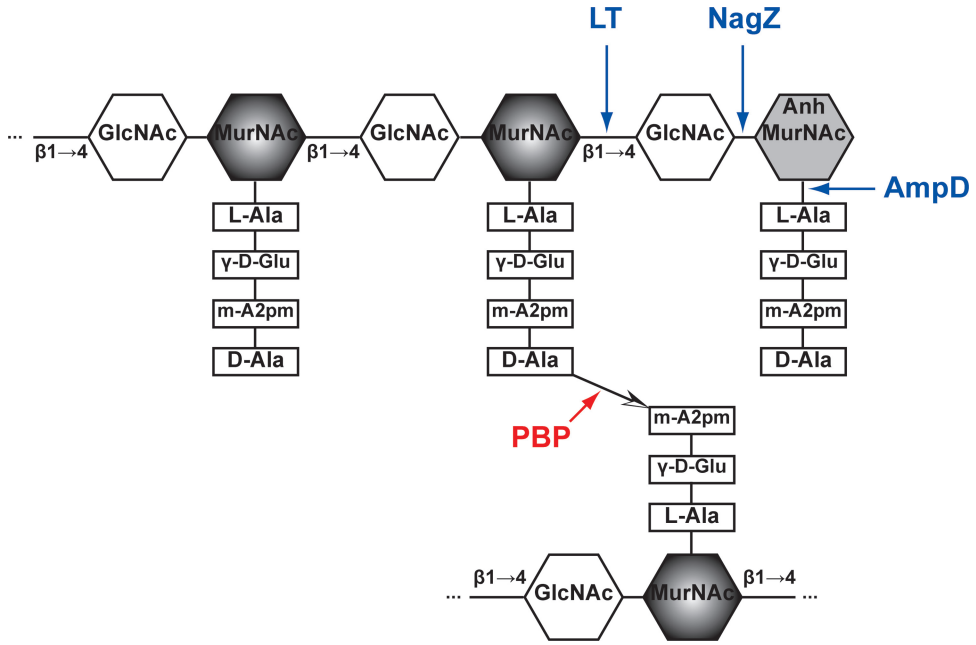

FIGURE 1 | Schematic structure of PG and target sites of different enzymes (pointed by color arrows). The synthetic enzyme (PBP) is highlighted in red while the lytic enzymes (NagZ, AmpD, and LT) are highlighted in blue. Notably, NagZ and AmpD catalyze the liberated muropeptides instead of intact PG. Hexagons denote sugars while rectangles denote stem amino acids. The cross-linkage (⟶) between the top and bottom glycan strands is D-Ala → meso-A2pm. LT, lytic transglycosylase; PBP, penicillin-binding protein, m-A2pm, meso-diaminopimelic acid; AnhMurNAc, 1,6-anhydro-MurNAc; β1 → 4, β-(1,4)-glycosidic bond.

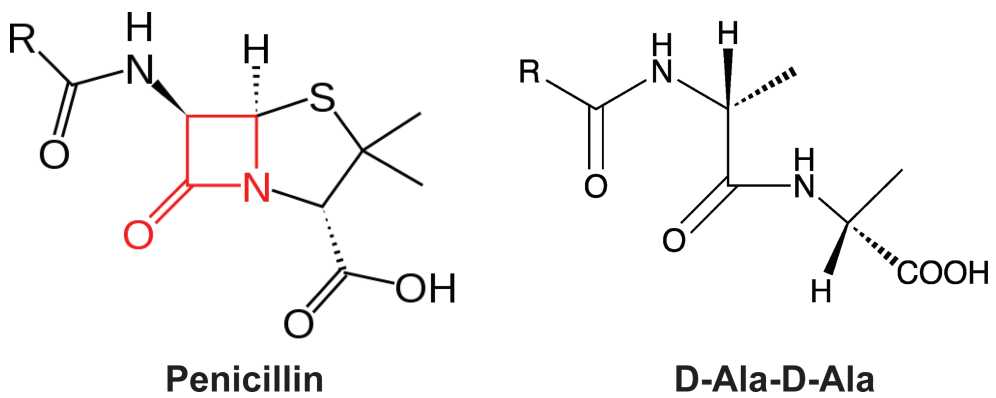

FIGURE 2 | The mimicry of beta-lactam antibiotics to D-alanyl-D-alanine (D-Ala-D-Ala). The four-member lactam ring in penicillin was highlighted in red.

Although numerous efforts have been placed on the discovery new generation of beta-lactam antibiotics to further improve their clinical efficacy, bacteria have been evolving with an unbeatable pace to fail those new beta-lactams (Culotta, 1994). To address this serious public health issue, it is imperative to study the molecular basis of beta-lactam resistance so that we can overcome beta-lactam resistance by targeting resistance mechanisms.

The molecular mechanisms of beta-lactam resistance have been widely studied (Ogawara, 1981; Fuda et al., 2004; Jovetic et al., 2010; Harris and Ferguson, 2012). To evade the bactericidal effects of beta-lactam antibiotics, Gram-negative bacteria have evolved multiple strategies, such as production of beta-lactamases (Korfmann and Wiedemann, 1988; Jacoby, 2009), production of novel PBPs with reduced affinity to beta-lactam antibiotics (Fuda et al., 2004), reducing beta-lactam antibiotics entry through mutations in porins, and expelling beta-lactam antibiotics out of cells using multi-drug efflux pumps (Kohler et al., 1999). Of these mechanisms, producing beta-lactamases, the enzymes that could hydrolyze beta-lactam ring, is still the most efficient strategy (Abraham and Chain, 1940; Jacoby and Munoz-Price, 2005). It has been proposed that beta-lactamases and the PBPs may share a common ancestor due to the presence of certain sequence homology (Massova and Mobashery, 1998). Recently, Fernandez et al. (2012) observed that overexpression beta-lactamases changed the

PG composition and affected bacterial fitness, likely due to the residual transpeptidase activity of the beta-lactamases.

Given the tight link between beta-lactam resistance and the beta-lactamase activity, it is not surprising that past studies were primarily focused on the structure, function, and ecology of beta-lactamases. Particularly, many epidemiological, clinical, and ecological studies are focused on the detection and characterization of specific beta-lactamase genes with little attention on the regulatory mechanism of beta-lactamases. The first "cryptic" beta-lactamase, AmpC (originally named AmpA), was identified in beta-lactam sensitive *E. coli* K-12 by stepwise selection on beta-lactam antibiotics containing medium (Eriksson-Grennberg et al., 1965; Eriksson-Grennberg, 1968). The beta-lactam resistant derivatives constitutively produced high-level of beta-lactamases, suggesting the presence of an inducible beta-lactamase gene in *E. coli* K-12 (Linstrom et al., 1970). Later, the AmpC gene was cloned and characterized as a beta-lactamase (Jaurin and Grundstrom, 1981). The expression of *ampC* normally is maintained at low level and dependent on growth rate (Jaurin et al., 1981). However, a single nucleotide mutation in the promoter region (likely an attenuator) of *ampC* led to overexpression of beta-lactamase, indicating that the *ampC* was subjected to regulation (Jaurin et al., 1981). Then the *ampC* was observed to be widely distributed in different enterobacterial species, such as *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Klebsiella pneumonia*; interestingly, the *ampC* was inducible under treatment of beta-lactam antibiotics (Bergstrom et al., 1982). However, the expression of *ampC* in *E. coli* was not induced by beta-lactam antibiotics due to the lack of a regulator gene *ampR* adjacent to the *ampC* in the chromosome (Honore et al., 1986). Complementation of *E. coli* with a plasmid containing the *ampR–ampC* operon from *Enterobacter cloacae* restored the phenotype of beta-lactamase induction (Kraft et al., 1999).

The induction of beta-lactamase is of great clinical importance. For example, prolonged administration of beta-lactam antibiotics could lead to emergence of *P. aeruginosa* mutants resistance to multiple beta-lactam antibiotics, eventually leading to treatment failure and patient death (Livermore, 1987; Sanders, 1987; Giwercman et al., 1990; Juan et al., 2005). Therefore, significant progresses have been made on the molecular basis of the beta-lactamase induction in Gram-negative bacteria in the past two decades.

MECHANISMS OF BETA-LACTAMASE INDUCTION

Understanding the molecular basis of beta-lactamase induction would facilitate us to develop effective combination therapy strategy by inhibiting the induction of beta-lactamase. Gram-negative bacteria have evolved two major mechanisms for beta-lactamase induction, the AmpG–AmpR–AmpC pathway and the two-component regulatory system (TCRS; **Figure 3**). Recent progresses in this significant research area are summarized below.

THE AmpG–AmpR–AmpC PATHWAY

As mentioned above, in many bacteria belonging to Enterobacteriaceae family, AmpC expression is induced by beta-lactam antibiotics. Since beta-lactam antibiotics treatment can trigger the release of large amount of muropeptides in periplasm, which could be subjected to cell wall recycling process, the relationship between cell wall recycling and beta-lactamase induction has been examined and confirmed in recent studies. Briefly, in the AmpG–AmpR–AmpC pathway, beta-lactam antibiotics treatment breaks the balance of PG biosynthesis (e.g., due to the inhibited PBP and the functional LT), consequently liberating GlcNAc-anhydro-MurNAc-oligopeptides in periplasm (Templin et al., 1992). The GlcNAc-anhydro-MurNAc-oligopeptides are further transported into cytoplasm through AmpG transporter (Park and Uehara, 2008). The GlcNAc moiety is removed by enzyme NagZ, leading the accumulated PG products (mainly anhydro-MurNAc-tetrapeptides). In cytoplasm, anhydro-MurNAc-oligopeptide are the inducer of beta-lactamase expression through the interaction with AmpR (Lindquist et al., 1989; Jacobs et al., 1997).

AmpR is a LysR type transcriptional regulator and is encoded immediately upstream of *ampC* with opposite direction (Lindquist et al., 1989; Jacobs et al., 1997). AmpR was demonstrated as an activator for *ampC* using *in vitro* transcription assay (Jacobs et al., 1997). However, production of *ampC* was still repressed even if bacterial host contains functional AmpR, unless exogenous beta-lactam antibiotic was added (Honore et al., 1986; Lindquist et al., 1989; Lodge et al., 1990; Jacobs et al., 1997). Therefore, it has been hypothesized that the activator function of AmpR was inhibited by certain cellular metabolite, which was demonstrated as the cell wall synthesis precursor, UDP-MurNAc-pentapeptide (Jacobs et al., 1997). This inhibition was abolished in the mutant with point mutation in AmpR (G102E; Bartowsky and Normark, 1991), indicating the role of the residue G for the association of UDP-MurNAc-pentapeptide. Upon the treatment of beta-lactam antibiotics, the accumulated intracellular anhydro-MurNAc-oligopeptides could displace the AmpR-associated UDP-MurNAc-pentapeptide, triggering conformational change of AmpR, and subsequently activating the transcription of *ampC* (Jacobs et al., 1997). The DNase I-protection assay showed the binding site of AmpR was in a 39-bp region upstream of the *ampC* transcription start site (−40 to −88; Jacobs et al., 1997). Interestingly, AmpR in *P. aeruginosa* is a global transcriptional factor whose regulon includes beta-lactamases, proteases, quorum sensing, and other virulence factors (Kong et al., 2005; Balasubramanian et al., 2012).

Among the PG cycling process, there is a negative effector to fine-tune the expression of AmpC. A cytoplasmic *N*-acetylmuramoyl-L-alanine amidase, named AmpD (Holtje et al., 1994), could dissociate stem peptides from the anhydro-MurNAc or GlcNAc-anhydro-MurNAc, therefore, reducing concentrations of the inducing muropeptides and mitigating the overexpression of AmpC (Jacobs et al., 1994).

Consistent with these observations on the relationship between PG cycling and beta-lactamase induction, perturbation of PG recycling also affected AmpC induction, suggesting potential pharmaceutical targets. For example, overproduction of the LT MltB stimulated beta-lactamase induction whereas specific inhibition of LT Slt70 by bulgecin repressed AmpC expression (Kraft et al., 1999). In addition, mutation of all six LT enzymes (Slt70, MltA, MltB, MltC, MltD, and EmtA) in *E. coli* decreased the beta-lactamase activities (Korsak et al., 2005).

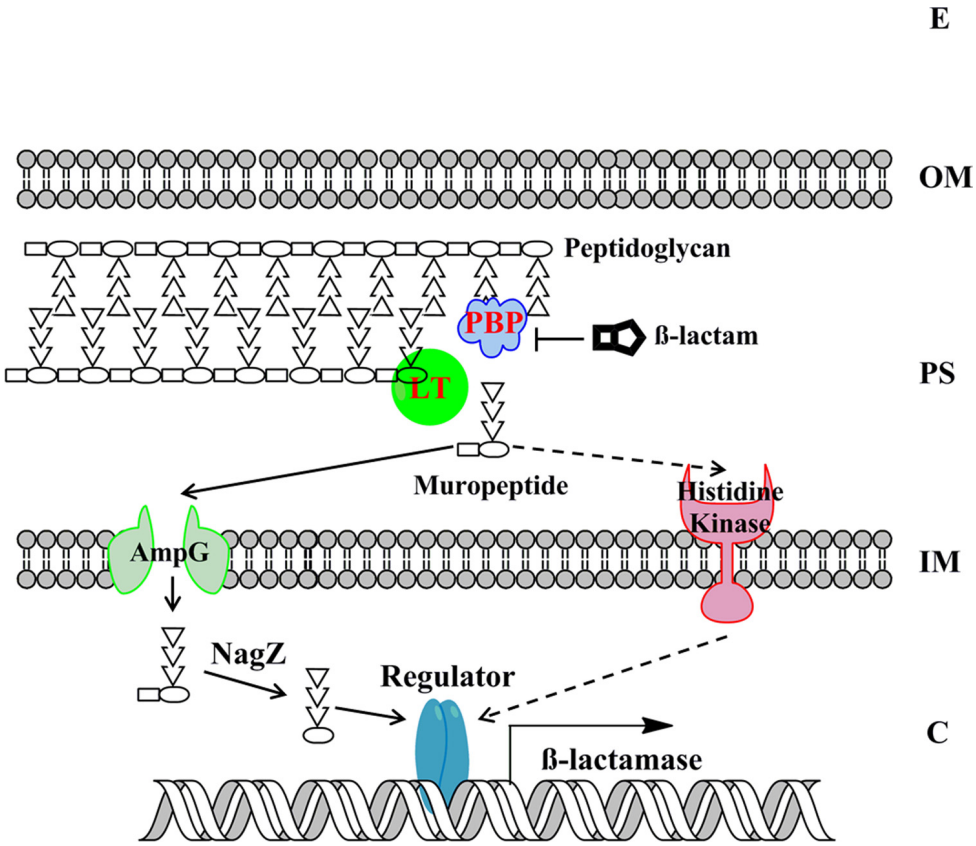

FIGURE 3 | The model of beta-lactamase induction in Gram-negative bacteria. The beta-lactamase induction by muropeptides via two major molecular mechanisms, the AmpG–AmpR–AmpC pathway and the BlrAB-like two-component regulatory system, are presented. The signaling pathway via two-component regulatory system is only supported by limited studies to date and is shown in dashed arrows. The "Regulator" denotes AmpR-like regulator or two-component response regulator. The "beta-lactamase" denotes the beta-lactamase that is subjected to induction. E, extracellular environment; OM, outer membrane; PS, periplasmic space; IM, inner membrane; C, cytoplasm.

Different versions of AmpG–AmpR–AmpC regulatory pathways exist in bacteria. For example, *E. coli* and *Shigella* spp. lacks an *ampR* gene (Bergstrom et al., 1982; Honore et al., 1986), leading to the low level, non-inducible expression of AmpC. The AmpC gene in *E. coli* was primarily regulated by an attenuator sequence in promoter region (Jaurin et al., 1981). The overexpression of AmpC can be achieved either by mutating attenuator (Jaurin et al., 1981) or by introducing an AmpR regulator (Kraft et al., 1999); the similar pathway was also observed in *Acinetobacter baumannii* (Bou and Martinez-Beltran, 2000). In *Salmonella*, the chromosomal AmpC–AmpR is usually absent, which may be due to unbearable production cost of AmpC (Morosini et al., 2000). However, clinical *Salmonella* strains can acquire AmpC–AmpR through horizontally transferred mobile elements (Barnaud et al., 1998). In *Serratia marcescens*, besides AmpR regulation, the post-transcriptional regulation also influences the expression of AmpC. Specifically, the half-life of *ampC* transcript could be affected by a 126-bp, non-encoding region that forms a stem-loop structure (Mahlen et al., 2003). In *P. aeruginosa* PAO1, interestingly, there are three copies of *ampD* genes, which contributed to the stepwise up-regulation of AmpC with the discrete mutation of each copy of *ampD* (Juan et al., 2006).

THE BlrAB-LIKE TWO-COMPONENT REGULATORY SYSTEM

The TCRS, which involves sensing specific environmental stimuli (Capra and Laub, 2012), was also observed to be involved in the induction of beta-lactamase. In *Aeromonas* spp., the AmpC and two other chromosomally encoded beta-lactamases were regulated by the response regulator BlrA of a TCRS instead of an AmpR-type regulator (Alksne and Rasmussen, 1997). Complementation study demonstrated that overexpression of BlrA in *E. coli* enhanced the expression of the *Aeromonas*-derived beta-lactamase in *E. coli* MC1061 while the beta-lactamase was expressed at low level in the absence of BlrA (Alksne and Rasmussen, 1997).

The closest TCRS homolog of BlrAB in *E. coli* is CreBC (Amemura et al., 1986; Wanner and Wilmes-Riesenberg, 1992). Interestingly, the beta-lactamases from *Aeromonas hydrophila* could be regulated by the CreBC TCRS system in the Cre^+ *E. coli* strain such as DH5α (Avison et al., 2000, 2001). The "*cre/blr*-tag" signature, which is the "TTCACnnnnnnTTCAC" motif located in the promoter of Cre-regulon, was identified in *E. coli* (Avison et al., 2001). These "cre/blr-tag" also reside in promoters of *Aeromonas*-derivative beta-lactamases (Niumsup et al., 2003), and the induction of those beta-lactamases by overexpressed BlrA was dependent on the presence of "*cre/blr*-tag" (Avison et al., 2004).

In *P. aeruginosa*, inactivation of a non-essential PBP was shown to trigger overproduction of a chromosomal AmpC gene and this overproduction is dependent on CreBC TCRS (Moya et al., 2009). Interestingly, among the 32 tested *E. coli* TCRS response regulators, overexpression of FimZ conferred increased level of beta-lactam resistance through the action of AmpC in *E. coli* (Hirakawa et al., 2003).

Despite above evidence showing that TCRS is also involved in the induction of beta-lactamase, the identity of the corresponding cues to which the TCRS respond for beta-lactamase induction is still unknown. We speculate that specific degraded PG components may serve as a signal for the response regulator to induce the production of beta-lactamase. This hypothesis needs to be examined in the future.

OTHER MECHANISMS

Another novel beta-lactamase induction pathway was discovered in *Ralstonia pickettii* (Girlich et al., 2006). The chromosomally encoded beta-lactamases (OXA-22 and OXA-60) were regulated by ORF-RP3 (short for RP3), a gene located at 192-bp upstream of the ATG codon of *oxa-60*. Inactivation of RP3 resulted in the abolishment of induction of the both beta-lactamases; complementation of the RP3 restored the inducible expression of OXA-22 and OXA-60 (Girlich et al., 2006). DNase I footprinting showed that RP3 specifically bound to tandem repeats upstream at the transcriptional start sites of OXA-22 and OXA-60 genes, suggesting RP3 is a novel positive-regulator for beta-lactamase induction (Girlich et al., 2009).

PHARMACEUTICAL IMPLICATIONS OF BETA-LACTAMASE INDUCTION MECHANISM

Discovery of beta-lactamase inhibitors is a promising strategy to combat the prevalent beta-lactam resistance (Bush and Macielag, 2010; Harris and Ferguson, 2012). However, this approach is challenged by the variable affinity of the inhibitors to different beta-lactamases and by the overwhelming quantity of the beta-lactamases produced in resistant cells. Based on the information reviewed here, we propose that the signaling pathways of beta-lactamase induction offer a broad array of promising targets for the discovery of new antibacterial drugs used for combination therapies. The inhibitors targeting beta-lactamase induction pathway may prevent the emergence of beta-lactam resistance and enhance the efficacy of clinical beta-lactam antibiotics, as what we have observed for the efflux pump inhibitors (Lomovskaya and Bostian, 2006). In supporting this hypothesis, the frequency of emergence of ceftazidime resistance in *blrAB* mutant in *P. aeruginosa* was below the detection limit ($<1 \times 10^{-11}$), which is far below that for the wild-type parent strain (3×10^{-8}; Moya et al., 2009).

The potential targets in the beta-lactamase induction pathway as well as the known inhibitors are summarized in **Table 1**. Several inhibitors have been identified for LTs that play a critical role in the initializing the PG cycling. The LT inhibitor bulgecin could induce cell lysis and morphology changes in the presence of beta-lactam antibiotics although bulgecin alone did not show any antibacterial activity against *E. coli* (Imada et al., 1982; Nakao et al., 1986; Bonis et al., 2012). The major molecular target of bulgecin was the soluble LT Slt70 (Templin et al., 1992). In a 2.8-Å resolution crystallographic structure of Slt70-bulgecin complex, one single bulgecin molecule was found to be located in the active site of Slt70, indicating that bulgecin may act as an analog of an oxocarbenium ion intermediate in the reaction catalyzed by Slt70 (Thunnissen et al., 1995). The beta-hexosaminidase inhibitor *N*-acetylglucosamine thiazoline (NAG-thiazoline) was also found to inhibit the LT sMltB from *P. aeruginosa* (Reid et al., 2004a,b). Another inhibitor, hexa-*N*-acetylchitohexaose, can inhibit the LT from bacteriophage lambda (Leung et al., 2001). Interestingly, a proteinaceous inhibitor of vertebrate lysozymes (Ivy), which has conserved CKPHDC motif, was also found to control the autolytic activity of bacterial LTs (Clarke et al., 2010).

Regarding other targets in beta-lactamase induction pathway, PUGNAc and modified EtBuPUG can inhibit the function of NagZ by the mimicry of the oxocarbenium ion-like transition state (Stubbs et al., 2007). Unlike PUGNAc that is also a potent inhibitor against human *O*-GlcNAcase and beta-hexosaminidase, EtBuPUG displayed 100-fold selectivity toward to NagZ. The function of inner membrane permease AmpG in laboratory strains of *P. aeruginosa* can be inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a general inhibitor of proton motive force, consequently leading to an increased susceptibility to beta-lactam antibiotics (Cheng and Park, 2002; Zhang et al., 2010). However, it is important to mention that CCCP also targets other energy-dependent systems, such as drug efflux pump; thus, the linkage between reduced beta-lactam resistance and AmpG inhibition was not clearly demonstrated in these studies.

Although a panel of inhibitors that target the PG recycling pathway have been identified (**Table 1**), it is still largely unknown if these inhibitors repress the inducible beta-lactam resistance effectively in Gram-negative bacteria, consequently enhancing the

Table 1 | The inhibitors targeting the beta-lactamase induction pathway.

Target	Function	Inhibitor
LT	Non-hydrolytic cleave PG with the concomitant formation of 1,6-anhydro-MurNAc	Bulgecin A (Templin et al., 1992); NAG-thiazoline (Reid et al., 2004a,b); hexa-*N*-acetylchitohexaose (Leung et al., 2001); Ivy (Clarke et al., 2010)[a]
NagZ	Cleave disaccharide oligopeptides to release 1,6-anhydro-MurNAc-peptide	PUGNAc, EtBuPUG (Stubbs et al., 2007)
AmpG	Inner membrane permease of the 1,6-GlcNAc-anhydro-MurNAc-peptides	CCCP (Zhang et al., 2010)
AmpR	Binary regulator of AmpC	UDP-*N*-acetylmuramic acid peptides (Jacobs et al., 1997)

[a] *Proteinaceous inhibitor, also the inhibitor of vertebrate lysozymes.*

efficacy of clinical beta-lactam antibiotics. This knowledge gap needs to be filled in the future. In addition, similar to all infectious disease drug developments, discovery of a promising inhibitor targeting the beta-lactamase induction pathway and conversion such inhibitor into a clinically useful therapeutic agent are likely a lengthy and challenging process. Some key issues, such as toxicity, stability, bioavailability, and production cost, must be addressed. Despite these challenges, it is imperative to develop clinically useful inhibitors to suppress beta-lactamase induction and enhance "shelf-life" of a broad spectrum of beta-lactam antibiotics against bacterial pathogens. To achieve this goal, in-depth structural and functional studies are needed for the potential targets (**Table 1**), which is critical for identifying corresponding inhibitors using various modern approaches, such as high-throughput screening of chemical compound library, homology modeling and molecular docking.

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