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LEGIONELLA: FROM PROTOZOA TO HUMANS

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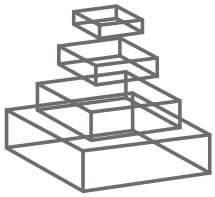

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LEGIONELLA: FROM PROTOZOA TO HUMANS

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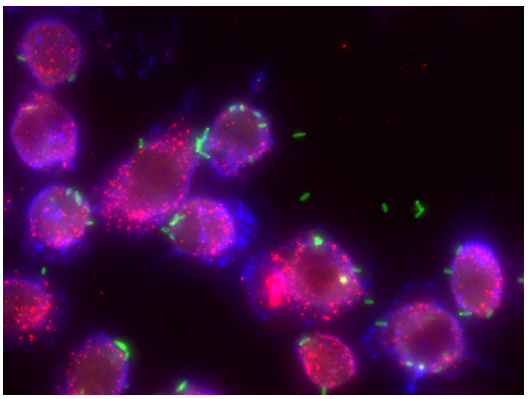

It is only a little more than 30 years since *Legionella pneumophila* was recognized as a human pathogen and the cause of a severe pneumonia known as Legionnaires' disease. The discovery that *L. pneumophila* is ubiquitous in aquatic environments and exists as an intracellular parasite of protozoa has provided a link between bacterial ecology and human disease. This Research Topic provides updated information on several important areas of *Legionella* research. Articles begin by discussing genomics of *Legionella* spp as it has significantly increased our knowledge of the pathogenesis of this disease by providing new insights into the evolution and genetic and physiological basis of *Legionella*–host interactions. Articles then further focus on different areas of host-*Legionella* interactions with protozoan or human cells. New findings on basic mechanisms of pathogen–host interactions, remarkable facts about the genetic basis of the intracellular lifestyle of *Legionella*, and its striking ability to manipulate host cell processes by molecular mimicry are discussed. Finally, knowledge of the host response to *Legionella* infection is presented.

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EDITORIAL
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Legionella: from protozoa to humans

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Pathogens that are able to enter and multiply within human cells are responsible for multiple diseases and millions of deaths worldwide. Thus, the challenge is to elucidate these pathogen-specific and cell biological mechanisms involved in intracellular growth and spread. Bacteria from the genus *Legionella* belong to this group of pathogens. They are environmental bacteria and ubiquitous in nature, where they parasitize protozoa. Strikingly, the capacity to grow intracellularly in protozoa like *Acanthamoeba castellanii*, *Hartmannella* sp., or *Naegleria* sp., has generated a pool of virulence traits during evolution, which allow *Legionella* to infect also human cells. Thus important human pathogens are present within the genus *Legionella*, the most prominent are *L. pneumophila* (Fraser et al., 1977; Mcdade et al., 1977) and *L. longbeachae* (Mckinney et al., 1981). These bacteria are the causative agents of Legionnaires' disease, a severe pneumonia diagnosed mainly in people whose immune defenses are weakened. *Legionella* is transmitted through breathing infected aerosols present in many artificial water systems like air conditioning systems, cooling towers, showers, and other aerosolizing devices. When reaching the alveolar parts of the lungs *Legionella* is engulfed by macrophages where it is able to multiply resulting in a severe, often fatal pneumonia. Intracellular infection is a consequence of the bacterium's capacity to manipulate host cellular processes using bacterial proteins that are delivered into the host cell by specialized secretion systems (Isberg et al., 2009; Hubber and Roy, 2010). In this Research Topic, we present a collection of Review, Opinion, Perspective, and Primary research articles that present both the well-established and the newly discovered strategies used by *Legionella* to achieve this intracellular lifestyle while escaping from the host immune response.

Recent advance in genome sequencing has had a tremendous impact on our understanding of the pathogenesis, evolution and diversity of *L. pneumophila* and *L. longbeachae* and the knowledge of the genome sequence has guided and facilitated the research on *Legionella* in many laboratories. Starting this issue, Gomez Valero et al. (2011) present a comprehensive review on what we have learned from the sequencing and analyses of six *L. pneumophila* and five *L. longbeachae* genomes published since 2004. In particular, genome sequence analysis revealed the presence of proteins with high similarity to eukaryotic proteins or proteins with domains preferentially or only present in eukaryotic genomes that are mimicking host functions helping the pathogen to replicate intracellularly (Cazalet et al., 2004, 2010). Further insight in the genetic basis of host differences and the evolution of the eukaryotic like proteins in these two pathogens are given.

The bacterial cell wall is at the forefront of the interaction with the host and is essential for cellular integrity and the resistance to external stress and aggressions. Shevchuk et al. (2011) provide an update on the structure, molecular composition, and virulence properties of the *L. pneumophila* cell envelope. In their review they show convincingly that lipopolysaccharide and several outer membrane proteins like Mip, a peptidyl-prolyl-*cis/trans* isomerase, a phospholipase A are essential virulence factors. It becomes clear that promising new fields of research will be the analyses of proteins, carbohydrates, and lipids of the cell envelope as they serve for both, structural and signaling roles. In the following review article Garduno et al. (2011) present us the current knowledge on the many functions that one of these outer membrane proteins, Hsp60 or HtpB plays in the *L. pneumophila* biology. This chaperonin is an unusual multifunctional protein which was discovered already as early as 1986 as an antigen that prominently reacted with patients diagnosed with Legionnaires' disease. Over 20 years later it has been shown to have protein folding as well as protein folding independent roles and that it is associated with virulence and survival of *L. pneumophila*.

Central to the pathogenesis of *L. pneumophila* and *L. longbeachae* is its Type IV secretion system (T4SS) called Dot/Icm. It is predicted to translocate over 270 effector proteins into the host cell which allow this bacterium to manipulate host cell functions to its advantage and to assure intracellular survival and replication. Nagai and Kubori (2011) recapitulate our present understanding of the T4SS apparatus and its components by taking advantage of genomic and structural information. Finally, using comparative genomics information of several bacteria and plasmids carrying similar systems a comparative analysis of T4SS components is presented. The following five reviews, research, and opinion articles discuss some of the secreted effectors of this T4SS and their divers roles in pathogenesis of *L. pneumophila*. Hilbi et al. (2011) present evidence that *L. pneumophila* subverts phosphoinositide (PI) lipids by anchoring specific effectors through distinct PIs to the cytosolic face of the *Legionella* containing vacuole (LCV) to promote the interaction with host vesicles and organelles, catalyze guanine nucleotide exchange of small GTPases, or bind to PI-metabolizing enzymes. Interestingly, *L. pneumophila* secretes also three glycosyltransferases through its T4SS. Belyi et al. (2011) report on this novel family of effector proteins that are structurally similar to clostridial glycosylating toxins. However, in *L. pneumophila* they do not produce toxic effects but modify the eukaryotic elongation factor EF1A to inhibit protein synthesis and subsequently to induce cell death. Another exciting strategy employed by *L. pneumophila* by secreting proteins encoding a eukaryotic F-box domain is the exploitation of the host's polyubiquitination and farnesylation machineries. In an original research article Al-Quadan and Kwaik (2011) discuss in detail how one of the *L. pneumophila* encoded F-box proteins uses these conserved eukaryotic signaling pathways to proliferate in *Dictyostelium discoideum*, a model ameba used as infection

model. Excitingly, this same effector also has another eukaryotic motif, which is used by the host's prenylation machinery for anchoring it in the outer leaflet of the LCV (Ivanov et al., 2010; Price et al., 2010). In another primary research article Price et al. (2010) show, that *L. pneumophila* contain several other proteins with this motif, that are also used by the host prenylation machinery to anchor proteins into cellular membranes contributing in this way to the evasion of lysosomal fusion by the LCV.

A further strategy of *L. pneumophila* to establish an environment beneficial to replication is to specifically targets and exploit the host phosphorylation system through T4SS effectors that act directly on phosphorylation cascades. Haenssler and Isberg (2011) present a comprehensive and exciting review on the different host kinases and phosphatases that are targeted during *L. pneumophila* infection and show how *L. pneumophila* modulates host cell signal transduction by phosphorylation at multiple levels. The part discussing the different strategies of *L. pneumophila* to modulate host functions through T4SS effectors of *L. pneumophila* finishes with an opinion article by Luo (2011b) that discusses a recent, stimulating finding. *L. pneumophila* has evolved an effector protein, which specifically target another bacterial effector protein for degradation to shutdown its action within the host cells at later stages of infection. This demonstrates a sophisticated level of coevolution between eukaryotic cells and *L. pneumophila* involving an effector that functions as a key regulator to temporally coordinate the function of a cognate effector protein and opens now the way for additional research into possible interaction of bacterial effectors among them and not only with host proteins.

To timely secret this large repertoire of different effectors and to adapt the metabolism and the physiology of *L. pneumophila* to the changing host environment, a tight regulation of the expression of the different genes coding these proteins is necessary. Thus *L. pneumophila* has evolved a complex regulatory system and many different regulatory elements governing its life cycle. One of the master regulators is CsrA that is regulated by binding to two small regulatory RNAs (Rasis and Segal, 2009; Sahr et al., 2009). Faucher and Shuman (2011) give an overview of what is known about small regulatory RNAs in *L. pneumophila* and present in a second article the first transcriptome analysis of *L. pneumophila* infecting human macrophages (Faucher et al., 2011). A model of the regulatory networks involving small RNA mediated control of virulence gene regulation in *L. pneumophila* is presented.

In the following part of this Research Topic Joshi and Swanson stress important aspects of *Legionella*–host interactions, by providing a critical view on the *Legionella* response to autophagy, a host defense against invading microbes (Joshi and Swanson, 2011). They propose that *L. pneumophila* is able to halt the autophagosome maturation by secreting different effector proteins. Once *L. pneumophila* adapted to an acidic environment another effector, LepB releases this blockage and the now acid resistant *L. pneumophila* are able to continue replicating in these autophagolysosomes. In the following article, Luo discusses recent progress in understanding the mechanisms *L. pneumophila* employs to interfere with apoptosis and how this modulation also contributes to the intracellular life cycle of this bacterium (Luo, 2011a).

Finally, infection by a pathogen and its outcome is always an interplay between the pathogen and the host. Thus, the host response to infection with *L. pneumophila* is a very important research question. This topic is the focus of a review by Massis and Zamboni (2011) that summarizes the current knowledge of the innate immune response to *L. pneumophila* infection. The implication of the different families of pattern recognition receptors (PRR) like TLRs, NLRs, and RLRs are discussed and a comprehensive analyses of the events triggered by the recognition of intracellular *L pneumophila* by these PRRs is presented. Schuelein et al. (2011) close this research topic by providing a thoughtful opinion article about the immune control of *Legionella* infection. The authors stress that macrophages play a pivotal role in initiating the host response to *L. pneumophila* infection, however, given the fact that the resolution of *L. pneumophila* infection needs multiple cell types and abundant cross talk between immune cells they propose that the role of other cell types such as dendritic cells and the mechanism of action of protective cytokines should be examined in the future.

The field of microbiology and the study of host pathogen interactions is moving fast ahead and, in conjunction with the avalanche of data provided by the application of new, powerful "omics" methods, the tremendous advances in imaging techniques allowing *in vivo*, and single cell analyses and improvements in analytical methods, will lead to many exciting new discoveries in the future.

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Comparative and functional genomics of *Legionella* identified eukaryotic like proteins as key players in host–pathogen interactions

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Although best known for its ability to cause severe pneumonia in people whose immune defenses are weakened, *Legionella pneumophila* and *Legionella longbeachae* are two species of a large genus of bacteria that are ubiquitous in nature, where they parasitize protozoa. Adaptation to the host environment and exploitation of host cell functions are critical for the success of these intracellular pathogens. The establishment and publication of the complete genome sequences of *L. pneumophila* and *L. longbeachae* isolates paved the way for major breakthroughs in understanding the biology of these organisms. In this review we present the knowledge gained from the analyses and comparison of the complete genome sequences of different *L. pneumophila* and *L. longbeachae* strains. Emphasis is given on putative virulence and *Legionella* life cycle related functions, such as the identification of an extended array of eukaryotic like proteins, many of which have been shown to modulate host cell functions to the pathogen's advantage. Surprisingly, many of the eukaryotic domain proteins identified in *L. pneumophila* as well as many substrates of the Dot/Icm type IV secretion system essential for intracellular replication are different between these two species, although they cause the same disease. Finally, evolutionary aspects regarding the eukaryotic like proteins in *Legionella* are discussed.

Keywords: *Legionella pneumophila, Legionella longbeachae,* evolution, comparative genomics, eukaryotic like proteins, virulence

INTRODUCTION

Genomics has the potential to provide an in depth understanding of the genetics, biochemistry, physiology, and pathogenesis of a microorganism. Furthermore comparative genomics, functional genomics, and related technologies, are helping to unravel the molecular basis of the pathogenesis, evolution, and phenotypic differences among different species, strains, or clones and to uncover potential virulence genes. Knowledge of the genomes provides the basis for the application of new powerful approaches for the understanding of the biology of the organisms studied.

Although *Legionella* are mainly environmental bacteria, several species are pathogenic to humans, in particular *Legionella pneumophila* (Fraser et al., 1977; Mcdade et al., 1977) and *Legionella longbeachae* (Mckinney et al., 1981). Legionnaires' disease has emerged in the second half of the twentieth century partly due to human alterations of the environment. The development of artificial water systems in the last decades like air conditioning systems, cooling towers, showers, and other aerosolizing devices has allowed *Legionella* to gain access to the human respiratory system. When inhaled in contaminated aerosols, pathogenic *Legionella* can reach the alveoli of the lung where they are subsequently engulfed by macrophages. In contrast to most bacteria, which are destroyed, some *Legionella* species can multiply within the phagosome and eventually kill the macrophage, resulting in a severe, often fatal pneumonia called legionellosis or Legionnaires' disease (mortality rate of 5–20%; up to 50% in nosocomial infections; Steinert et al., 2002; Marrie, 2008; Whiley and Bentham, 2011). To replicate intracellularly *L. pneumophila* manipulates host cellular processes using bacterial proteins that are delivered into the cytosolic compartment of the host cell by a specialized type IV secretion system called Dot/Icm. The proteins delivered by the Dot/Icm system target host factors implicated in controlling membrane transport in eukaryotic cells, which enables *L. pneumophila* to create an endoplasmic reticulum-like vacuole that supports intracellular replication in both protozoan and mammalian host cells (for a review see Hubber and Roy, 2010).

An interesting epidemiological observation is, that among the over 50 *Legionella* species described today, strains belonging to the species *L. pneumophila* are responsible for over 90% of the legionellosis cases worldwide and strains belonging to the species *L. longbeachae* are responsible for about 5% of human legionellosis cases worldwide (Yu et al., 2002). Surprisingly, this distribution is very different in Australia and New Zealand where *L. pneumophila* accounts for "only" 45.7% of the cases but *L. longbeachae* is implicated in 30.4% of the human cases. Furthermore, among the strains causing Legionnaires' disease, *L. pneumophila* serogroup 1 (Sg1) alone is responsible for over 85% of cases (Yu et al., 2002; Doleans et al., 2004) despite the description of 15 different Sg within this species. In addition, the characterization of over 400

different *L. pneumophila* Sg1 strains has shown that only a minority among these is responsible for causing most of the human disease (Edelstein and Metlay, 2009). Some of these clones are distributed worldwide like *L. pneumophila* strain Paris (Cazalet et al., 2008) others have a more restricted geographical distribution, like the recently described endemic clone, prevalent in Ontario, Canada (Tijet et al., 2010). For the species *L. longbeachae* two serogroups are described to date (Bibb et al., 1981; Mckinney et al., 1981). *L. longbeachae* Sg1 is predominant in human disease as it causes up to 95% of the cases of legionellosis worldwide and most outbreaks and sporadic cases in Australia (Anonymous, 1997; Montanaro-Punzengruber et al., 1999). The two main human pathogenic *Legionella* species, *L. pneumophila* and *L. longbeachae* cause the same disease and symptoms in humans (Amodeo et al., 2009), however, there exist major differences between both species in niche adaptation and host susceptibility.

(i) They are found in different environmental niches, as *L. pneumophila* is mainly found in natural and artificial water circuits and *L. longbeachae* is principally found in soil and therefore associated with gardening and use of potting compost (O'Connor et al., 2007). However, although less common, the isolation of *L. pneumophila* from potting soil in Europe has also been reported (Casati et al., 2009; Velonakis et al., 2009). Human infection due to *L. longbeachae* is particularly common in Australia but cases have been documented also in other countries like the USA, Japan, Spain, England, or Germany (MMWR, 2000; Garcia et al., 2004; Kubota et al., 2007; Kumpers et al., 2008; Pravinkumar et al., 2010).

(ii) As described for other *Legionella* species, person to person transmission of *L. longbeachae* has not been documented, however, the primary transmission mode seems to be inhalation of dust from contaminated compost or soil that contains the organism (Steele et al., 1990; MMWR, 2000; O'Connor et al., 2007).

(iii) Furthermore, for *L. pneumophila* a biphasic life cycle was observed *in vitro* and *in vivo* as exponential phase bacteria do not express virulence factors and are unable to replicate intracellularly. The ability of *L. pneumophila* to replicate intracellularly is triggered at the post-exponential phase by a complex regulatory cascade (Molofsky and Swanson, 2004; Sahr et al., 2009). In contrast, less is known on the *L. longbeachae* intracellular life cycle and its virulence factors. It was recently shown that unlike *L. pneumophila* the ability of *L. longbeachae* to replicate intracellularly is independent of the bacterial growth phase (Asare and Abu Kwaik, 2007) and that phagosome biogenesis is different. Like *L. pneumophila*, the *L. longbeachae* phagosome is surrounded by endoplasmic reticulum and does not mature to a phagolysosome; however it acquires early and late endosomal markers (Asare and Abu Kwaik, 2007).

(iv) Another interesting difference between these two species is their ability to colonize the lungs of mice. While only A/J mice are permissive for replication of *L. pneumophila*, A/J, C57BL/6, and BALB/c mice are all permissive for replication of *L. longbeachae* (Asare et al., 2007; Gobin et al., 2009). Resistance of C57BL/6 and BALB/c mice to *L. pneumophila* has been attributed to polymorphisms in Nod-like receptor apoptosis inhibitory protein 5 (*naip5*) allele that recognizes the C-terminus of flagellin (Wright et al., 2003; Molofsky et al., 2006; Ren et al., 2006; Lightfield et al., 2008). The current model is that *L. pneumophila* replication is restricted due to flagellin dependent caspase-1 activation through Naip5-Ipaf and early macrophage cell death by pyroptosis. However, although depletion or inhibition of caspase-1 activity leads to decreased targeting of bacteria to lysosomes, the mechanism of caspase-1-dependent restriction of *L. pneumophila* replication in macrophages and *in vivo* is not fully understood (Schuelein et al., 2011).

In the last years, six genomes of different *L. pneumophila* strains (Paris, Lens, Philadelphia, Corby, Alcoy, and 130b (Cazalet et al., 2004; Chien et al., 2004; Steinert et al., 2007; D'Auria et al., 2010; Schroeder et al., 2010) have been published. The genome sequences of all but strain 130b were completely finished. Furthermore, the sequencing and analysis of four genomes of *L. longbeachae* have been carried out recently (Cazalet et al., 2010). *L. longbeachae* strain NSW150 of Sg1 isolated in Australia from a patient was sequenced completely, and for the remaining three strains (ATCC33462, Sg1 isolated from a human lung, C-4E7 and 98072, both of Sg2 isolated from patients) a draft genome sequence was reported. A fifth *L. longbeachae* strain (D-4968 of Sg1, isolated in the US from a patient) was recently sequenced and the analysis of the genome sequences assembled into 89 contigs was reported (Kozak et al., 2010).

Here we will describe what we learned from the analysis and comparison of the sequenced *Legionella* strains. We will discuss their general characteristics and then highlight the specific features or common traits with respect to the different ecological niches and the differences in host susceptibility of these two *Legionella* species. Emphasis will be put on putative virulence and *Legionella* life cycle related functions. In the last part we will analyze and discuss the possible evolution of the identified virulence factors. Finally, future perspectives in *Legionella* genomics are presented.

GENERAL FEATURES OF THE *L. PNEUMOPHILA* AND *L. LONGBEACHAE* GENOMES

Legionella pneumophila and *L. longbeachae* each have a single, circular chromosome with a size of 3.3–3.5 Mega bases (Mb) for *L. pneumophila* and 3.9–4.1 Mb for *L. longbeachae*. For both the average G + C content is 38% (**Tables 1A,B**). The *L. pneumophila* strains Paris and Lens each contain different plasmids, 131.9 kb and 59.8 kb in size, respectively. In strain Philadelphia-1, 130b, Alcoy, and Corby no plasmid was identified. The *L. longbeachae* strains NSW10 and D-4986 carry highly similar plasmids of about 70 kb and DNA identity of 99%, strains C-4E7 and 98072 also contain each a highly similar plasmid of 133.8 kb in size. Thus similar plasmids circulate among *L. longbeachae* strains, but they seem to be different from those found in *L. pneumophila*.

A total of ∼3000 and 3500 protein-encoding genes are predicted in the *L. pneumophila* and *L. longbeachae* genomes, respectively. No function could be predicted for about 40% of these genes and about 20% are unique to the genus *Legionella*. Comparative analysis of the genome structure of the *L. pneumophila* genomes showed

Table 1 | General features of the sequenced *Legionella* genomes.

A. Complete and draft genomes of *L. pneumophila* obtained by classical or new generation sequencing

L. pneumophila						
	Paris	Lens	Philadelphia	Corby	Alcoy	130b[c]
Chromosome size (kb)[a]	3504 (131.9)[b]	3345 (59.8)	3397	3576	3516	3490
G + C content (%)	38.3 (37.4)	38.4 (38)	38.3	38	38.4	38.2
No. of genes[a]	3123 (142)	2980 (60)	3031	3237	3197	3288
No. of protein coding genes[a]	3078 (140)	2921 (60)	2999	3193	3097	3141
Percentage of CDS (%)	87.9	88.0	90.2	86.8	86.0	87.9
No. of specific genes	225	181	213	144	182	386[c]
No. of 16S/23S/5S	03/03/03	03/03/03	03/03/03	03/03/03	03/03/03	ND
No. transfer RNA	44	43	43	43	43	42
Plasmids	1	1	0	0	0	0

B. Complete and draft genomes of *L. longbeachae* obtained by classical or new generation sequencing

L. longbeachae					
	NSW 150	D-4968	ATCC33462	98072	C-4E7
Chromosome size (Kb)	4077 (71)	4016 (70)	4096	4018 (133.8)	3979 (133.8)
G + C content (%)	37.1 (38.2)	37.0	37.0	37.0 (37.8)	37 (37.8)
No. of genes	3660 (75)	3557 (61)	–	–	–
No. of 16S/23S/5S	04/04/04	04/04/04	04/04/04	04/04/04	04/04/04
No. of contigs > 0.5–300 kb	Complete	13	64	65	63
N50 contig size*	Complete	–	138 kb	129 kb	134 kb
Percentage of coverage**	100%	96.3	96.3	93.4	93.1
Number of SNP with NSW150	0	1900	1611	16 853	16 820
Plasmids	1	1	0	1	1

*[a]Updated annotation; CDS, coding sequence; [b]data from plasmids in parenthesis; [c]The 130b sequence is not a manually corrected and finished assembly, thus the high number of specific genes might be due to not corrected sequencing errors; ND, not determined; *N50 contig size, calculated by ordering all contig sizes and adding the lengths (starting from the longest contig) until the summed length exceeds 50% of the total length of all contigs (half of all bases reside in a contiguous sequence of the given size or more); SNP, single nucleotide polymorphism; **for SNP detection; – not determined.*

high colinearity, with only few translocations, duplications, deletions, or inversions (**Figures 1A,B**) and identified between 6 and 11% of genes as specific to each *L. pneumophila* strain. Principally, the genomes contain three large plasticity zones, where the synteny is disrupted: a 260-kb inversion in strain Lens with respect to strains Paris and Philadelphia-1, a 130-kb fragment which is inserted in a different genomic location in strains Paris and Philadelphia-1 and the about 50 kb chromosomal region carrying the Lvh type IV secretion system, previously described in strain Philadelphia-1 (Segal et al., 1999). Furthermore, deletions and insertions of several smaller regions were identified in each strain, as well as regions with variable gene content. In contrast, comparison of the completed chromosome sequences of *L. pneumophila* and *L. longbeachae* shows that the two *Legionella* species have a significantly different genome organization (**Figure 1C**). Moreover only about 65% of the *L. longbeachae* genes are orthologous to *L. pneumophila* genes, whereas about 34% of all genes are specific to *L. longbeachae* with respect to *L. pneumophila* Paris, Lens, Philadelphia, and Corby (defined by less than 30% amino acid identity over 80% of the length of the smallest protein).

Analysis of single nucleotide polymorphisms (SNP) revealed a very low SNP number of less than 0.4% among the four *L. longbeachae* genomes, which is significantly lower than the polymorphism of about 2% between *L. pneumophila* Sg1 strains Paris and Philadelphia (**Table 1B**). Comparison of the two *L. longbeachae* Sg1 genomes (NSW150, ATCC33462) identified 1611 SNPs of which 1426 are located in only seven chromosomal regions mainly encoding putative mobile elements, whereas the remaining 185 SNPs were evenly distributed around the chromosome. A similar number of about 1900 SNPs were identified when comparing strains NSW150 to strain D-4968 (**Table 1B**). In contrast, the SNP number between two strains of different Sg was higher, with about 16000 SNPs present between Sg1 and Sg2 strains (**Table 1B**). This low SNP number and relatively homogeneous distribution of the SNPs around the chromosome suggest recent expansion for the species *L. longbeachae* (Cazalet et al., 2010). The sequences and their analysis are accessible at http://genolist.pasteur.fr/LegioList/.

To investigate the phylogenetic relationship among the *L. pneumophila* and *L. longbeachae* strains we here used the nucleotide sequence of *recN* (recombination and repair protein-encoding gene) aligned based on the protein alignment. Based on an analysis of 32 protein-encoding genes widely distributed among bacterial genomes, RecN was described as the gene with the greatest potential for predicting genome relatedness at the genus or subgenus level (Zeigler, 2003). As depicted in **Figure 2**, the phylogenetic relationship among the four *L. pneumophila* strains is very high, and *L. longbeachae* is clearly more distant.

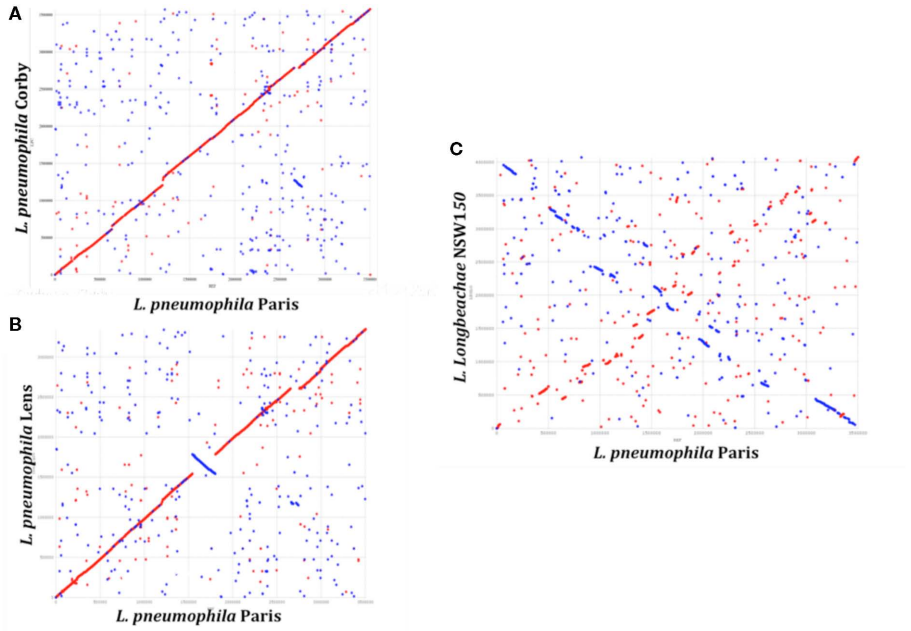

FIGURE 1 | Synteny plot of the chromosomes of *L. pneumophila* strains Paris, Lens, Corby, and *L. longbeachae* NSW150. The plot was created using the mummer software package. **(A)** Synteny plot of the chromosomes of strains *L. pneumophila* Paris and Corby **(B)** and strains *L. pneumophila* Paris and Lens and **(C)** strains *L. pneumophila* Paris and *L. longbeachae* NSW150. Inversions between the genomic sequences are represented in blue. Genome-wide synteny is disrupted by a 260 kb inversion (blue) and a 130 kb plasticity zone between strain *L. pneumophila* Paris and Lens. In contrast, synteny between *L. pneumophila* and *L. longbeachae* is highly conserved.

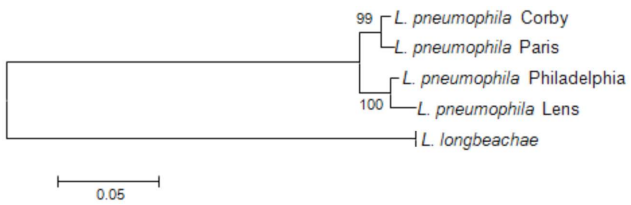

FIGURE 2 | Phylogenetic tree showing the relationship of the sequenced *L. pneumophila* and *L. longbeachae* strains based on the *recN* sequence. The tree was constructed using the *recN* sequences of each genome and the Neighbor joining method in MEGA. *L. longbeachae* is indicated without strain designation, as the RecN sequence of all sequenced strains is identical and thus only one representative strain is indicated on the tree. Numbers at branching nodes are percentages of 1000 bootstrap replicates.

DIVERSITY IN SECRETION SYSTEMS AND THEIR SUBSTRATES MAY CONTRIBUTE TO DIFFERENCES IN INTRACELLULAR TRAFFICKING AND NICHE ADAPTATION

The capacity of pathogens like *Legionella* to infect eukaryotic cells is intimately linked to the ability to manipulate host cell functions to establish an intracellular niche for their replication. Essential for the ability of *Legionella* to subvert host functions are its different secretion systems. The two major ones, known to be involved in virulence of *L. pneumophila* are the Dot/Icm type IV secretion system (T4BSS) and the Lsp type II secretion system (T2SS; Marra et al., 1992; Berger and Isberg, 1993; Rossier and Cianciotto, 2001).

For *L. pneumophila* type II protein secretion is critical for infection of amebae, macrophages and mice. Analyses of the *L. longbeachae* genome sequences showed, that it contains all genes to encode a functional Lsp type II secretion machinery (Cazalet et al., 2010; Kozak et al., 2010). Several studies, including the analysis of the *L. pneumophila* type II secretome indicated that *L. pneumophila* encodes at least 25 type II secreted substrates (Debroy et al., 2006; Cianciotto, 2009). Although this experimentally defined repertoire of type II secretion-dependent proteins is the largest known in bacteria, it may contain even more than 60 proteins as 35 additional proteins with a signal sequence were identified by *in silico* analyses (Cianciotto, 2009). A search for homologs of these substrates in the *L. longbeachae* genome sequences revealed that 9 (36%) of the 25 type II secretion system substrates described for *L. pneumophila* are absent from *L. longbeachae* (**Table 2**). For example the phospholipase C encoded by *plcA* and the *chiA*-encoded chitinase, which was shown to promote *L. pneumophila* persistence in the lungs of A/J mice are not present in *L. longbeachae* (Debroy et al., 2006). Thus over a third of the T2SS substrates seem to differ between *L. pneumophila* and *L. longbeachae*, a feature probably related to the different ecological niches occupied, but also to different virulence properties in the hosts.

Indispensible for replication of *L. pneumophila* in the eukaryotic host cells is the Dot/Icm T4SS (Nagai and Kubori, 2011), which translocate a large repertoire of bacterial effectors into the host cell. These effectors modulate multiple host cell processes and in particular, redirect trafficking of the *L. pneumophila* phagosome and mediate its conversion into an ER-derived organelle competent for

Table 2 | Distribution of type II secretion-dependent proteins of *L. pneumophila* in *L. longbeachae*.

L. pneumophila						*L. longbeachae*		Name	Product
Phila	**Paris**	**Lens**	**Corby**	**Alcoy**	**130b***	**NSW**	**D-4968**		
lpg0467	*lpp0532*	*lpl0508*	*lpc2877*	*lpa00713*	*lpw05741*	*llo2721*	*llb2607*	*proA*	Zinc metalloprotease, promotes amebal infection
lpg1119	*lpp1120*	*lpl1124*	*lpc0577*	*lpa01742*	–	*llo1016*	*llb0700*	*map*	Tartrate-sensitive acid phosphatase
lpg2343	*lpp2291*	*lpl2264*	*lpc1811*	*lpa03353*	*lpw25361*	*llo2819*	*llb2504*	*plaA*	Lysophospholipase A
lpg2837	*lpp2894*	*lpl2749*	*lpc3121*	*lpa04118*	*lpw30971*	*llo0210*	*llb1661*	*plaC*	Glycerophospholipid:cholestrol transferase
lpg0502	*lpp0565*	*lpl0541*	*lpc2843*	*lpa00759*	*lpw05821*	–	–	*plcA*	Phospholipase C
lpg0745	*lpp0810*	*lpl0781*	*lpc2548*	*lpa01148*	*lpw08251*	*llo2076*	*llb3335*	*lipA*	Mono- and triacylglycerol lipase
lpg1157	*lpp1159*	*lpl1164*	*lpc0620*	*lpa01801*	*lpw12111*	*llo2433*	*llb2928*	*lipB*	Triacylglycerol lipase
lpg2848	*lpp2906*	*lpl2760*	*lpc3133*	*lpa04141*	*lpw31111*	*llo0201*	*llb1671*	*srnA*	Type 2 ribonuclease, promotes amebal infection
lpg1116	*lpp1117*	*lpl1121*	*lpc0574*	*lpa01738*	*lpw11641*	–	–	*chiA*	Chitinase, promotes lung infection
lpg2814	*lpp2866*	*lpl2729*	*lpc3100*	*lpa04088*	*lpw30701*	*llo0255*	*llb1611*	*lapA*	Leucine, phenylalanine, and tyrosine aminopeptidase
lpg0032	*lpp0031*	*lpl0032*	*lpc0032*	*lpa00041*	*lpw00321*	–	–	*lapB*	Lysine and arginine aminopeptidase
lpg0264	*lpp0335*	*lpl0316*	*lpc0340*	*lpa00461*	*lpw03521*	*llo3103*	*llb2271*		Weakly similar to bacterial amidase
lpg2622	*lpp2675*	*lpl2547*	*lpc0519*	*lpa03836*	*lpw28341*	–	–		Weakly similar to bacterial cysteine protease
lpg1918	*lpp1893*	*lpl1882*	*lpc1372*	*lpa02774*	*lpw19571*	*llo3308*	*llb2032*	*celA*	Endoglucanase
lpg2999	*lpp3071*	*lpl2927*	*lpc3315*	*lpa04395*	*lpw32851*	–	–		Predicted astacin-like zink endopeptidase
lpg2644	*lpp2697*	*lpl2569*	*lpc0495*	*lpa03870*	–	–	–		Some similarity to collagen like protein
lpg1809	*lpp1772*	*lpl1773*	*lpc1253*	*lpa02614*	*lpw18401*	*llo1104*	*llb0603*		Unknown
lpg1385	*lpp1340*	*lpl1336*	*lpc0801*	*lpa02037*	*lpw13951*	*llo1474*	*llb0177*		Unknown
lpg0873	*lpp0936*	*lpl0906*	*lpc2419*	*lpa01320*	*lpw09571*	*llo2475*	*llb2883*		Unknown
lpg0189	*lpp0250*	*lpl0249*	*lpc0269*	*lpa00360*	*lpw02811*	–	–		Unknown
lpg0956	*lpp1018*	*lpl0958*	*lpc2331*	*lpa01443*	*lpw10421*	*llo1935*	*llb3498*		Unknown
lpg2689	*lpp2743*	*lpl2616*	*lpc0447*	*lpa03925*	*lpw29431*	*llo0361*	*llb1497*	*icmX*	Linked to Dot/Icm type IV secretion genes
lpg1244	*lpp0181*	*lpl0163*	–	–	*lpw01541*	–	–	*lvrE*	Linked to Lvh type IV secretion genes
lpg1832	*lpp1795*	*lpl1796*	*lpc1276*	*lpa02647*	*lpw18641*	*llo1152*	*llb0546*		Weakly similar to VirK
lpg1962	*lpp1946*	*lpl1936*	*lpc1440*	*lpa02861*	*lpw20131*	–	–		Putative peptidyl-prolyl cis-trans isomerase
lpg0422	*lpp0489*	*lpl0465*	*lpc2921*	*lpa0657*	*lpw05041*	*llo2801*	*llb2523*	*gamA*	Glucoamylase

*Substrates in this list are according to Cianciotto (2009); *strain 130b is not a finished sequence and not manually curated. Thus absence of a substrate can also be due to gaps in the sequence; – means not present; NSW means L. longbeachae NSW150.*

intracellular bacterial replication (Shin and Roy, 2008; Cianciotto, 2009). The Dot/Icm system is conserved in *L. longbeachae* with a similar gene organization and protein identities of 47–92% with respect to *L. pneumophila* (**Figure 3**). This is similar to what has been reported previously for other *Legionella* species (Morozova et al., 2004). The only major differences identified are that in *L. longbeachae* the *icmR* gene is replaced by the *ligB* gene, however, the encoded proteins have been shown to perform similar functions (Feldman and Segal, 2004; Feldman et al., 2005) and that the DotG/IcmE protein of *L. longbeachae* (1525 aa) is 477 amino acids larger than that of *L. pneumophila* (1048 aa; Cazalet et al., 2010). DotG of *L. pneumophila* is part of the core transmembrane complex of the secretion system and is composed of three domains: a transmembrane N-terminal domain, a central region composed of 42 repeats of 10 amino acid and a C-terminal region homologous to VirB10. In contrast, the central region of *L. longbeachae* DotG is composed of approximately 90 repeats. Among the many VirB10 homologs present in bacteria, the *Coxiella* DotG and the *Helicobacter pylori* Cag7 are the only ones, which also have multiple repeats of 10 aa (Segal et al., 2005). It will be challenging to understand the impact of this modification on the function of the type IV secretion system. A *L. longbeachae* T4SS mutant obtained by deleting the *dotA* gene is strongly attenuated for intracellular growth in *Acanthamoeba castellanii* and human macrophages (Cazalet et al., 2010, and unpublished data), is outcompeted by the wild type strain 24 and 72 h after infection of lungs of A/J mice and is also dramatically attenuated for replication in lungs of A/J mice upon single infections (Cazalet et al., 2010). Thus, similar to what is seen for *L. pneumophila*, the Dot/Icm T4SS of *L. longbeachae* is also central for its pathogenesis and the capacity to replicate in eukaryotic host cells.

This T4SS is crucial for intracellular replication for *Legionella* as it secretes an exceptionally large number of proteins into the host cell. Using different methods, 275 substrates have been shown to be translocated in the host cell in a Dot/Icm T4SS dependent manner (Campodonico et al., 2005; De Felipe et al., 2005, 2008; Shohdy et al., 2005; Burstein et al., 2009; Heidtman et al., 2009; Zhu et al., 2011). **Table 3** shows the distribution of the 275 Dot/Icm substrates identified in *L. pneumophila* strain Philadelphia and their distribution in the six *L. pneumophila* and five *L. longbeachae* genomes sequenced. Their conservation among different *L. pneumophila* strains is very high, as over 80% of the substrates are present in all *L. pneumophila* strains analyzed here. In contrast, the search for homologs of these *L. pneumophila* Dot/Icm

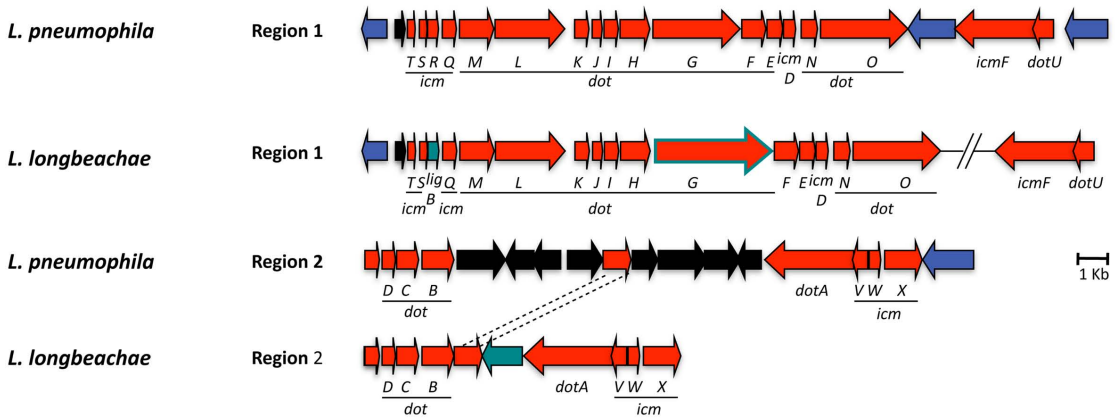

FIGURE 3 | Alignment of the chromosomal regions of *L. pneumophila* and *L. longbeachae* coding the Dot/Icm type 4 secretion system genes. The comparison shows that all genes are highly conserved (47–92% identity) between *L. pneumophila* Paris and *L. longbeachae*. Red arrows, genes conserved between *L. pneumophila* and *L. longbeachae* (>47% identity); black arrows, *L. pneumophila* specific genes compared to *L. longbeachae* (<35% identity); blue arrows, genes conserved between *L. pneumophila* and *L. longbeachae* but located in different places of the genome; green arrows, *L. longbeachae* specific genes compared to *L. pneumophila*. Red arrow boxed in green depicts *dotG*. N-terminal and C-terminal parts of *dotG* are highly conserved while the central part composed of repeated sequences differs between *L. pneumophila* and *L. longbeachae*.

substrates in *L. longbeachae* showed that even more pronounced differences are present than in the repertoire of type II secreted substrates. Only 98 of these 275 *L. pneumophila* Dot/Icm substrates have homologs in the *L. longbeachae* genomes (**Table 3**). However, the repertoire of *L. longbeachae* substrates seems also to be quite large, as a search for proteins that encode eukaryotic like domains and contain the secretion signal described by Nagai et al. (2005) and the additional criteria defined by Kubori et al. (2008) predicted 51 putative Dot/Icm substrates specific for *L. longbeachae* NSW150 (Cazalet et al., 2010) indicating that at least over 140 proteins might be secreted by the Dot/Icm T4SS of *L. longbeachae*. A similar number of *L. longbeachae* specific putative eukaryotic like proteins and effectors was predicted for strain D-4968 (Kozak et al., 2010). Examples of effector proteins conserved between the two species are RalF, VipA, VipF, SidC, SidE, SidJ, YlfA LepA, and LepB, which contribute to trafficking or recruitment and retention of vesicles to *L. pneumophila* (Nagai et al., 2002; Chen et al., 2004; Luo and Isberg, 2004; Campodonico et al., 2005; Shohdy et al., 2005; Liu and Luo, 2007). It is interesting to note that homologs of SidM/DrrA and SidD are absent from *L. longbeachae* but a homolog of LepB is present. For *L. pneumophila* it was shown that SidM/DrrA, SidD, and LepB act in cooperation to manipulate Rab1 activity in the host cell. DrrA/SidM possesses three domains, an N-terminal AMP-transfer domain (AT), a nucleotide exchange factor (GEF) domain in the central part and a phosphatidylinositol-4-Phosphate binding domain (P4M) in its C-terminal part. After association of DrrA/SidM with the membrane of the *Legionella*-containing vacuole (LCV) via P4M (Brombacher et al., 2009), it recruits Rab1 via the GEF domain and catalyzes the GDP–GTP exchange (Ingmundson et al., 2007; Machner and Isberg, 2007). Rab1 is then adenylated by the AT domain leading to inhibition of GAP-catalyzed Rab1-deactivation (Müller et al., 2010). LepB cannot bind AMPylated Rab1 (Ingmundson et al., 2007). Recently it was shown that SidD deAMPylates Rab1 and enables LepB to bind Rab1 to promote its GTP–GDP exchange (Neunuebel et al., 2011; Tan and Luo, 2011). One might assume that other proteins of *L. longbeachae* not yet identified may perform the functions of DrrA/SidM and SidD. Another interesting observation is, that all except four of the effector proteins of *L. pneumophila* that are conserved in *L. longbeachae* are also conserved in all sequenced *L. pneumophila* genomes (**Table 3**).

Taken together the T2SS Lsp and the T4SS Dot/Icm are highly conserved between *L. pneumophila* and *L. longbeachae*. However, more than a third of the known *L. pneumophila* type II- and over 70% of type IV-dependent substrates differ between both species. These species specific, secreted effectors might be implicated in the different niche adaptations and host susceptibilities. Most interestingly, of the 98 *L. pneumophila* substrates conserved in *L. longbeachae* 87 are also present in all *L. pneumophila* strains sequenced to date. Thus, these 87 Dot/Icm substrates might be essential for intracellular replication of *Legionella* and represent a minimal toolkit for intracellular replication that has been acquired before the divergence of the two species.

MOLECULAR MIMICRY IS A MAJOR VIRULENCE STRATEGY OF *L. PNEUMOPHILA* AND *L. LONGBEACHAE*

The *L. pneumophila* genome sequence analysis has revealed that many of the predicted or experimentally verified Dot/Icm secreted substrates are proteins similar to eukaryotic proteins or contain motifs mainly or only found in eukaryotic proteins (Cazalet et al., 2004; De Felipe et al., 2005). Thus comparative genomics suggested that *L. pneumophila* encodes specific virulence factors that have evolved during its evolution with eukaryotic host cells such as fresh-water ameba (Cazalet et al., 2004). The protein-motifs predominantly found in eukaryotes, which were identified in the *L. pneumophila* genomes are ankyrin repeats, SEL1 (TPR), Set domain, Sec7, serine threonine kinase domains (STPK), U-box, and F-box motifs. Examples for eukaryotic like proteins of *L. pneumophila* are two secreted apyrases, a

Table 3 | Distribution of 275 Dot/Icm substrates identified in strain *L. pneumophila* Philadelphia in the 5 sequenced *L. pneumophila* and 5 sequenced *L. longbeachae* strains.

L. pneumophila						*L. longbeachae*					Name	Product
Phila	**Paris**	**Lens**	**Corby**	**Alcoy**	**130b**	**NSW 150**	**D-4968**	**AT**	**98072**	**C-4E7**		
lpg0008	*lpp0008*	*lpl0008*	*lpc0009*	*lpa0011*	*lpw00071*	–	–	–	–	–	*ravA*	Unknown
lpg0012	*lpp0012*	*lpl0012*	*lpc0013*	*lpa0016*	*lpw00111*	–	–	–	–	–	*cegC1*	Ankyrin
lpg0021	*lpp0021*	*lpl0022*	*lpc0022*	*lpa0030*	*lpw00221*	*llo0047*	*llb1841*	+	+	+	–	Unknown
lpg0030	*lpp0030*	*lpl0031*	*lpc0031*	*lpa0040*	*lpw00311*	–	–	–	–	–	*ravB*	Unknown
lpg0038	*lpp0037*	*lpl0038*	*lpc0039*	*lpa0049*	*lpw00381*	–	–	–	–	–	*ankQ/ legA10*	Ankyrin repeat
lpg0041	–	–	*lpc0042*	*lpa0056*	–	–	–	–	–	–	–	Putative metalloprotease
lpg0045	*lpp0046*	*lpl0044*	*lpc0047*	*lpa0060*	*lpw00441*	–	–	–	–	–	–	Unknown
lpg0046	*lpp0047*	*lpl0045*	*lpc0048*	*lpa0062*	*lpw00451*	–	–	–	–	–	–	Unknown
lpg0059	*lpp0062*	*lpl0061*	*lpc0068*	*lpa0085*	*lpw00621*	–	–	–	–	–	*ceg2*	Unknown
lpg0080	*lpp0094*	–	–	*lpa3018*	*lpw00781*	–	–	–	–	–	*ceg3*	Unknown
lpg0081	*lpp0095*	–	–	–	*lpw00791*	–	–	–	–	–	–	Unknown
lpg0090	*lpp0104*	*lpl0089*	*lpc0109*	*lpa0132*	*lpw00881*	–	–	–	–	–	*lem1*	Unknown
lpg0096	*lpp0110*	*lpl0096*	*lpc0115*	*lpa0145*	*lpw00961*	*llo1322*	*llb0347*	+	+	+	*ceg4*	Unknown
lpg0103	*lpp0117*	*lpl0103*	*lpc0122*	*lpa0152*	*lpw01031*	*llo3312*	*llb2028*	+	+	+	*vipF*	N-terminal acetyl-transferase, GNAT
lpg0126	*lpp0140*	*lpl0125*	*lpc0146*	*lpa0185*	*lpw01261*	–	–	–	–	–	*cegC2*	Ninein
lpg0130	*lpp0145*	*lpl0130*	*lpc0151*	*lpa0194*	*lpw01311*	*llo3270*	*llb2073*	+	+	+	–	Unknown
lpg0135	*lpp0150*	*lpl0135*	*lpc0156*	*lpa0204*	*lpw01361*	*llo2439*	*llb2921*	+	+	+	*sdhB*	Unknown
lpg0160	*lpp0224*	*lpl0224*	*lpc0242*	*lpa0322*	*lpw02541*	–	–	–	–	–	*ravD*	Unknown
lpg0170	*lpp0232*	*lpl0233*	*lpc0251*	*lpa0335*	*lpw02641*	*llo1378*	*llb0280*	+	+	+	*ravC*	Unknown
lpg0171	*lpp0233*	*lpl0234*	–	–	*lpw02651*	–	–	–	–	–	*legU1*	F-box motif
lpg0172	*lpp0234*	–	*lpc0253*	*lpa0339*	*lpw02661*	–	–	–	–	–	–	Unknown
lpg0181	*lpp0245*	*lpl0244*	*lpc0265*	*lpa0388*	*lpw02761*	*llo2453*	*llb2907*	+	+	+	–	Unknown
lpg0191	*lpp0251*	–	–	–	*lpw02821*	–	–	–	–	–	*ceg5*	Unknown
lpg0195	*lpp0253*	*lpl0251*	*lpc0272*	*lpa0339*	*lpw02851*	–	–	–	–	–	*ravE*	Unknown
lpg0196	*lpp0254*	*lpl0252*	–	–	*lpw02861*	*llo2549*	*llb2798*	+	+	+	*ravF*	Unknown
lpg0210	*lpp0269*	*lpl0264*	*lpc0285*	*lpa0388*	*lpw02981*	–	–	–	–	–	*ravG*	Unknown
lpg0227	*lpp0286*	*lpl0281*	*lpc0303*	*lpa0412*	*lpw03151*	*llo2491*	*llb2864*	+	+	+	*ceg7*	Unknown
lpg0234	*lpp0304*	*lpl0288*	*lpc0309*	*lpa0419*	*lpw03221*	*llo0425*	*llb1431*	+	+	+	*sidE/laiD*	Unknown
lpg0240	*lpp0310*	*lpl0294*	*lpc0316*	*lpa0428*	*lpw03291*	*llo1601*	*llb0040*	+	+	+	*ceg8*	Unknown
lpg0246	*lpp0316*	*lpl0300*	*lpc0323*	*lpa0436*	*lpw03361*	–	–	–	–	–	*ceg9*	Unknown
lpg0257	*lpp0327*	*lpl0310*	*lpc0334*	*lpa0450*	*lpw03461*	*llo2362*	*llb3009*	+	+	+	*sdeA*	Multidrug resistance protein
lpg0260	*lpp0332*	*lpl0313*	*lpc0337*	*lpa0456*	*lpw03491*	–	–	–	–	–	–	Unknown
lpg0275	*lpp0349*	*lpl0327*	*lpc0351/ 3529*	*lpa0477*	*lpw03641*	–	–	–	–	–	*sdbA*	Unknown
lpg0276	*lpp0350*	*lpl0328*	*lpc0353*	*lpa0479*	*lpw03651*	*llo0327*	*llb1533*	+	+	+	*legG2*	Ras guanine nucleotide exchange factor
lpg0284	*lpp0360*	*lpl0336*	*lpc0361*	*lpa0490*	*lpw03741*	–	–	–	–	–	*ceg10*	Unknown
lpg0285	*lpp0361*	*lpl0337*	*lpc0362*	*lpa0492*	*lpw03751*	–	–	–	–	–	*lem2*	Unknown
lpg0294	*lpp0372*	*lpl0347*	*lpc0373*	*lpa0508*	*lpw03861*	*llo0464*	*llb1386*	+	+	+	–	Unknown
lpg0364	*lpp0429*	*lpl0405*	*lpc2980*	*lpa0578*	*lpw04431*	–	–	–	–	–	–	Unknown
lpg0365	*lpp0430*	*lpl0406*	*lpc2979*	*lpa0580*	*lpw04441*	*llo0525*	*llb1334*	+	+	+	–	Unknown
lpg0375	*lpp0442*	*lpl0418*	*lpc2968*	*lpa0596*	–	–	–	–	–	–	–	Unknown
lpg0376	*lpp0443*	*lpl0419*	*lpc2967*	*lpa0597*	*lpw04591*	*llo0548*	*llb1307*	+	+	+	*sdhA*	GRIP, coiled-coil
lpg0390	*lpp0457*	*lpl0433*	*lpc2954*	*lpa0613*	*lpw04721*	–	–	–	–	–	*vipA*	Unknown
lpg0401	*lpp0468*	*lpl0444*	*lpc2942*	*lpa0629*	*lpw04831*	*llo2582*	*llb2763*	+	+	+	*legA7/ceg*	Unknown

(Continued)

Table 3 | Continued

L. pneumophila						*L. longbeachae*					Name	Product
Phila	**Paris**	**Lens**	**Corby**	**Alcoy**	**130b**	**NSW 150**	**D-4968**	**AT**	**98072**	**C-4E7**		
lpg0402	–	–	–	–	–	–	–	–	–	–	*ankY/legA9*	Ankyrin, STPK
lpg0403	*lpp0469*	*lpl0445*	*lpc2941*	*lpa0630*	*lpw04841*	–	–	–	–	–	*ankG/ankZ/legA7*	Ankyrin
lpg0405	*lpp0471*	*lpl0447*	*lpc2939*	*lpa0633*	*lpw04861*	*llo2845*	*llb2472*	+	+	+	–	Spectrin domain
lpg0422	*lpp0489*	*lpl0465*	*lpc2921*	*lpa0657*	*lpw05041*	*llo2801*	*llb2523*	+	+	+	*legY*	Putative Glucan 1,4-alpha-glucosidase
lpg0436	*lpp0503*	*lpl0479*	*lpc2906*	*lpa0673*	*lpw05181*	–	–	–	–	–	*ankJ/legA11*	Ankyrin
lpg0437	*lpp0504*	*lpl0480*	*lpc2905*	*lpa0674*	*lpw05191*	–	–	–	–	–	*ceg14*	Unknown
lpg0439	*lpp0505*	*lpl0481*	*lpc2904*	*lpa0678*	*lpw05201*	*llo2983*	*llb2392*	+	+	+	*ceg15*	Unknown
lpg0483	*lpp0547*	*lpl0523*	*lpc2861*	*lpa0739*	*lpw05631*	*llo2705*	*llb2623*	+	+	+	*ankC/legA12*	Ankyrin
lpg0515	*lpp0578*	*lpl0554*	*lpc2829*	*lpa0776*	*lpw05951*	*llo3224*	*llb2129*	+	+	+	*legD2*	Phytanoyl-CoA dioxygenase domain
lpg0518	*lpp0581*	*lpl0557*	*lpc2826*	*lpa0781*	*lpw05981*	–	–	–	–	–	–	Unknown
lpg0519	–	–	–	–	–	–	–	–	–	–	*ceg17*	Unknown
lpg0621	*lpp0675*	*lpl0658*	*lpc2673*	*lpa0975*	*lpw06951*	–	–	–	–	–	*sidA*	Unknown
lpg0634	*lpp0688*	*lpl0671*	*lpc2660*	*lpa0996*	*lpw07081*	*llo2574*	*llb2771*	+	+	+	–	Unknown
lpg0642	*lpp0696/97*	*lpl0679*	*lpc2651*	*lpa1005*	*lpw07161*	–	–	–	–	–	*wipB*	Unknown
lpg0695	*lpp0750*	*lpl0732*	*lpc2599*	*lpa1082*	*lpw07721*	–	–	–	–	–	*ankN/ankX legA8*	Ankyrin
lpg0696	*lpp0751*	*lpl0733*	*lpc2598*	*lpa1084*	*lpw07731*	–	–	–	–	–	*lem3*	Unknown
lpg0716	*lpp0782*	*lpl0753*	*lpc2577*	*lpa1108*	*lpw07931*	–	–	–	+	+	–	Unknown
lpg0733	*lpp0799*	*lpl0770*	*lpc2559*	*lpa1135*	*lpw08111*	*llo0831*	*llb0892*	+	+	+	*ravH*	Unknown
lpg0796	*lpp0859*	–	–	–	–	–	–	–	–	–	–	Unknown
lpg0898	*lpp0959*	*lpl0929*	*lpc2395*	*lpa1360*	*lpw09801*	–	–	–	–	–	*ceg18*	Unknown
lpg0926	*lpp0988*	*lpl0957*	*lpc2365*	*lpa1397*	*lpw10111*	–	–	–	–	–	*ravI*	Unknown
lpg0940	*lpp1002*	*lpl0971*	*lpc2349*	*lpa1415*	*lpw10251*	–	–	–	–	–	*lidA*	Unknown
lpg0944	*lpp1006*	–	*lpc2345*	*lpa1421*	–	–	–	–	–	–	*ravJ*	Unknown
lpg0945	*lpp1007*	*lpl1579*	*lpc2344*	*lpa1423*	*lpw10311*	–	–	–	–	–	*legL1*	LLR
lpg0963	*lpp1025*	*lpl0992*	*lpc2324*	*lpa1453*	*lpw10491*	*llo0934*	*llb0782*	+	+	+	–	Unknown
lpg0967	*lpp1029*	–	*lpc2320*	*lpa1459*	*lpw10531*	–	–	–	–	–	–	Unknown
lpg0968	*lpp1030*	*lpl0997*	*lpc2319*	*lpa1460*	*lpw10541*	–	–	–	–	–	*sidK*	Unknown
lpg0969	*lpp1031*	*lpl0998*	*lpc2318*	*lpa1461*	*lpw10551*	*llo3265*	*llb2078*	+	+	+	*ravK*	Unknown
lpg1083	–	–	–	–	–	–	–	–	–	–	–	Unknown
lpg1101	*lpp1101*	*lpl1100*	*lpc2154**	*lpa1709*	*lpw11451*	–	–	–	–	–	*lem4*	Unknown
lpg1106	*lpp1105*	*lpl1105*	*lpc2149*	*lpa1719*	*lpw11501*	*llo1414*	*llb0239/4*	+	+	+	–	Unknown
lpg1108	*lpp1108*	*lpl1108*	*lpc2146*	*lpa1724*	*lpw11531*	*llo3030*	*llb2350*	+	+	+	*ravL*	Unknown
lpg1109	*lpp1109*	–	*lpc2145*	*lpa1725*	–	–	–	–	–	–	*ravM*	Unknown
lpg1110	*lpp1111*	*lpl1114*	*lpc2142*	*lpa1728*	*lpw11571*	–	–	–	–	–	*lem5*	Unknown
lpg1111	*lpp1112*	*lpl1115*	*lpc2141*	*lpa1730*	*lpw11581*	*llo3126*	*llb2244*	+	+	+	*ravN*	Unknown
lpg1120	–	–	–	–	*lpw11681*	–	–	–	–	–	*lem6*	Unknown
lpg1121	*lpp1121*	*lpl1126*	*lpc0578*	*lpa1743*	*lpw11691*	*llo1321*	*llb0348*	+	+	+	*ceg19*	Unknown
lpg1124	*lpp1125*	*lpl1129*	*lpc0582*	*lpa1748*	*lpw11741*	*llo3206*	*llb2150*	+	+	+	–	Unknown
lpg1129	*lpp1130*	–	–	–	*lpw11801*	–	–	–	–	–	*ravO*	Unknown
lpg1137	*lpp1139*	*lpl1144*	*lpc0601*	*lpa1776*	*lpw11901*	*llo2404*	*llb2962*	+	+	+	–	Unknown
lpg1144	*lpp1146*	*lpl1150*	*lpc0607*	*lpa1785*	*lpw11971*	–	–	–	–	–	*cegC3*	Unknown
lpg1145	*lpp1147*	*lpl1151*	*lpc0608*	*lpa1787*	*lpw11981*	–	–	–	–	–	*lem7*	Unknown
lpg1147	*lpp1149*	*lpl1153*	*lpc0610*	*lpa1789*	*lpw12001*	–	–	–	–	–	–	GCN5-related *N*-acetyltransferase
lpg1148	*lpp1150*	*lpl1154*	*lpc0611*	*lpa1790*	*lpw12011*	–	–	–	–	–	–	Unknown
lpg1152	*lpp1154*	*lpl1159*	*lpc0615*	*lpa1795*	*lpw12061*	–	–	–	–	–	*ravP*	Unknown

(Continued)

Table 3 | Continued

L. pneumophila						*L. longbeachae*					Name	Product
Phila	**Paris**	**Lens**	**Corby**	**Alcoy**	**130b**	**NSW 150**	**D-4968**	**AT**	**98072**	**C-4E7**		
lpg1154	*lpp1156*	*lpl1161*	*lpc0617*	*lpa1797*	*lpw12081*	*llo2487*	*llb2868*	+	+	+	*ravQ*	Unknown
lpg1158	*lpp1160*	*lpl1165**	*lpc0621*	*lpa1802*	*lpw12121*	–	–	–	–	–	–	Unknown
lpg1166	*lpp1168*	*lpl1174*	*lpc0631*	*lpa1819*	*lpw12211*	*llo1034*	*llb0680*	+	+	+	*ravR*	Unknown
lpg1171	*lpp1173*	*lpl1179*	*lpc0637*	*lpa1826*	–	–	–	–	–	–	–	Spectrin domain
lpg1183	*lpp1186*	*lpl1192*	*lpc0650*	*lpa1839*	*lpw12401*	*llo2390*	*llb2978*	+	+	+	*ravS*	Unknown
lpg1227	*lpp1235*	*lpl1235*	*lpc0696*	*lpa1899*	*lpw12861*	–	–	–	–	–	*vpdB*	Unknown
lpg1273	*lpp1236*	*lpl1236*	*lpc0698*	*lpa1901*	*lpw12871*	–	–	–	–	–	–	Unknown
lpg1290	*lpp1253*	–	–	–	–	–	–	–	–	–	*lem8*	Unknown
lpg1312	–	–	–	–	*lpw13261*	–	–	–	–	–	*legC1*	Unknown
lpg1316	–	–	–	–	–	*llo1389*	*llb0269*	+	+	+	*ravT*	Unknown
lpg1317	–	–	–	–	–	–	–	–	–	–	*ravW*	Unknown
lpg1328	*lpp1283*	*lpl1282*	*lpc0743*	*lpa1958*	–	–	–	–	–	–	*legT*	Thaumatin domain
lpg1355	*lpp1309*	–	–	–	–	–	–	–	–	–	*sidG*	Coiled-coil
lpg1426	*lpp1381*	*lpl1377*	*lpc0842*	*lpa2090*	*lpw14431*	*llo1791*	*llb3606*	+	+	+	*vpdC*	Patatin domain
lpg1449	*lpp1404*	–	–	–	*lpw14671*	–	–	–	–	–	–	Unknown
lpg1453	*lpp1409*	*lpl1591*	*lpc0868*	*lpa2119*	*lpw14711*	–	–	–	–	–	–	Unknown
lpg1483	*lpp1439*	*lpl1545*	*lpc0898*	*lpa2161*	*lpw15031*	*llo1682*	*llb3727*	+	+	+	*legK1*	STPK
lpg1484	*lpp1440*	*lpl1544*	*lpc0899*	*lpa2162*	*lpw15041*	–	–	–	–	–	–	Unknown
lpg1488	*lpp1444*	*lpl1540*	*lpc0903**	*lpa2168*	*lpw15081*	–	–	–	–	–	*lgt3/legc5*	Coiled-coil
lpg1489	*lpp1445*	*lpl1539*	*lpc0905*	*lpa2169*	*lpw15091*	–	–	–	–	–	*ravX*	Unknown
lpg1491	*lpp1447*	–	–	–	–	–	–	–	–	–	*lem9*	Unknown
lpg1496	*lpp1453*	*lpl1530*	*lpc0915*	*lpa2185*	*lpw15181*	–	–	–	–	–	*lem10*	Unknown
lpg1551	*lpp1508*	*lpl1475*	*lpc0972*	*lpa2253*	–	–	–	–	–	–	*ravY*	Unknown
lpg1578	*lpp4178*	*lpl4143*	*lpc1002*	*lpa2292*	*lpw16011*	*llo1503*	*llb0148*	+	+	+	–	Unknown
lpg1588	*lpp1546*	*lpl1437*	*lpc1013*	*lpa2305*	*lpw16131*	–	–	–	–	–	*legC6*	Coiled–coil
lpg1598	*lpp1556*	*lpl1427*	*lpc1025*	*lpa2317*	*lpw16231*	–	–	–	–	–	*lem11*	Unknown
lpg1602	*lpp1567*	*lpl1423/26**	*lpc1028*	*lpa2318*	*lpw16241*	–	–	–	–	–	*legL2*	LRR
lpg1621	*lpp1591*	*lpl1402*	*lpc1048*	*lpa2346*	*lpw16461*	*llo1014*	*llb0702*	+	+	+	*ceg23*	Unknown
lpg1625	*lpp1595*	*lpl1398*	*lpc1052*	*lpa2350*	*lpw16511*	*llo0719*	*llb1016*	+	+	+	*lem23*	Unknown
lpg1639	*lpp1609*	*lpl1387*	*lpc1068*	*lpa2367*	*lpw16651*	–	–	–	–	–	–	Unknown
lpg1642	*lpp1612a/b*	*lpl1384*	*lpc1071*	*lpa2371*	*lpw16681*	–	–	–	–	–	*sidB*	Putative hydrolase
lpg1654	*lpp1625*	–	*lpc1084*	*lpa2390*	–	*llo0791*	*llb0935*	+	+	+	–	Unknown
lpg1660	*lpp1631*	*lpl1625*	*lpc1090*	*lpa2398*	*lpw16861*	–	–	–	–	–	*legL3*	LRR
lpg1661	*lpp1632*	*lpl1626*	*lpc1091*	*lpa2399*	*lpw16871*	*llo1691*	*llb3715*	+	+	+	–	Putative *N*-acetyl transferase
lpg1666	*lpp1637*	*lpl1631*	*lpc1096*	*lpa2408*	*lpw16921*	–	–	–	–	–	–	Unknown
lpg1667	*lpp1638*	*lpl1632*	*lpc1097*	*lpa2409*	*lpw16931*	–	–	–	–	–	–	Unknown
lpg1670	*lpp1642*	*lpl1635*	*lpc1101*	*lpa2413*	*lpw16971*	–	–	–	–	–	–	Unknown
lpg1683	–	–	*lpc1114*	*lpa2431*	–	*llo2508*	*llb2843*	+	+	+	*ravZ*	Unknown
lpg1684	–	–	*lpc1115*	*lpa2432*	–	*llo2267*	*llb3113*	+	+	+	–	Unknown
lpg1685	–	–	*lpc1116*	*lpa2433*	–	*llo3208*	*llb2147*	+	+	+	–	Unknown
lpg1687	*lpp1656*	*lpl1650*	*lpc1118*	*lpa2437*	*lpw17121*	–	–	–	–	–	*mavA*	Unknown
lpg1689	*lpp1658*	*lpl1652*	*lpc1120*	*lpa2439*	*lpw17141*	*llo1697*	*llb3708*	+	+	+	–	Unknown
lpg1692	–	–	*lpc1123*	*lpa2442*	–	–	–	–	–	–	–	Unknown
lpg1701	*lpp1666*	*lpl1660*	*lpc1130*	*lpa2455*	*lpw17231*	–	–	–	–	–	*ppeA/legC3*	Coiled-coil
lpg1702	*lpp1667*	*lpl1661*	*lpc1131*	*lpa2456*	*lpw17241*	–	–	–	–	–	*ppeB*	Unknown
lpg1716	*lpp1681*	*lpl1675*	*lpc1146*	*lpa2474*	*lpw17391*	–	–	–	–	–	–	Unknown
lpg1717	*lpp1682*	–	–	–	*lpw17401*	–	–	–	–	–	–	Unknown

(Continued)

Table 3 | Continued

L. pneumophila						*L. longbeachae*					Name	Product
Phila	**Paris**	**Lens**	**Corby**	**Alcoy**	**130b**	**NSW 150**	**D-4968**	**AT**	**98072**	**C-4E7**		
lpg1718	*lpp1683*	*lpl1682*	*lpc1152*	*lpa2484*	*lpw17411*	–	–	–	–	–	*ankI/legAS4*	Ankyrin
lpg1751	*lpp1715*	*lpl1715*	*lpc1191*	*lpa2538*	*lpw17761*	*llo2314*	*llb3061*	+	+	+	–	Unknown
lpg1752	*lpp1716*	*lpl1716*	*lpc1192*	*lpa2539*	*lpw17771*	*llo2315*	*llb3060*	+	+	+	–	Unknown
lpg1776	*lpp1740*	*lpl1740*	*lpc1217*	*lpa2570*	*lpw18031*	*llo1437*	*llb0214**	+	+	+	–	Unknown
lpg1797	–	–	*lpc1239*	*lpa2599*	*lpw32931*	–	–	–	–	–	*rvfA*	Unknown
lpg1798	*lpp1761*	*lpl1761*	*lpc1241*	*lpa2600*	*lpw18281*	*llo0991*	*llb0731*	+	+	+	*marB*	Unknown
lpg1803	*lpp1766*	*lpl1766*	*lpc1246*	*lpa2606*	*lpw18331*	*llo2611*	*llb2729*	+	+	+	–	Unknown
lpg1836	*lpp1799*	*lpl1800*	*lpc1280*	*lpa2652*	*lpw18691*	–	–	–	–	–	*ceg25*	Unknown
lpg1851	*lpp1818*	*lpl1817*	*lpc1296*	*lpa2675*	*lpw18871*	*llo1047*	*llb0666*	+	+	+	*lem14*	Unknown
lpg1884	*lpp1848*	*lpl1845*	*lpc1331*	*lpa2714*	*lpw19161*	–	–	–	–	–	*ylfB/legC2*	Coiled-coil
lpg1888	*lpp1855*	*lpl1850*	*lpc1336*	*lpa2723*	*lpw19211*	–	–	–	–	–	–	Unknown
lpg1890	–	*lpl1852*	*lpc1338*	*lpa2726*	*lpw19231*	–	–	–	–	–	*legLC8*	LRR, coiled-coil
lpg1907	*lpp1882*	*lpl1871*	*lpc1361*	*lpa2762*	*lpw19461*	*llo1240*	*llb0452*	+	+	+	–	Unknown
lpg1924	*lpp1899*	*lpl1888*	*lpc1378*	*lpa2783*	*lpw19631*	–	–	–	–	–	–	Unknown
lpg1933	*lpp1914*	*lpl1903*	*lpc1406*	*lpa2811*	*lpw19721*	–	–	–	–	–	*lem15*	Unknown
lpg1947	*lpp1930*	*lpl1917**	–	*lpa2835*	*lpw19951*	–	–	–	–	–	*lem16*	Spectrin domain
lpg1948	–	–	–	–	–	–	–	–	–	–	*legLC4*	LRR, coiled-coil
lpg1949	*lpp1931*	*lpl1918*	*lpc1422*	*lpa2837*	*lpw19961*	–	–	–	–	–	*lem17*	Unknown
lpg1950	*lpp1932*	*lpl1919*	*lpc1423*	*lpa2838*	*lpw19971*	*llo1397*	*llb0259*	+	+	+	*ralF*	Sec7 domain
lpg1953	*lpp1935*	*lpl1922*	*lpc1426*	*lpa2842*	*lpw20041*	–	–	–	–	–	*legC4*	Coiled-coil
lpg1958	*lpp1940*	–	–	–	–	–	–	–	–	–	*legL5*	LRR
lpg1959	*lpp1941*	–	–	*lpa2857*	*lpw20101*	–	–	–	–	–	–	Unknown
lpg1960	*lpp1942*	*lpl1934**	*lpc1437*	*lpa2859*	*lpw20111*	*llo0565*	*llb1288*	+	+	+	*lirA*	Unknown
lpg1962	*lpp1946*	*lpl1936*	*lpc1440*	*lpa2861*	*lpw20131*	–	–	–	–	–	*lirB*	Rotamase
lpg1963	–	–	*lpc1441/42*	*lpa2863*	–	–	–	–	–	–	*pieA/lirC*	Unknown
lpg1964	–	–	–	–	–	–	–	–	–	–	*pieB/lirD*	Unknown
lpg1965	–	–	*lpc1443/45*	*lpa2865*	*lpw20141*	–	–	–	–	–	*pieC/lirE*	Unknown
lpg1966	*lpp1947*	–	*lpc1446*	*lpa2867*	*lpw20151*	–	–	–	–	–	*pieD/lirF*	Unknown
lpg1969	*lpp1952*	*lpl1941*	*lpc1452*	*lpa2874*	*lpw20201*	*llo3131*	*llb2239*	+	+	+	*pieE*	Unknown
lpg1972	*lpp1955*	*lpl1950*	*lpc1459*	*lpa2884*	*lpw20291*	–	–	–	–	–	*pieF*	Unknown
lpg1975	*lpp1959*	*lpl1953*	*lpc1462*	*lpa2889(1)*	*lpw20351*	–	–	–	–	–	–	Unknown
lpg1976	*lpp1959*	*lpl1953*	*lpc1462*	*lpa2889(2)*	*lpw20351*	–	–	–	–	–	*pieG/legG1*	Regulator of chromosome condensation
lpg1978	*lpp1961*	*lpl1955*	*lpc1464*	*lpa2892*	*lpw20371*	–	–	–	–	–	*setA*	Putative Glyosyltransferase
lpg1986	*lpp1967*	*lpl1961*	*lpc1469*	*lpa2898*	*lpw20431*	–	–	–	–	–	–	Unknown
lpg2050	*lpp2033*	*lpl2028*	*lpc1536*	*lpa2992*	*lpw21141*	–	–	–	–	–	–	Unknown
lpg2131	–	–	–	–	–	–	–	–	–	–	*legA6*	Unknown
lpg2137	*lpp2076*	*lpl2066*	*lpc1586*	*lpa3060*	*lpw23101*	–	–	–	–	–	*legK2*	STPK
lpg2144	*lpp2082*	*lpl2072*	*lpc1593*	*lpa3071*	*lpw23181*	–	–	–	–	–	*ankB/legAU13/ceg27*	Ankyrin, F-box
lpg2147	*lpp2086*	*lpl2075*	*lpc1596*	*lpa3076*	*lpw23211*	–	–	–	–	–	*mavC*	Unknown
lpg2148	*lpp2087*	*lpl2076*	*lpc1597*	*lpa3077*	*lpw23221*	–	–	–	–	–	–	Unknown
lpg2149	*lpp2088*	*lpl2077*	*lpc1598*	*lpa3078*	*lpw23231*	–	–	–	–	–	–	Unknown
lpg2153	*lpp2092*	*lpl2081*	*lpc1602*	*lpa3083*	*lpw23271*	–	–	–	–	–	*sdeC*	Unknown
lpg2154	*lpp2093*	*lpl2082*	*lpc1603*	*lpa3086*	*lpw23281*	*llo3097*	*llb2278*	+	+	+	*sdeC*	Unknown
lpg2155	*lpp2094*	*lpl2083*	*lpc1604*	*lpa3087*	*lpw23291*	*llo3096*	*llb2279*	+	+	+	*sidJ*	Unknown
lpg2156	*lpp2095*	*lpl2084*	*lpc1605*	*lpa3088*	*lpw23301*	*llo3095*	*llb2280*	+	+	+?	*sdeB*	Unknown
lpg2157	*lpp2096*	*lpl2085*	*lpc1618*	*lpa3037*	*lpw23331*	–	–	–	–	–	*sdeC*	Unknown
lpg2166	*lpp2104*	*lpl2093*	*lpc1626*	*lpa3107*	*lpw23451*	*llo2398*	*llb2969*	+	+	+	*lem19*	Unknown

(Continued)

Table 3 | Continued

L. pneumophila						*L. longbeachae*					Name	Product
Phila	**Paris**	**Lens**	**Corby**	**Alcoy**	**130b**	**NSW 150**	**D-4968**	**AT**	**98072**	**C-4E7**		
lpg2160	*lpp2099*	*lpl2088*	*lpc1621*	*lpa3100*	*lpw23361*	*llo2645*	*llb2690*	+	+	+	–	Unknown
lpg2176	*lpp2128*	*lpl2102*	*lpc1635*	*lpa3118*	*lpw23561*	–	–	–	–	–	*legS2*	Sphingosine-1-phosphate lyase
lpg2199	*lpp2149*	*lpl2123*	*lpc1663*	*lpa3157*	*lpw23811*	–	–	–	–	–	*cegC4*	Unknown
lpg2200	*lpp2150*	*lpl2124*	*lpc1664*	*lpa3158*	*lpw23821*	–	–	–	–	–	*cegC4*	Unknown
lpg2215	*lpp2166*	*lpl2140*	*lpc1680*	*lpa3179*	*lpw24011*	–	–	–	–	–	*legA2*	Ankyrin
lpg2216	*lpp2167*	*lpl2141*	*lpc1681*	*lpa3180*	*lpw24021*	–	–	–	–	–	*lem20*	Unknown
lpg2222	*lpp2174*	*lpl2147*	*lpc1689*	*lpa3191*	*lpw24081*	*llo1443*	*llb0208*	+	+	+	*lpnE*	Putative beta-lactamase (SEL1 domain)
lpg2223	*lpp2175*	*lpl2149**	*lpc1691*	*lpa3196*	*lpw24091*	–	–	–	–	–	–	Unknown
lpg2224	–	–	–	–	–	–	–	–	–	–	*ppgA*	Regulator of chromosome condensation
lpg2239	*lpp2192*	–	–	–	*lpw24261*	–	–	–	–	–	–	Unknown
lpg2248	*lpp2202*	*lpl2174*	*lpc1717*	*lpa3237*	*lpw24371*	–	–	–	–	–	*lem21*	Unknown
lpg2271	*lpp2225*	*lpl2197*	*lpc1740*	*lpa3268*	*lpw24611*	*llo2530*	*llb2821*	+	+	+	–	Unknown
lpg2298	*lpp2246*	*lpl2217*	*lpc1763*	*lpa3296*	*lpw24841*	*llo1707*	*llb3696*	+	+	+	*ylfA/legC7*	Coiled-coil
lpg2300	*lpp2248*	*lpl2219*	*lpc1765*	*lpa3298*	*lpw24871*	*llo0584*	*llb1266*	+	+	+	*ankH/ legA3, ankW*	Ankyrin, NfkappaB inhibitor
lpg2311	*lpp2259*	*lpl2230*	*lpc1776*	*lpa3312*	*lpw24981*	–	–	–	–	–	*ceg28*	Unknown
lpg2322	*lpp2270*	*lpl2242*	*lpc1789*	*lpa3328*	*lpw25121*	*llo0570*	*llb1282*	+	+	+	*ankK/legA5*	Ankyrin
lpg2327	*lpp2275*	*lpl2247*	*lpc1794*	*lpa3335*	*lpw25181*	–	–	–	–	–	–	Unknown
lpg2328	*lpp2276*	*lpl2248*	*lpc1795*	*lpa3336*	*lpw25191*	–	–	–	–	–	*lem22*	Unknown
lpg2344	*lpp2292*	*lpl2265*	*lpc1812*	*lpa3355*	*lpw25371*	–	–	–	–	–	*mavE*	Unknown
lpg2351	*lpp2300*	*lpl2273*	*lpc1820*	*lpa3367*	*lpw25461*	*llo2850*	*llb2466*	+	+	+	*mavF*	Unknown
lpg2359	*lpp2308*	*lpl2281*	*lpc1828*	*lpa3376*	*lpw25561*	*llo2856*	*llb2460*	+	+	+	–	Unknown
lpg2370	–	–	–	–	–	–	–	–	–	–	–	HipA fragment
lpg2372	*lpp3009*	–	*lpc3248*	*lpa4300*	–	–	–	–	–	–	–	Unknown
lpg2382	*lpp2444*	*lpl2300*	*lpc2108*	*lpa3446*	*lpw25841*	*llo1576*	*llb0071*	+	+	+	–	Unknown
lpg2391	*lpp2458*	*lpl2315*	*lpc2086*	*lpa3485*	*lpw26021*	–	–	–	–	–	*sdbC*	Unknown
lpg2392	*lpp2459*	*lpl2316*	*lpc2085*	*lpa3486*	*lpw26041*	–	–	–	–	–	*legL6*	LRR
lpg2400	–	*lpl2323*	–	–	*lpw26121*	–	–	–	–	–	*legL6*	LRR
lpg2406	*lpp2472*	*lpl2329*	*lpc2070*	*lpa3506*	*lpw26191*	*llo2172*	*llb3225*	+	+	+	*lem23*	Unknown
lpg2407	*lpp2474*	–	*lpc2069*	*lpa3507*	–	–	–	–	–	–	–	Unknown
lpg2409	*lpp2476*	*lpl2332*	*lpc2067*	*lpa3511*	*lpw26241*	–	–	–	–	–	*ceg29*	Unknown
lpg2410	*lpp2479*	*lpl2334*	*lpc2065*	*lpa3513*	*lpw26261*	–	–	–	–	–	*vpdA*	Patatin domain
lpg2411	*lpp2480*	*lpl2335*	*lpc2064*	*lpa3515*	*lpw26281*	*llo2227*	*llb3158*	+	+	+	*lem24*	Unknown
–	*lpp2486*	–	–	–	–				–	–	–	F-box
lpg2416	–	*lpl2339*	*lpc2057*	*lpa3527*	*lpw26351*	–	–	–	–	–	*legA1*	Unknown
lpg2420	–	*lpl2343*	*lpc2056*	*lpa3529*	*lpw26391*	–	–	–	–	–	–	Unknown
lpg2422	*lpp2487*	*lpl2345*	*lpc2055*	*lpa3530*	*lpw26401*	*llo1650*	*llb3763/6*	+	+	+	*lem25*	Unknown
lpg2424	*lpp2489*	*lpl2347*	*lpc2053*	*lpa3532*	*lpw26421*	–	–	–	–	–	*mavG*	Unknown
lpg2425	*lpp2491*	*lpl2348*	*lpc2051*	*lpa3537*	*lpw26431*	–	–	–	–	–	*mavH*	Unknown
lpg2433	*lpp2500*	*lpl2353*	*lpc2043*	*lpa3548*	*lpw26521*	–	–	–	–	–	*ceg30*	Unknown
lpg2434	*lpp2501*	*lpl2355*	*lpc2042*	*lpa3550*	*lpw26531*	–	–	–	–	–	–	Unknown
lpg2443	*lpp2510*	*lpl2363*	*lpc2033*	*lpa3562*	–	–	–	–	–	–	–	Unknown
lpg2444	*lpp2511*	*lpl2364*	*lpc2032*	*lpa3563*	*lpw26641*	–	–	–	–	–	*mavI*	Unknown

(Continued)

Table 3 | Continued

L. pneumophila						*L. longbeachae*					Name	Product
Phila	**Paris**	**Lens**	**Corby**	**Alcoy**	**130b**	**NSW 150**	**D-4968**	**AT**	**98072**	**C-4E7**		
lpg2452	*lpp2517*	*lpl2370*	*lpc2026*	*lpa3574*	*lpw26701*	–	–	–	–	–	*ankF/legA14/ceg31*	Ankyrin
lpg2456	*lpp2522*	*lpl2375*	*lpc2020*	*lpa3583*	*lpw26751*	*llo0365*	*llb1493*	+	+	+	*ankD/legA15*	Ankyrin
lpg2461	*lpp2527*	*lpl2380*	*lpc2015*	*lpa3589*	*lpw26801*	*llo1991*	*llb3433*	+	+	+	–	Unknown
lpg2464	–	*lpl2384*	–	–	*lpw26851*	–	–	–	–	–	*sidM/drrA*	Unknown
lpg2465	–	*lpl2385*	–	–	*lpw26861*	–	–	–	–	–	*sidD*	Unknown
lpg2490	*lpp2555*	*lpl2411*	*lpc1987*	*lpa3628*	*lpw27131*	–	–	–	–	–	*lepB*	Coiled-coil, Rab1 GAP
lpg2482	*lpp2546*	*lpl2402*	*lpc1996*	*lpa3615*	*lpw27041*	–	–	–	–	–	*sdbB*	Unknown
lpg2498	*lpp2566*	*lpl2420*	*lpc1975*	*lpa3646*	*lpw27241*	–	–	–	–	–	*mavJ*	Unknown
lpg2504	*lpp2572*	*lpl2426*	*lpc1967*	*lpa3658*	*lpw27301*	*llo2525*	*llb2826*	+	+	+	*sidI/ceg32*	Unknown
lpg2505	*lpp2573*	*lpl2427*	*lpc1966*	*lpa3659*	*lpw27311*	*llo2526*	*llb2825*	+	+	+	–	Unknown
lpg2508	*lpp2576*	*lpl2430*	*lpc1962/63**	*lpa3666*	*lpw27341*	–	–	–	–	–	*sdjA*	Unknown
lpg2509	*lpp2577*	*lpl2431*	*lpc1961*	*lpa3667*	*lpw27351*	*llo3097*	*llb2278*	+	+	+	*sdeD*	Unknown
lpg2510	*lpp2578*	*lpl2432*	*lpc1960*	*lpa3668*	–	*llo3098*	*llb2276*	+	+	+	*sdcA*	Unknown
lpg2511	*lpp2579*	*lpl2433*	*lpc1959*	*lpa3669*	*lpw27371*	–	–	–	–	–	*sidC*	PI(4)P binding domain
lpg2523	–	–	–	–	*lpw27501*	–	–	–	–	–	*lem26*	Unknown
lpg2525	–	–	–	–	–	–	–	–	–	–	*mavK*	Unknown
lpg2526	*lpp2591*	*lpl2446*	*lpc1946*	*lpa3687*	*lpw27521*	–	–	–	–	–	*mavL*	Unknown
lpg2527	*lpp2592*	*lpl2447*	*lpc1944*	*lpa3688*	*lpw27531*	*llo3335*	*llb2002*	+	+	+	–	Unknown
lpg2529	*lpp2594*	*lpl2449*	*lpc1942*	*lpa3692*	*lpw27551*	*llo2238*	*llb3146*	+	+	+	*lem27*	Unknown
lpg2538	*lpp2604*	*lpl2459*	*lpc1930*	*lpa3706*	*lpw27671*	–	–	–	–	–	–	Unknown
lpg2539	*lpp2605*	*lpl2460*	*lpc1929*	*lpa3707*	*lpw27681*	*llo1348*	*llb0317*	+	+	+	–	Unknown
lpg2541	*lpp2607*	*lpl2462*	*lpc1927*	*lpa3710*	*lpw27701*	–	–	–	–	–	–	Unknown
lpg2546	*lpp2615*	–	*lpc1919*	*lpa3727*	*lpw27791*	–	–	–	–	–	–	Unknown
lpg2552	*lpp2622*	*lpl2473*	*lpc1911*	*lpa3738*	*lpw27871*	*llo1062*	*llb0648*	+	+	+	–	Unknown
lpg2555	*lpp2625*	*lpl2480*	*lpc1908*	*lpa3743*	*lpw27901*	*llo2220*	*llb3170*	+	+	+	–	Unknown
lpg2556	*lpp2626*	*lpl2481*	*lpc1906*	*lpa3745*	*lpw27911*	*llo2218*	*llb3172*	+	+	+	*legK3*	STPK
lpg2577	*lpp2629*	*lpl2499*	*lpc0570*	*lpa3768*	*lpw28241*	–	–	–	–	–	*mavM*	Unknown
lpg2584	*lpp2637*	*lpl2507*	*lpc0561*	*lpa3779*	*lpw28321*	–	–	–	–	–	*sidF*	Unknown
lpg2588	*lpp2641*	*lpl2511*	*lpc0557*	*lpa3784*	*lpw28361*	*llo2622*	*llb2718*	+	+	+	*legS1*	Unknown
lpg2591	*lpp2644*	*lpl2514*	*lpc0551*	*lpa3790*	*lpw28391*	*llo0626*	*llb1219*	+	+	+	*ceg33*	Unknown
lpg2603	*lpp2656*	*lpl2526*	*lpc0539*	*lpa3807*	*lpw28521*	–	–	–	–	–	*lem28*	Unknown
lpg2628	*lpp2681*	*lpl2553*	*lpc0513*	*lpa3846*	*lpw28781*	–	–	–	–	–	–	Unknown
lpg2637	*lpp2690*	*lpl2562*	*lpc0503*	*lpa3859*	*lpw28871*	–	–	–	–	–	–	Unknown
lpg2638	*lpp2691*	*lpl2563*	*lpc0502*	*lpa3861*	*lpw28891*	*llo2645*	*llb2690*	+	+	+	*mavV*	Unknown
lpg2692	*lpp2746*	*lpl2619*	*lpc0444*	*lpa3929*	*lpw29461*	–	–	–	–	–	–	Unknown
lpg2694	*lpp2748*	*lpl2621*	*lpc0442*	*lpa3931*	*lpw29481*	–	–	–	–	–	*legD1*	Phyhd1 protein
lpg2718	*lpp2775*	*lpl2646*	*lpc0415*	*lpa3966*	*lpw29771*	–	–	–	–	–	*wipA*	Unknown
lpg2720	*lpp2777*	*lpl2648*	*lpc0413*	*lpa3968*	*lpw29791*	–	–	–	–	–	*legN*	cAMP-binding protein
lpg2744	*lpp2800*	*lpl2669*	*lpc0386*	*lpa4004*	*lpw30031*	–	–	–	–	–	–	Unknown
lpg2745	*lpp2801*	*lpl2670*	*lpc0385*	*lpa4005*	*lpw30041*	*llo0308*	*llb1553*	+	+	+	–	Unknown
lpg2793	*lpp2839*	*lpl2708*	*lpc3079*	*lpa4063*	*lpw30471*	–	–	–	–	–	*lepA*	Effector protein A
lpg2804	*lpp2850*	*lpl2719*	*lpc3090*	*lpa4076*	*lpw30591*	*llo0267*	*llb1598*	+	+	+	*lem29*	Unknown
lpg2815	*lpp2867*	*lpl2730*	*lpc3101*	*lpa4089*	*lpw30711*	*llo0254*	*llb1612*	+	+	+	*mavN*	Unknown
lpg2826	–	*lpl2741*	*lpc3113*	*lpa4104*	*lpw30831*	–	–	–	–	–	*ceg34*	Unknown
lpg2828	*lpp2882*	*lpl2743*	*lpc3115*	*lpa4109*	*lpw30851*	*llo0783*	*llb0944*	+	+	+	–	Unknown
lpg2829	*lpp2883/86**	–	–	–	*lpw30861*	–	–	–	–	–	*sidH*	Unknown
lpg2830	*lpp2887*	–	–	–	*lpw30881*	–	–	–	–	–	*lubX/legU2*	U-box motif
lpg2831	*lpp2888*	–	–	–	*lpw30891*	–	–	–	–	–	*VipD*	Patatin-like phopholipase

(Continued)

Table 3 | Continued

L. pneumophila						*L. longbeachae*					Name	Product
Phila	**Paris**	**Lens**	**Corby**	**Alcoy**	**130b**	**NSW 150**	**D-4968**	**AT**	**98072**	**C-4E7**		
lpg2832	*lpp2889*	*lpl2744*	*lpc3116*	*lpa4110*	*lpw30921*	*llo0214*	*llb1656*	+	+	+	–	Putative hydrolase
lpg2844	*lpp2903*	*lpl2756*	*lpc3128*	*lpa4133*	–	–	–	–	–	–	–	Unknown
lpg2862	–	–	–	–	–	–	–	–	–	–	*Lgt2/legC8*	Coiled-coil
lpg2874	*lpp2933*	*lpl2787*	*lpc3160*	*lpa4176*	*lpw31411*	–	–	–	–	–	–	Unknown
lpg2879	*lpp2938*	*lpl2792*	*lpc3165*	*lpa4186*	*lpw31471*	*llo0192*	*llb1681*	+	+	+	–	Unknown
lpg2884	*lpp2943*	*lpl2797*	*lpc3170*	*lpa4193*	*lpw31531*	*llo0197*	*llb1676*	+	+	+	–	Unknown
lpg2885	*lpp2944*	*lpl2798*	*lpc3171*	–	*lpw31541*	–	–	–	–	–	–	Unknown
lpg2888	*lpp2947*	*lpl2801*	*lpc3174*	*lpa4199*	*lpw31571*	*llo0200*	*llb1672*	+	+	+	–	Unknown
lpg2912	*lpp2980*	*lpl2830*	*lpc3214*	*lpa4255*	*lpw31931*	–	–	–	–	–	–	Unknown
lpg2936	*lpp3004*	*lpl2865*	*lpc3243*	*lpa4293*	*lpw32251*	*llo0081*	*llb1804*	+	+	+	–	rRNA small subunit methyltransferase E
lpg2975	*lpp3047*	*lpl2904*	*lpc3290*	*lpa4358*	–?	*llo3405*	*llb1930*	+	+	+	–	Unknown
lpg2999	*lpp3071*	*lpl2927*	*lpc3315*	*lpa4395*	*lpw32851*	–	–	–	–	–	*legP*	Astacin protease
lpg3000	*lpp3072*	*lpl2928*	*lpc3316*	*lpa4397*	*lpw32861*	*llo3444*	*llb1887*	+	+	+	–	Unknown

*List of substrates is based on Isberg et al. (2009), De Felipe et al. (2008), Ninio et al. (2009), Zhu et al. (2011); AT = ATCC33462; *pseudogene, +? or –? strains 130b, C-4E7 and 98072 are not a finished sequence and not manually curated. Thus absence of a substrate can also be due to gaps in the sequence; shaded in gray, substrates conserved in all L. pneumophila and L. longbeachae genomes.*

sphingosine-1-phosphate lyase and sphingosine kinase, eukaryotic like glycoamylase, cytokinin oxidase, zinc metalloprotease, or an RNA binding precursor (Cazalet et al., 2004; De Felipe et al., 2005; Bruggemann et al., 2006). Function prediction based on similarity searches suggested that many of these proteins are implicated in modulating host cell functions to the pathogens advantage (Cazalet et al., 2004). Recent functional studies confirm these predictions.

As a first example, it was shown that *L. pneumophila* is able to interfere with the host ubiquitination pathway. The *L. pneumophila* U-box containing protein LubX was shown to be a secreted effector of the Dot/Icm secretion system that mediates polyubiquitination of a host kinase Clk1 (Kubori et al., 2008). Recently, LubX was described as the first example of an effector protein, which targets and regulates another effector within host cells, as it functions as an E3 ubiquitin ligase that hijacks the host proteasome to specifically target the bacterial effector protein SidH for degradation. Delayed delivery of LubX to the host cytoplasm leads to the shutdown of SidH within the host cells at later stages of infection. This demonstrates a sophisticated level of co-evolution between eukaryotic cells and *L. pneumophila* involving an effector that functions as a key regulator to temporally coordinate the function of a cognate effector protein (Kubori et al., 2010; Luo, 2011). Furthermore, AnkB/Lpp2028, one of the three F-box proteins of *L. pneumophila*, was shown to be a T4SS effector that is implicated in virulence of *L. pneumophila* and in recruiting ubiquitinated proteins to the LCV (Al-Khodor et al., 2008; Price et al., 2009; Habyarimana et al., 2010; Lomma et al., 2010).

A second example is the apyrases (Lpg1905 and Lpg0971) encoded in the *L. pneumophila* genomes. Indeed, both are secreted enzymes important for intracellular replication of *L. pneumophila*. Lpg1905 is a novel prokaryotic ecto-NTPDase, similar to CD39/NTPDase1, which is characterized by the presence of five apyrase-conserved regions and enhances the replication of *L. pneumophila* in eukaryotic cells (Sansom et al., 2007). Apart from ATP and ADP, Lpg1905 also cleaves GTP and GDP with similar efficiency to ATP and ADP, respectively (Sansom et al., 2008). A third example is a *L. pneumophila* homolog of the highly conserved eukaryotic enzyme sphingosine-1-phosphate lyase (Spl). In eukaryotes, SPL is an enzyme that catalyzes the irreversible cleavage of sphingosine-1-phosphate (S1P). S1P is implicated in various physiological processes like cell survival, apoptosis, proliferation, migration, differentiation, platelet aggregation, angiogenesis, lymphocyte trafficking and development. Despite the fact that the function of the *L. pneumophila* Spl remains actually unknown, the hypothesis is that it plays a role in autophagy and/or apoptosis (Cazalet et al., 2004; Bruggemann et al., 2006). Recently it has been shown that the *L. pneumophila* Spl is a secreted effector of the Dot/Icm T4SS, that it is able to complement the sphingosine-sensitive phenotype of *Saccharomyces cerevisiae*. Moreover, *L. pneumophila* Spl co-localizes to the host cell mitochondria (Degtyar et al., 2009).

Taken together, the many different functional studies undertaken based on the results of the genome sequence analyses deciphering the roles of the eukaryotic like proteins have clearly established that they are secreted virulence factors that are involved in host cell adhesion, formation of the LCV, modulation of host cell functions, induction of apoptosis and egress of *Legionella* (Nora et al., 2009; Hubber and Roy, 2010). Most of these effector proteins are expressed at different stages of the intracellular life cycle of *L. pneumophila* (Bruggemann et al., 2006) and are delivered to the host cell by the Dot/Icm T4SS. Thus molecular mimicry of eukaryotic proteins is a major virulence strategy of *L. pneumophila*.

As expected, eukaryotic like proteins and proteins encoding domains mainly found in eukaryotic proteins are also present in the *L. longbeachae* genomes. However, between the two species a

considerable diversity in the repertoire of these proteins exists. For example Spl, LubX, the three *L. pneumophila* F-box proteins, and the homolog of one (Lpg1905) of the two apyrases are missing in all sequenced *L. longbeachae* genomes. In contrast a glycoamylase (Herrmann et al., 2011) and an uridine kinase homolog are present also in *L. longbeachae* (Cazalet et al., 2010; Kozak et al., 2010; **Table 3**). However, other proteins encoded by the *L. longbeachae* genome contain U-box and F-box domains and might therefore fulfill similar functions. Thus, although the specific proteins may not be conserved, the eukaryotic like protein–protein interaction domains found in *L. pneumophila* are also present in *L. longbeachae*.

The differences in trafficking between *L. longbeachae* and *L. pneumophila* mentioned above might be related to specific effectors encoded by *L. longbeachae*. A search for such specific putative effectors of *L. longbeachae* identified several proteins that might contribute to these differences like a family of Ras-related small GTPases (Cazalet et al., 2010; Kozak et al., 2010). These proteins may be involved in vesicular trafficking and thus may account at least partly for the specificities of the *L. longbeachae* life cycle. *L. pneumophila* is also known to exploit monophosphorylated host phosphoinositides (PI) to anchor the effector proteins SidC, SidM/DrrA, LpnE, and LidA to the membrane of the replication vacuole (Machner and Isberg, 2006; Murata et al., 2006; Weber et al., 2006, 2009; Newton et al., 2007; Brombacher et al., 2009). *L. longbeachae* may employ an additional strategy to interfere with the host PI as a homolog of the mammalian PI metabolizing enzyme phosphatidylinositol-4-phosphate 5-kinase was identified in its genome. One could speculate that this protein allows direct modulation of the host cell PI levels.

Interestingly, although 23 of the 29 ankyrin proteins identified in the *L. pneumophila* strains are absent from the *L. longbeachae* genome, *L. longbeachae* encodes a total of 23 specific ankyrin repeat proteins (**Table 3**). For example, *L. pneumophila* AnkX/AnkN that was shown to interfere with microtubule-dependent vesicular transport is missing in *L. longbeachae* (Pan et al., 2008). However, *L. longbeachae* encodes a putative tubulin–tyrosine ligase (TTL). TTL catalyzes the ATP-dependent post-translational addition of a tyrosine to the carboxy terminal end of detyrosinated alpha-tubulin. Although the exact physiological function of alpha-tubulin has so far not been established, it has been linked to altered microtubule structure and function (Eiserich et al., 1999). Thus this protein might take over this function in *L. longbeachae*.

Legionella longbeachae is the first bacterial genome encoding a protein containing an Src Homology 2 (SH2) domain. SH2 domains, in eukaryotes, have regulatory functions in various intracellular signaling cascades. Furthermore, *L. longbeachae* encodes two proteins with pentatricopeptide repeat (PPR) domains. This family seems to be greatly expanded in plants, where they appear to play essential roles in organellar RNA metabolism (Lurin et al., 2004; Nakamura et al., 2004; Schmitz-Linneweber and Small, 2008). Only 12 bacterial PPR domain proteins have been identified to date, all encoded by two species, the plant pathogens *Ralstonia solanacearum* and the facultative photosynthetic bacterium *Rhodobacter sphaeroides*. Thus, genome analysis revealed a particular feature of the *Legionella* genomes, the presence of many eukaryotic like proteins and protein domains, some of which are common to the two *Legionella* species, others which are specific and may thus account for the species specific features in intracellular trafficking and niche adaptation in the environment.

SURFACE STRUCTURES – A CLUE TO MOUSE SUSCEPTIBILITY TO INFECTION WITH *LEGIONELLA*

Despite the presence of many different species of *Legionella* in aquatic reservoirs, the vast majority of human disease is caused by a single serogroup (Sg) of a single species, namely *L. pneumophila* Sg1, which is responsible for about 84% of all cases worldwide (Yu et al., 2002). Similar results are obtained for *L. longbeachae*. Two serogroups are described, but *L. longbeachae* Sg1 is predominant in human disease. Lipopolysaccharide (LPS) is the basis for the classification of serogroups but it is also a major immunodominant antigen of *L. pneumophila* and *L. longbeachae*. Interestingly, it has also been shown that membrane vesicles shed by virulent *L. pneumophila* containing LPS are sufficient to inhibit phagosome–lysosome fusion (Fernandez-Moreira et al., 2006). Results obtained from large-scale genome comparisons of *L. pneumophila* suggested that LPS of Sg1 itself might be implicated in the predominance of Sg1 strains in human disease compared to other serogroups of *L. pneumophila* and other *Legionella* species (Cazalet et al., 2008). A comparative search for LPS coding regions in the genome of *L. longbeachae* NSW 150 identified two gene clusters encoding proteins that could be involved in production of lipopolysaccharide (LPS) and/or capsule. Neither shared homology with the *L. pneumophila* LPS biosynthesis gene cluster suggesting considerable differences in this major immunodominant antigen between the two *Legionella* species. However, homologs of *L. pneumophila* lipidA biosynthesis genes (LpxA, LpxB, LpxD, and WaaM) are present. Electron microscopy also demonstrated that, in contrast to *L. pneumophila*, *L. longbeachae* produces a capsule-like structure, suggesting that one of the aforementioned gene cluster encodes LPS and the other the capsule (Cazalet et al., 2010).

As mentioned in the introduction, only A/J mice are permissive for replication of *L. pneumophila*, in contrast A/J, C57BL/6, and BALB/c mice are all permissive for replication of *L. longbeachae*. In C57BL/6 mice cytosolic flagellin of *L. pneumophila* triggers Naip5-dependent caspase-1 activation and subsequent proinflammatory cell death by pyroptosis rendering them resistant to infection (Diez et al., 2003; Wright et al., 2003; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Lamkanfi et al., 2007; Lightfield et al., 2008). Genome analysis shed light on the reasons for these differences. *L. longbeachae* does not carry any flagellar biosynthesis genes except the sigma factor FliA, the regulator FleN, the two-component system FleR/FleS and the flagellar basal body rod modification protein FlgD (Cazalet et al., 2010; Kozak et al., 2010). Analysis of the genome sequences of strains *L. longbeachae* D-4968, ATCC33642, 98072, and C-4E7 as well as a PCR-based screening of 50 *L. longbeachae* isolates belonging to both serogroups by Kozak et al. (2010) and of 15 additional isolates by Cazalet et al. (2010) did not detect flagellar genes in any isolate confirming that *L. longbeachae*, in contrast to *L. pneumophila* does not synthesize flagella. Interestingly, all genes bordering flagellar gene clusters are conserved between *L. longbeachae* and *L. pneumophila*, suggesting deletion of these regions from the *L. longbeachae* genome. This

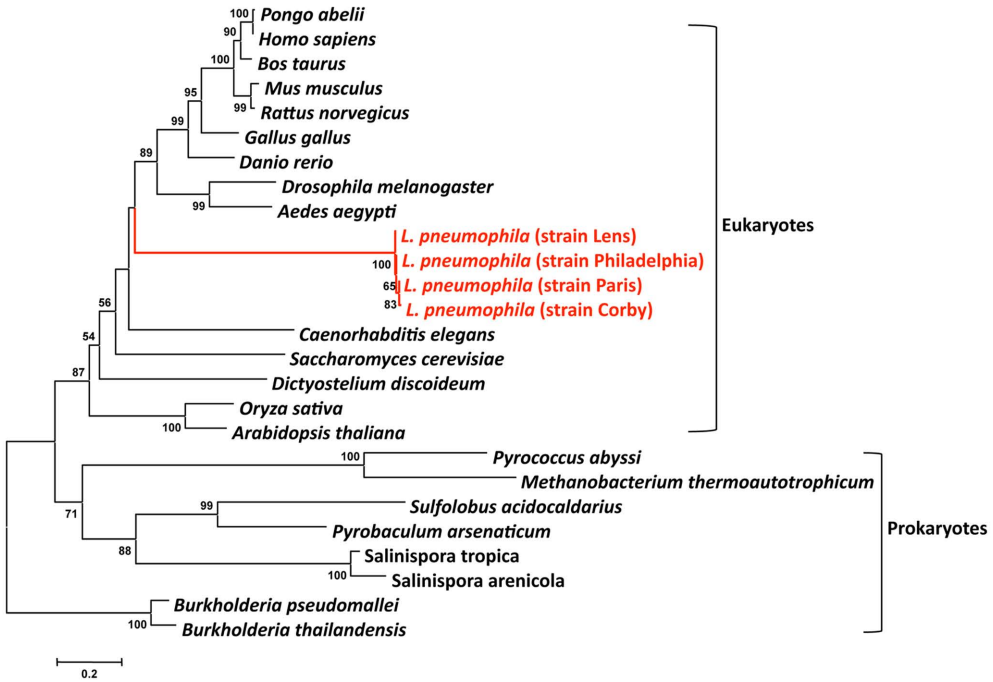

FIGURE 4 | Phylogenetic tree of a multiple sequence comparison of sphingosine-phosphate lyase proteins present in eukaryotic and prokaryotic genomes. Phylogenetic reconstruction was done with MEGA using the Neighbor-Joining method. Numbers indicate bootstrap values after 1000 bootstrap replicates. The red lines indicate the *L. pneumophila* sequences that are embedded in the eukaryotic clade. The bar at the bottom represents the estimated evolutionary distance.

result suggests, that *L. longbeachae* fails to activate caspase-1 due to the lack of flagellin, which may also partly explain the differences in mouse susceptibility to *L. pneumophila* and *L. longbeachae* infection. The putative *L. longbeachae* capsule may also contribute to this difference.

Quite interestingly, although *L. longbeachae* does not encode flagella, it encodes a putative chemotaxis system. Chemotaxis enables bacteria to find favorable conditions by migrating toward higher concentrations of attractants. In many bacteria, the chemotactic response is mediated by a two-component signal transduction pathway, comprising a histidine kinase CheA and a response regulator CheY. Homologs of this regulatory system are present in the *L. longbeachae* genomes sequenced (Cazalet et al., 2010; Kozak et al., 2010). Furthermore, two homologs of the "adaptor" protein CheW that associate with CheA or cytoplasmic chemosensory receptors are present. Ligand-binding to receptors regulates the autophosphorylation activity of CheA in these complexes. The CheA phosphoryl group is subsequently transferred to CheY, which then diffuses away to the flagellum where it modulates motor rotation. Adaptation to continuous stimulation is mediated by a methyltransferase CheR. Together, these proteins represent an evolutionarily conserved core of the chemotaxis pathway, common to many bacteria and archea (Kentner and Sourjik, 2006; Hazelbauer et al., 2008). Homologs of all these proteins are present in the *L. longbeachae* genomes (Cazalet et al., 2010; Kozak et al., 2010) and a similar chemotaxis system is present in *Legionella drancourtii* LLAP12 (La Scola et al., 2004) but it is absent from *L. pneumophila*. The flanking genomic regions are highly conserved among *L. longbeachae* and all *L. pneumophila* strains sequenced, suggesting that *L. pneumophila*, although it encodes flagella has lost the chemotaxis system encoding genes by deletion events.

Thus these two species differ markedly in their surface structures. *L. longbeachae* encodes a capsule-like structure, synthesizes a very different LPS, does not synthesize flagella but encodes a chemotaxis system. These differences in surface structures seem to be due to deletion events leading to the loss of flagella in *L. longbeachae* and the loss of chemotaxis in *L. pneumophila* leading in part to the adaptation to their different main niches, soil, and water.

EVOLUTION OF EUKARYOTIC EFFECTORS – ACQUISITION BY HORIZONTAL GENE TRANSFER FROM EUKARYOTES?

Human to human transmission of *Legionella* has never been reported. Thus humans have been inconsequential in the evolution of these bacteria. However, *Legionella* have co-evolved with freshwater protozoa allowing the adaptation to eukaryotic cells. The idea that protozoa are training grounds for intracellular pathogens was born with the finding by Rowbotham (1980) that *Legionella* has the ability to multiply intracellularly. This lead to a new percept in microbiology: bacteria parasitize protozoa and can utilize the same process to infect humans. Indeed, the long co-evolution of *Legionella* with protozoa is reflected in its genome by the presence of eukaryotic like genes, many of which are clearly virulence factors used by *L. pneumophila* to subvert host functions. These genes may have been acquired either through horizontal gene transfer (HGT) from the host cells (e.g., aquatic protozoa) or from bacteria or may have evolved by convergent evolution. Recently it has

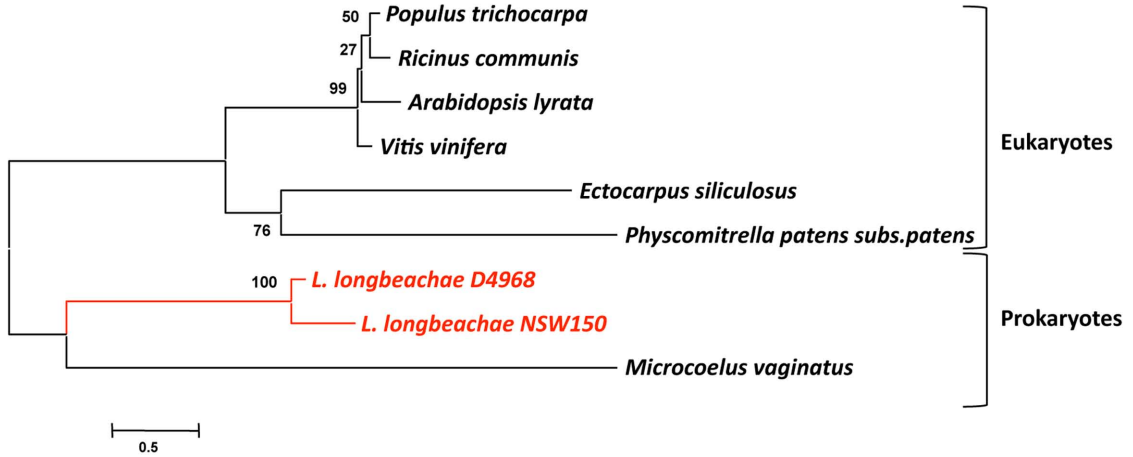

FIGURE 5 | Phylogenetic tree of the protein Llo2643 and their homologs after blastp search. The tree was constructed by the Neighbor-joining method using the program MEGA. The red lines indicate the *L. longbeachae* sequences that are close to sequences derived from plant genomes. Numbers indicate bootstrap support for nodes from 1000 NJ bootstrap replicates. The bar at the bottom represents the estimated evolutionary distance.

been reported that *L. drancourtii* a relative of *L. pneumophila* has acquired a sterol reductase gene from the *Acanthamoeba polyphaga Mimivirus* genome, a virus that grows in ameba (Moliner et al., 2009). Thus, the acquisition of some of the eukaryotic like genes of *L. pneumophila* by HGT from protozoa is plausible. *ralF* was the first gene suggested to have been acquired by *L. pneumophila* from eukaryotes by HGT, as RalF carries a eukaryotic Sec 7 domain (Nagai et al., 2002). In order to study the evolutionary origin of eukaryotic *L. pneumophila* genes, we have undertaken a phylogenetic analysis of the eukaryote-like sphingosine-1-phosphate lyase of *L. pneumophila* that is encoded by *lpp2128* described earlier. The phylogenetic analyses shown in **Figure 4** revealed that it was most likely acquired from a eukaryotic organism early during *Legionella* evolution (Degtyar et al., 2009; Nora et al., 2009) as the Lpp2128 protein sequence of *L. pneumophila* clearly falls into the eukaryotic clade of SPL sequences.

We then tested the hypothesis that *L. longbeachae* might have acquired genes also from plants, which is conceivable as it is found in soil. We thus undertook here a phylogenetic analysis similar to that described above for the *L. longbeachae* protein Llo2643 that contains PPR repeats, a protein family typically present in plants. A Blast search in the database revealed that homologs of Llo2643 are only found in eukaryotes, in particular in plants and algae. The only prokaryotes encoding this protein are the cyanobacteria *Microcoelus vaginatus* and *Cylindrospermopsis rasiborskii*. This rare presence in bacteria is suggestive of a horizontal transfer event from eukaryotes to these bacteria. **Figure 5** shows the phylogenetic tree we obtained. The fact that the bacterial proteins group together may also be due to a phenomenon of long branch attraction. Thus, the Llo2643 protein of *L. longbeachae* appears closer to plant proteins than prokaryotic ones. Once more plant proteins, perhaps from algae, will be in the database, it might become possible to evaluate whether *L. longbeachae* indeed acquired genes from plants.

Legionella is not the only prokaryote whose genome shows an enrichment of proteins with eukaryotic domains. Another example is the genome of "*Ca. Amoebophilus asiaticus*" a Gram-negative, obligate intracellular ameba symbiont belonging to the *Bacteroidetes*, which has been discovered within an ameba isolated from lake sediment (Schmitz-Esser et al., 2008) has been reported (Schmitz-Esser et al., 2010). In a recent report Schmitz-Esser et al. (2010) show that the genome of this organism also encodes an arsenal of proteins with eukaryotic domains. To further investigate the distribution of these protein domains in other bacteria the authors have undertaken an enrichment analysis comparing the fraction of all functional protein domains among 514 bacterial proteomes (Schmitz-Esser et al., 2010). This showed that the genomes of bacteria for which the replication in ameba has been demonstrated were enriched in protein domains that are predominantly found in eukaryotic proteins. Interestingly, the domains potentially involved in host cell interaction described above, such as ANK repeats, LRR, SEL1 repeats, and F- and U-box domains, are among the most highly enriched domains in proteomes of ameba-associated bacteria. Bacteria that can exploit amebae as hosts thus share a set of eukaryotic domains important for host cell interaction despite their different lifestyles and their large phylogenetic diversity. This suggests that bacteria thriving within ameba use similar mechanisms for host cell interaction to facilitate survival in the host cell. Due to the phylogenetic diversity of these bacteria, it is most likely that these traits were acquired independently during evolutionary early interaction with ancient protozoa.

CONCLUSION

Legionella pneumophila and *L. longbeachae* are two human pathogens that are able to modulate, manipulate, and subvert many eukaryotic host cell functions to their advantage, in order to enter, replicate, and evade protozoa or human alveolar macrophages during disease. In the last years genome analyses, as well as comparative and functional genomics have demonstrated that genome plasticity plays a major role in differences in host cell exploitation and niche adaptation of *Legionella*. The genomes of these environmental pathogens are shaped by HGT between

eukaryotes and prokaryotes, allowing them to mimic host cell functions and to exploit host cell pathways. Genome plasticity and HGT lead in each strain and species to a different repertoire of secreted effectors that may allow subtle adaptations to, e.g., different protozoan hosts. Plasmids can be exchanged among strains and phages and deletions of surface structures like flagella or chemotaxis systems has taken place. Thus genome plasticity is major mechanism by which *Legionella* may adapt to different niches and hosts.

Access to genomic data has revealed many potential virulence factors of *L. pneumophila* and *L. longbeachae* as well as metabolic capacities of these bacteria. The increasing information in the genomic database will allow a better identification of the origin and similarity of eukaryotic like proteins or eukaryotic protein domains and other virulence factors. New eukaryotic genomes like that of the natural host of *Legionella*, *A. castellanii* are in progress. These additional data will allow studying possible transfer events of genes from the eukaryotic host to *Legionella* more in depth. Taken together, the progressive increase of information on *Legionella* as well as on protozoa will allow more complete comparative and phylogenetic studies to shed light on the evolution of virulence in *Legionella*. However, much work remains to be done to translate the basic findings from genomics research into improved understanding of the biology of this organism. As data are accumulating, new fields of investigation will emerge. Without doubt the investigation and characterization of regulatory ncRNAs will be one such field. Manipulation of host-epigenetic information and investigating host susceptibility to disease will be another. In particular development of high throughput techniques for comparative and functional genomics as well as more and more powerful imaging techniques will accelerate the pace of knowledge acquisition.

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Virulence properties of the *Legionella pneumophila* cell envelope

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The bacterial envelope plays a crucial role in the pathogenesis of infectious diseases. In this review, we summarize the current knowledge of the structure and molecular composition of the *Legionella pneumophila* cell envelope. We describe lipopolysaccharides biosynthesis and the biological activities of membrane and periplasmic proteins and discuss their decisive functions during the pathogen–host interaction. In addition to adherence, invasion, and intracellular survival of *L. pneumophila*, special emphasis is laid on iron acquisition, detoxification, key elicitors of the immune response and the diverse functions of outer membrane vesicles. The critical analysis of the literature reveals that the dynamics and phenotypic plasticity of the *Legionella* cell surface during the different metabolic stages require more attention in the future.

Keywords: *Legionella pneumophila*, bacterial envelope, phospholipids, membrane proteins, LPS, outer membrane vesicles

Bacterial cell envelopes fulfill several basic functions: They protect the bacterium from environmental hazards, they allow a selective passage of nutrients into and a specific export of waste products and secretion system substrates out of the cell. Additionally, they mediate the direct contact with other organisms. This holds particularly true for pathogenic bacteria, whose often highly specific interactions with host organisms depend largely on their surface structures. Accordingly, the ability of the Gram-negative facultative intracellular bacterium *Legionella pneumophila* to cause Legionnaires' disease hinges predominantly on the components and characteristics of its cell envelope.

The cytoplasm of Gram-negative bacteria is bordered by the inner membrane. It consists of a bilayer of two phospholipid leaflets with integral and peripheral proteins and lipoproteins. It harbors metabolic enzymes, components of the respiratory chain and parts of the iron acquisition machinery (**Figure 1**).

The periplasm contains a relatively thin layer of peptidoglycan and different proteins. *Legionella* peptidoglycan is strongly crosslinked (Amano and Williams, 1983). The periplasm is the location of many detoxifying enzymes which degrade harmful substances from the environment. Secretion machineries which cross two membranes also go through the periplasmic space.

The outer membrane is asymmetric with an inner leaflet of mostly phospholipids and an outer leaflet of mostly lipopolysaccharides (LPS). It harbors proteins with diverse functions in virulence such as adhesion and uptake into host cells. *Legionella* LPS has a unique architecture, particularly concerning the hydrophobic O-antigen.

Certain types of surface appendages such as pili and flagella, which are required for bacterial motility and pathogenicity, are anchored in the inner membrane and protrude into the extracellular space (Liles et al., 1998; Stone and Abu Kwaik, 1998; Heuner and Steinert, 2003).

Virulence properties of outer membrane components are particularly important in regard to outer membrane vesicles (OMVs). Like most bacteria, *L. pneumophila* sheds these vesicles from its outer membrane. OMVs are spherical lipid bilayers and contain outer membrane components and periplasmic proteins.

The actual structure of the *L. pneumophila* cell envelope was assessed in detail by electron microscopy shortly after the discovery of the bacterium (Rodgers and Davey, 1982). Both membranes and the peptidoglycan layer were visualized by different methods, resulting in vivid images of the components that are, nowadays, analyzed mostly biochemically. The authors are also the first to show the existence of OMVs of *L. pneumophila*, even though they are termed "blebs" and explained as "condensed pili-related proteins or random structural proteins of the outer membranes." An extensive study of *L. pneumophila* morphology including envelope architecture was performed by Faulkner and Garduño (2002). They hypothesize the existence of several morphological variants, each corresponding to a certain growth phase or stage of the infection cycle. Interestingly, five different envelope structures are presented which vary in thickness, number of membrane layers, and electron density of individual components. As some of the morphological variants only occurred during intracellular growth, the authors propose that the development of these variants depends on host metabolites. This notion can explain the absence of these forms during extracellular growth in liquid media. The impact of processing artifacts arising during the preparation of the samples, however, remains to be clarified.

Many secretion systems and outer membrane proteins with roles in virulence have been excellently reviewed elsewhere and are not within the focus of this work. This includes T1SS and twin-arginine translocation (Tat) secretion (Lammertyn and Anne, 2004), T2SS (Cianciotto, 2009), T4SS as well as their respective translocated effectors (Ninio and Roy, 2007). Finally, secreted phospholipases connect *Legionella* virulence to host lipids (Banerji et al., 2008).

Less attention was paid to other components of the *Legionella* cell envelope which are not part of the aforementioned complexes. This review concentrates on these envelope components and how they mediate *Legionella* virulence properties.

THE INNER MEMBRANE OF *L. PNEUMOPHILA*

Starting from the inside and proceeding outward, the first layer is the inner membrane, also termed cytoplasmic or plasma membrane. It is a lipid bilayer with integrated components of various systems, including the iron uptake machinery, the respiratory chain, and the detoxification system (**Table 1**).

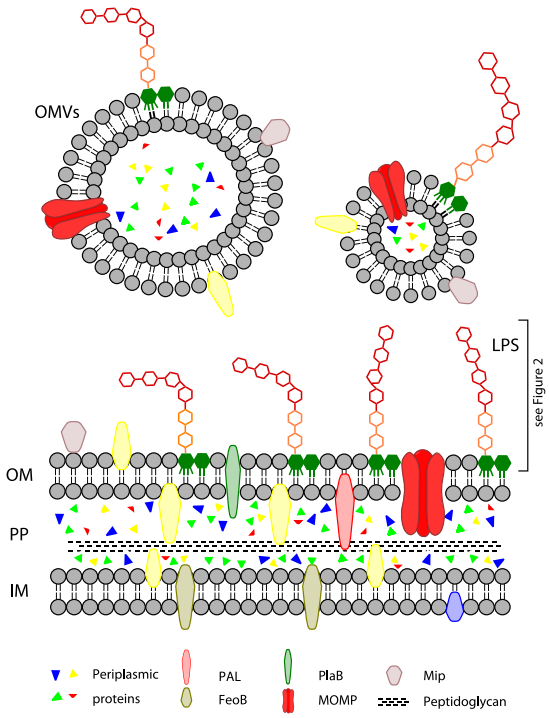

FIGURE 1 | Overview of the *L. pneumophila* cell envelope. CP, cytoplasm; IM, inner membrane; PP, periplasm; OM, outer membrane; OMVs, outer membrane vesicles; LPS, lipopolysaccharides; PAL, peptidoglycan-associated lipoprotein; FeoB, iron transporter; PlaB, phospholipase A/lysophospholipase A; MOMP, major outer membrane protein; Mip, macrophage infectivity potentiator.

The lipid composition of a crude inner membrane preparation of *L. pneumophila* was analyzed shortly after the discovery of the bacteria (Hindahl and Iglewski, 1984). They described it to contain mainly phosphatidylethanolamine and phosphatidylcholine with smaller amounts of cardiolipin and phosphatidylglycerol. Contamination with outer membrane components cannot be excluded due to methodical reasons. Thus, these data should be interpreted carefully.

An important function of the inner membrane of *L. pneumophila* is the regulation of iron acquisition, summarized elsewhere (Cianciotto, 2007). Iron uptake is a crucial process during all phases of *L. pneumophila* growth. It is mainly carried out by the GTP-dependent iron transporter FeoB, which mediates the uptake of Fe(II) (Robey and Cianciotto, 2002; Petermann et al., 2010). The protein is required for optimal growth under iron-limiting conditions in liquid media as well as in iron-restricted amoeba and macrophages. FeoB is required for efficient killing of macrophages and full virulence in a mouse model of Legionnaires' disease.

Another iron acquisition mechanism involves the proteins IraA and IraB. IraA is described as a small-molecule methyltransferase. It mediates iron uptake and is required for the infection of human macrophages and guinea pigs. IraB is an integral protein of the inner membrane with homology to bacterial peptide transporters. It is suggested to mediate the uptake of iron ions into the cell, possibly chelated by small peptides. The potential significance of the *iraAB* locus for virulence is underlined by the fact that it is found almost exclusively in pathogenic *Legionella,* but not in avirulent species (Viswanathan et al., 2000).

The multi-copper oxidase MCO is tethered to the cytoplasmic membrane of *L. pneumophila*. Recently, this enzyme was suggested to oxidize ferrous iron, which could otherwise lead to the formation of hydroxyl radicals under aerobic conditions (Huston et al., 2008). An MCO-negative mutant displays normal intracellular replication within macrophages. Therefore the function of MCO seems to be limited to extracellular growth, during which the authors hypothesize it to be involved in the protection against iron-related oxidative stress.

Inner membrane proteins often regulate cytoplasmic processes such as gene expression and the synthesis of signal transduction molecules. One example of this is LadC, a putative adenylate cyclase. *L. pneumophila* expresses the corresponding gene exclusively during

Table 1 | Inner membrane proteins of *L. pneumophila* associated with virulence and survival.

Protein	Molecular function	Role in infection/required for	Reference
FeoB	GTP-dependent Fe(II) transporter	Macrophage killing, full virulence in mouse	Petermann et al. (2010), Robey and Cianciotto (2002)
IraA/IraB	Small-molecule methyl transferase/ peptide transporter	Iron uptake, infection of human macrophages, and guinea pigs	Viswanathan et al. (2000)
Multi-copper oxidase	Potential oxidation of ferrous iron	Extracellular replication	Huston et al. (2008)
LadC	Putative adenylate cyclase	Adhesion to macrophages, intracellular replication, putative modification of protein functions via cAMP	Newton et al. (2008)
TatB	T2S, additional function(s)	Intracellular replication in human macrophages, growth under iron-limiting conditions, cytochrome c-dependent respiration, export of PLC activity to supernatant	Rossier and Cianciotto (2005)

intracellular infection. Its importance for virulence is underlined by the finding that a LadC-negative mutant adheres to macrophages less effectively and replicates less within mammalian cells and amoeba. In contrast to most other bacterial adenylate cyclases, LadC does not alter transcriptional profiles, so it is assumed that LadC produces cAMP which, in turn, modifies protein–protein interactions or regulates protein activities (Newton et al., 2008).

The inner membrane is crossed by the Tat complex which can be involved in type II secretion. One of the Tat components, TatB, was found to have additional, unexpected functions (Rossier and Cianciotto, 2005). Firstly, intracellular replication in human macrophages is impaired in the absence of TatB independently of type II secretion. Secondly, TatB-negative mutants are defective in growth under iron-limiting conditions, both extracellularly and within amoeba. Moreover, TatB of *L. pneumophila* is also required for cytochrome c-dependent respiration and finally for the export of a specific phospholipase C activity to the culture supernatant – possibly executed by PlcA.

The inner membrane is also the starting point of several secretion systems. The unique Dot/Icm system of *Legionella* has been reviewed very well elsewhere as has the type II secretion system (Cianciotto, 2009; Hubber and Roy, 2010).

In summary, the inner membrane of *L. pneumophila* influences virulence functions rather indirectly via the mediation of iron acquisition and other cellular processes such as protein secretion. The contribution of inner membrane components to virulence may emerge from enhanced survival under hostile conditions – and infection processes may just be an example for this. From this perspective, findings which relate survival factors to virulence may simply be due to the fact that host cells and tissues are examples of hostile environments to most bacteria.

PERIPLASM

The periplasmic space is a gel-like layer composed of soluble proteins and strongly crosslinked peptidoglycan located between the outer and inner membranes. It is enriched in proteases and nucleases and other degradative enzymes. Thus, the periplasm has been called an "evolutionary precursor of the lysosomes of eukaryotic cells" (Silhavy et al., 2010).

The presence of digestive enzymes was confirmed by recent *L. pneumophila* membrane proteome data. It has been shown that mainly enzymes were found in the periplasm, such as metalloproteases, phosphatases, isomerases, the periplasmic components of the Dot/Icm machinery and other proteins involved in *L. pneumophila* virulence that promote penetration of host cells (Cirillo et al., 2000; Khemiri et al., 2008).

Legionella pneumophila peptidoglycan was shown to contain muramic acid, glucosamine, glutamic acid, alanine, and meso-diaminopimelic acid (meso-DAP). Interestingly, extremely strong crosslinking was observed, with approximately 85% of meso-DAP and 90% of alanine residues contributing to these crosslinks. Peptidoglycan was found to be partially resistant to lysozyme treatment. The stable peptidoglycan layer is likely to promote survival in hostile environments (Amano and Williams, 1983). The important role of peptidoglycan in virulence is underlined by the finding that DAP-auxotroph *L. pneumophila* mutants display impaired survival within macrophages and amoeba (Amano and Williams, 1983).

Peptidoglycan fragments of many bacteria are recognized by members the NLR family of receptors (Nucleotide-binding domain, Leucine-Rich repeat-containing proteins) in the host cytosol. Their activation leads to inflammatory responses. Interestingly, *L. pneumophila* cell extracts and culture supernatants activate two members of this family, NOD1 and NOD2 (Nucleotide-binding, Oligomerization Domain-containing proteins 1 and 2), only to a very small extent (Hasegawa et al., 2006). Why *L. pneumophila* is only weakly detected by NLRs and the details of its recognition by the host has to be the subject of future investigations.

The periplasm harbors many enzymes which degrade harmful substances that enter the bacterial cell. One example is the *L. pneumophila* copper–zinc–superoxide dismutase (Cu–Zn–SOD). It was shown that this enzyme is essential for survival in the stationary growth phase. As *Legionella* has to survive for long periods when no host is available, the Cu–Zn–SOD may aid the bacteria to overcome oxidative stress encountered during this period. Interestingly, copper–zinc oxidases occur in many eukaryotes, but only in very few bacteria such as *Haemophilus influenzae*, *Brucella abortus*, and *Escherichia coli*. A general involvement of this enzyme class in microbial virulence is discussed (Schnell and Steinman, 1995; St John and Steinman, 1996).

The hydrogen peroxide which is produced by the Cu–Zn–SOD can be converted to H_2O and O_2 by the periplasmic katalase KatA. KatA and its cytoplasmic counterpart KatB are both required for optimal infection cycles in primary macrophages and amoeba (Bandyopadhyay et al., 2003). The authors propose a model in which KatA and KatB maintain a low intracellular H_2O_2 level, which is required for optimal function of the Dot/Icm apparatus and many other processes.

One of the Dot/Icm machinery components, IcmX, was localized to the *L. pneumophila* periplasm. This protein is required for the establishment of the *Legionella*-containing vacuole and pore formation in macrophage cell membranes, yet these effects are independent of an intact Dot/Icm apparatus. A truncated form of IcmX is secreted into culture supernatants, but not into the cytoplasm of host cells (Matthews and Roy, 2000). Intriguingly, a sequence near the C terminus of the IcmX gene is annotated to contain a DNA polymerase domain of the POLBc superfamily. If this holds true, the purpose of a periplasmic DNA polymerase remains to be clarified.

Many periplasmic components contribute to *L. pneumophila* virulence and some may be involved in bacterial protection against immune defense mechanisms (see **Table 2**).

THE OUTER MEMBRANE

The OM is the distinguishing feature of all Gram-negative bacteria. It is a lipid bilayer composed of phospholipids, lipoproteins, LPS, and proteins. Phospholipids are located mainly in the inner leaflet of the outer membrane, as are the lipoproteins that connect the outer membrane to peptidoglycan. The outer membrane is the location of mature LPS molecules and the shedding of OMVs.

The phospholipids of *L. pneumophila* were analyzed shortly after the discovery of the bacteria (Finnerty et al., 1979). They are, in decreasing order of concentration, phosphatidylcholine, phosphatidylethanolamine, cardiolipin, monomethylphosphatidylethanolamine, phosphatidylglycerol, and dimethylphosphati-

dylethanolamine. It remains unclear whether the lipid composition of the outer membrane differs significantly from that of the inner membrane (Hindahl and Iglewski, 1984; Gabay and Horwitz, 1985).

The discovery of phosphatidylcholine is striking as only about 10% of all known bacteria contain this lipid in their membranes – mostly those bacteria that are closely associated with eukaryotes. Examples include *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, and *B. abortus*. Nevertheless the exact function of this phospholipid in bacterial cell envelopes remains unknown (Sohlenkamp et al., 2003). Intriguingly, the loss of phosphatidylcholine from the *L. pneumophila* envelope causes reduced cytotoxicity and lower yields of bacteria within macrophages (Conover et al., 2008). Additionally the strains lacking this lipid bind to macrophages less effectively. Recently it was shown that *Legionella bozemanae* synthesizes phosphatidylcholine from exogenous choline (Palusinska-Szysz et al., 2011).

In addition to phospholipid species, the fatty acid composition of membranes also influences bacterial properties. In the stationary growth phase of *L. pneumophila*, the proportion of branched-chain fatty acids rises to over 60% and the average length of fatty acids in phospholipid molecules decreases compared to exponential growth. This change in fatty acid composition leads to an increased tolerance to the antimicrobial peptide warnericin RK (Verdon et al., 2011). The contribution of fatty acids to *Legionella* infection processes is still unknown. Future studies will shed more light on the influence of lipids on membrane protein structures, localization, and functions. In addition, the existence of distinct lipid domains in *L. pneumophila* membranes has not been described so far.

A bioinformatic approach proposed around 250 proteins in the *L. pneumophila* OM, however, most of their functions still need to be elucidated (Khemiri et al., 2008). With few exceptions, the proteins of the OM can be divided into two classes, lipoproteins and β-barrel proteins. Lipoproteins have lipid moieties attached to an amino-terminal cysteine residue (Sankaran and Wu, 1994). β-barrel proteins are nearly all integral membrane proteins of the outer membrane. Most outer membrane proteins are involved in either attachment or invasion of host cells. Both classes of proteins are in direct contact with the environment and host cells. They are therefore preferential targets for vaccine development as well as for diagnosis (Silhavy et al., 2010).

One such example is the 19-kDa peptidoglycan-associated lipoprotein (PAL) which is a species-common immunodominant antigen for the diagnosis of Legionnaires' disease (Kim et al., 2003; Shim et al., 2009). This protein activates murine macrophages via toll-like receptor 2 (TLR2) and induces the secretion of proinflammatory cytokines such as IL-6 and TNF-α (**Table 3**).

Three of the T4SS components (DotD, DotC, IcmN) contain a lipobox motif at their N terminus and are predicted to be lipoproteins. DotD and DotC are essential for bacterial intracellular survival (Yerushalmi et al., 2005; Nakano et al., 2010).

For *L. pneumophila*, several outer membrane proteins are characterized as important virulence factors. An example of an outer membrane-associated and at least partially surface-exposed protein with virulence functions is PlaB (major cell-associated phospholi-

Table 2 | Periplasmic proteins of *L. pneumophila* associated with virulence and survival.

Protein	Molecular function	Role in infection/required for	Reference
Copper–zinc–superoxide dismutase	Detoxification of superoxide radicals	Stationary growth survival	St John and Steinman (1996)
KatA	Degradation of H_2O_2	Optimal infection of macrophages and amoeba (optimal function of the Dot/Icm apparatus)	Bandyopadhyay et al. (2003)
IcmX	Putative DNA polymerase (POLBc superfamily)	Establishment of the *Legionella*-containing vacuole, pore formation in macrophage cell membranes	Matthews and Roy (2000)

Table 3 | Outer membrane proteins of *L. pneumophila* associated with virulence and survival.

Protein	Molecular function	Role in infection/required for	Reference
PAL		Activation of murine macrophages via TLR2, induction of the secretion of proinflammatory cytokines such as IL-6 and TNF-α	Kim et al. (2003), Shim et al. (2009)
DotD, DotC, IcmN		Intracellular survival	Nakano et al. (2010), Yerushalmi et al. (2005)
PlaB	Phospholipase A/ lysophospholipase A	Contact-dependent hemolytic activity and plays an important role in guinea pig infection	Schunder et al. (2010)
MOMP	Porin	attachment to host cells	Bellinger-Kawahara and Horwitz (1990), Krinos et al. (1999)
Hsp60		Attachment to and invasion of a HeLa cell	Garduño et al. (1998), Hoffman and Garduño (1999)
Mip	Peptidyl–prolyl *cis/trans* isomerase	Efficient replication within host cells and transmigration across an *in vitro* model of the lung epithelial barrier	Wagner et al. (2007), Debroy et al. (2006)
Lcl	Collagen-like protein	Adherence to and invasion of host cells	Vandersmissen et al. (2010)

pase A/lysophospholipase A). It displays contact-dependent hemolytic activity and plays an important role in guinea pig infection (Schunder et al., 2010).

The *L. pneumophila* major outer membrane protein (MOMP) is involved in the attachment to host cells (Gabay et al., 1985; Bellinger-Kawahara and Horwitz, 1990; Mintz et al., 1995; Krinos et al., 1999). The heat shock protein Hsp60 is also important for attachment to and invasion of a HeLa cell model (Garduño et al., 1998; Hoffman and Garduño, 1999).

Mip, the macrophage infectivity potentiator, is a membrane-associated homodimeric protein that is mainly found on the bacterial surface (Riboldi-Tunnicliffe et al., 2001). The C-terminal domain of Mip displays peptidyl–prolyl *cis/trans* isomerase (PPIase) activity. It is related to the human FK506-binding protein and binds to collagen of types I, II, III, IV, V, and VI. The protein is necessary for efficient replication within host cells. Interestingly, it is also required for the transmigration of *L. pneumophila* across an *in vitro* model of the lung epithelial barrier (Wagner et al., 2007).

The substrate of Mip and its exact function in virulence have not been identified yet. A step toward this goal was the finding that Mip is required for the extracellular release of an phospholipase C-like activity. Mip may mediate this by activating the secreted enzyme – and potentially other proteins – directly after secretion of one of the secretion machinery components by its PPIase activity (Debroy et al., 2006).

The *Legionella* collagen-like protein Lcl contains an outer membrane motif and was shown to contribute to the adherence and invasion of host cells. Interestingly, the number of repeat units present in the *lcl* gene has an influence on these adhesion characteristics (Vandersmissen et al., 2010).

In summary, the outer membrane is the direct interface between *L. pneumophila* and its host organisms. Some of its proteinaceous components are directly involved in adhesion and invasion processes (**Table 3**). The influence of lipid composition on the functions of OM virulence factors remains to be elucidated.

L. PNEUMOPHILA LPS

Lipopolysaccharides are located in the outer leaflet of the outer membrane and they are a major immunodominant antigen of *Legionella*. Based on O-antigen architecture, the species *L. pneumophila* can be divided into at least 15 serogroups (Helbig and Amemura-Maekawa, 2009). Within each serogroup, so-called monoclonal subgroups can be defined. For example, serogroup 1 can be divided into 10 subgroups (Ciesielski et al., 1986). The species *L. pneumophila* accounts for about 90% of the cases of legionellosis, and about 85% of these are caused by members of serogroup 1 (Helbig et al., 2002; Doleans et al., 2004; Gosselin et al., 2010; Napoli et al., 2010). For this reason, most researchers focus on serogroup 1, and this chapter, too, describes the chemical structure and functions of *L. pneumophila* serogroup 1 LPS.

In comparison to the LPS of other Gram-negative bacteria, the *L. pneumophila* LPS has a unique structure. Due to high levels of long, branched fatty acids, and elevated levels of *O*- and *N*-acetyl groups, this LPS is highly hydrophobic (**Figure 2**). LPS molecules consist of the O-specific chain, the core region, and the lipid A component, which is also called endotoxin. The O-chain and the core constitute the polysaccharide region of the LPS, whereas lipid A represents the part of the molecule which anchors the LPS in the outer membrane.

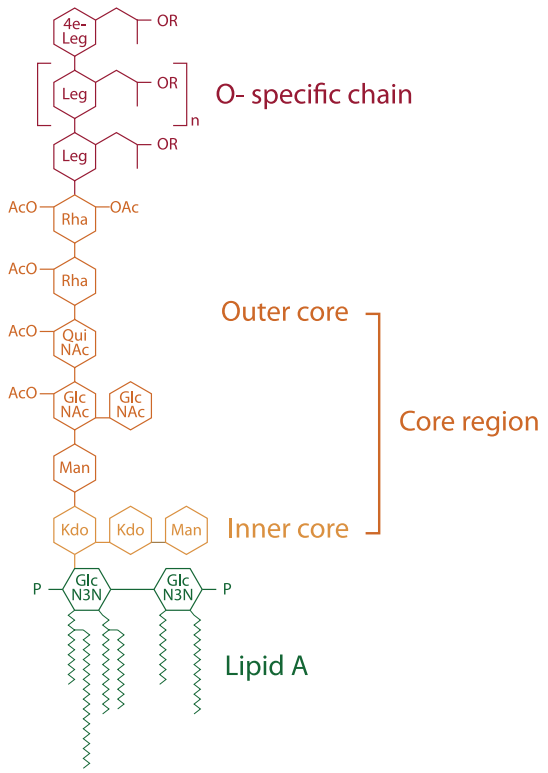

FIGURE 2 | Chemical structure of *L. pneumophila* LPS (modified from Kooistra et al., 2002b). Structure indicates its various regions: O-specific chain, core region consisting of the outer core and inner core and lipid A. Leg, derivatives of legionaminic acid; 4e-Leg, derivatives of 4-epilegionaminic acid; Rha, rhamnose; Man, mannose; QuiNAc, acetylquinovosamine; GlcNAc, acetylglucosamine; Kdo, 3-deoxy-d-*manno*-oct-2-ulosonic acid; P, phosphate; OAc, *O*-acetyl.

The O-chain of *L. pneumophila* LPS is a homopolymer of the unusual sugar 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid, termed legionaminic acid (Palusinska-Szysz and Russa, 2009). This sugar molecule completely lacks free hydroxyl groups and is therefore very hydrophobic (Knirel et al., 1994; Helbig et al., 1995; Zähringer et al., 1995; Kooistra et al., 2002a). The core region consists of the outer core and the inner core. The outer core of *L. pneumophila* is a oligosaccharide composed of rhamnose (Rha), mannose (Man), acetylquinovosamine (QuiNAc), and acetylglucosamine GlcNAc (Knirel et al., 1996, 1997). Like the O-chain it also exhibits hydrophobic properties, in contrast to the inner core, which is hydrophilic. The inner core oligosaccharide of *L. pneumophila* LPS is characterized by a 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) disaccharide [α-Kdo-(2α4)-α-Kdo-(2α6)] linked to lipid A which is conserved within many Gram-negative bacteria and is essential for microbial growth (Moll et al., 1997).

Lipid A of *L. pneumophila* serogroup 1 contains unusual long-chain and branched fatty acids, which may be responsible for its low endotoxic potential (Moll et al., 1992; Neumeister et al., 1998). The structural function of lipid A is anchoring the LPS in the bacterial

membrane. Unlike in other Gram-negative bacteria, *L. pneumophila* lipid A does not function as a classical endotoxin. It was demonstrated that *L. pneumophila* LPS is about 1000 times less potent in its ability to induce the secretion of proinflammatory cytokines from human monocytic cells than LPS of members of the family *Enterobacteriaceae* (Neumeister et al., 1998).

LPS biosynthesis and transport

The biosynthesis of LPS is a complex process involving various steps that occur at the inner membrane, following by assembly in the periplasm and translocation of LPS molecules to the bacterial cell surface.

The genes involved in core oligosaccharide and O-chain biosynthesis are mainly localized on a 30-kb gene locus (Lüneberg et al., 2000). The excision of this region from the chromosome leads to an alteration of the LPS epitope and a loss of virulence (Lüneberg et al., 1998, 2001). The *L. pneumophila* LPS gene locus includes genes with products which are likely to be involved in LPS core oligosaccharide biosynthesis *(rmlA-D,* glycosyltransferases, acetyltransferase) as well as O-chain biosynthesis and translocation *(mnaA, neuB, neuA, wecA, wzt, wzm)*. The genes involved in LPS biosynthesis and translocation and its distribution among five sequenced and annotated *L. pneumophila* genomes are summarized in **Table 4**. Interestingly, the gene cluster coding for the determinants of serogroup 1 LPS is present in diverse serogroups, suggesting that it is mobile and can be exchanged by horizontal gene transfer (Cazalet et al., 2008). The region encoding proteins involved in LPS biosynthesis can be subdivided in two blocks of 13 and 20 kb. Most of the genes in the 13-kb block are present in all *L. pneumophila* strains, whereas the majority of genes in the 20-kb block are specific for serogroup 1. Three genes, coding for two O-antigen transporters (*wzt* and *wzm*) and one hypothetical protein, might be used as markers for *Legionella* serogroup 1 (Cazalet et al., 2008; Merault et al., 2010).

Lipid A can be modified, a process which alters the physical properties of the outer membrane (Albers et al., 2007). Some of these modifications are known to be under the control of the PmrA/PmrB and/or the PhoP/PhoQ two-component systems in other Gram-negative organisms (Miller et al., 1989; Guo et al., 1997, 1998; Gunn et al., 1998). Despite the detailed characterization of the PmrA/PmrB two-component system of *L. pneumophila* and its influence on gene expression of most of virulence determinants, the role of the this system in lipid A modification in *Legionella* has not yet been analyzed (Al-Khodor et al., 2009; Hovel-Miner et al., 2009; Rasis and Segal, 2009).

PhoP/PhoQ is a two-component system which regulates a number of lipid A-modifying enzymes in *Salmonella enterica* serovar Typhimurium. It was not detected in genomes of the genus *Legionella* (Gibbons et al., 2005). Nevertheless, it is conceivable that analogs with low protein homology or other two-component systems regulate lipid A-modifying enzymes. One of the genes which is transcriptionally activated by the PhoP/PhoQ system in *Salmonella* is *pagP* (Kawasaki et al., 2004). The inactivation of this gene leads to a decreased resistance to cationic antimicrobial peptides (CAMPs). The *Legionella* homolog of *pagP* is called resistance to CAMPs (*rcp*) and functions as a palmitoyl transferase, which transfers palmitate to lipid A molecules. The increased palmitoylation is believed to promote resistance to CAMPs by decreasing membrane fluidity and preventing insertion of the peptides (Guo et al., 1998; Bishop et al., 2000; Robey et al., 2001; Soderberg and Cianciotto, 2010). The structural modification of lipid A might help the bacteria to resist CAMPs released by the host immune system, or to evade recognition by TLR4, the innate immune receptor. *L. pneumophila rcp* influences virulence and the adaptation to Mg^{2+}-limiting conditions (Wang and Quinn, 2010).

After synthesis on the cytoplasmic face, both core-lipid A and O-antigen need to be transported to the periplasmic face of the inner membrane. Little is known about the mechanisms of LPS polymerization and translocation in *Legionella*. After attachment of the core, nascent core-lipid A is most probably flipped to the periplasmic face of the inner membrane by the ABC transporter MsbA, where the O-antigen polymer is attached (Doerrler et al., 2004). Transport of the O-antigen may occur through an Wzt/Wzm ABC transporter. In all analyzed *L. pneumophila* genomes, we have found Wzt and Wzm genes (**Table 4**). Wzm forms a channel in the inner membrane for the passage of the lipid-linked O-antigen, and Wzt provides energy through its ATPase activity (Lüneberg et al., 2000).

It is not known how *Legionella* LPS is transported from the periplasm to the outer leaflet of the OM. Recently it was shown that in *E. coli* the LptD/LptE complex performs this function (Ma et al., 2008). The homolog of LptD/LptE was found in *L. pneumophila*, therefore it can be speculated that the transport of LPS occurs by a related mechanism.

Functions of the Legionella LPS

Members of the TLR family in cells of the innate immune system recognize specific conserved components of microbes, including LPS. This initiates the cascade of the inflammatory response and activates adaptive immunity through the induction of cytokine production and synthesis of co-stimulatory molecules. LPS can be recognized by TLR4, a receptor found on the surface of different immune cells such as macrophages, neutrophils, and dendritic cells (Mintz et al., 1992; Akira et al., 2001). The correlation between a TLR4 polymorphism and its influence on susceptibility to Legionnaires' disease was reported (Hawn et al., 2005). It is interesting to note that *L. pneumophila* requires TLR2 rather than TLR4 to elicit the expression of CD14, which acts as a co-receptor for the detection of bacterial LPS. It is hypothesized that long-chain fatty acids and the high hydrophobicity of *L. pneumophila* lipid A can abolish the interaction with the soluble LPS receptor CD14 and the ability of LPS molecules to activate bone marrow cells (Neumeister et al., 1998; Girard et al., 2003). Remarkably, *L. pneumophila* is known to up-regulate both, TLR2 and TLR4, and to activate CD40, CD86, and MHC class I/II molecules on dendritic cells (Rogers et al., 2007).

Recently it was demonstrated that LPS of *L. pneumophila* shed in liquid culture is able to arrest phagosome maturation in amoeba and human macrophages. In particular, the presence of high-molecular-weight LPS correlates with the inhibition of phagosome–lysosome fusion (Seeger et al., 2010). Another group has shown that *L. pneumophila* LPS specifically interacts with pulmonary collectins and surfactant proteins A and D, which play important roles in innate immunity in the lung. The authors also propose that

Table 4 | Paralogs of LPS biosynthesis and translocation proteins in *L. pneumophila* strains.

Enzyme	Molecular function	*L. pneumophila* strains				
		Corby	Philadelphia-1	Lens	Paris	2300/99 Alcoy
LIPID A BIOSYNTHESIS						
LpxA	UDP–*N*-acetylglucosamine acyltransferase	LPC_2835	Lpg0511	Lpl0549	Lpp0573	Lpa_00769
		LPC_3254	Lpg2943	Lpl2874	Lpp3016	Lpa_04308
LpxC	UDP–3-*O*-[3-hydroxymyristoyl] *N*-acetylglucosamine deacetylase	LPC_0533	Lpg2608	Lpl2531	Lpp2661	Lpa_03814
LpxD	UDP–3-*O*-[3-hydroxymyristoyl] glucosamine *N*-acyltransferase	LPC_0119	Lpg0100	Lpl0100	Lpp0114	Lpa_00149
		LPC_2837	Lpg0508	Lpl0547	Lpp0571	Lpa_00766
		LPC_3255	Lpg2944	Lpl2873	Lpp3015	Lpa_04309
LpxH	UDP–2,3-diacylglucosamine hydrolase	LPC_0973	Lpg1552	Lpl1474	Lpp1509	Lpa_02254
LpxB	Lipid A disaccharide synthase	LPC_0787	Lpg1371	Lpl1322	Lpp1325	Lpa_02021
		LPC_3256	Lpg2945	Lpl2872	Lpp3014	Lpa_04311
LpxK	Tetraacyldisaccharide 4′-kinase	LPC_1262*	Lpg1818*	Lpl1782*	Lpp1781*	Lpa_02629*
	Tetraacyldisaccharide-1-P-4′-kinase	LPC_1374	Lpg1920	Lpl1884	Lpp1895	Lpa_02777
KdtA (WaaA)	3-Deoxy-d-manno-oct-2-ulosonic acid transferase	LPC_1808	Lpg2340	Lpl2261	Lpp2288	Lpa_03350
LpxL (WaaM)	Lipid A acyltransferase	LPC_2981	Lpg0363	Lpl0404	Lpp0428	Lpa_00577
		LPC_3251#	Lpg2940#	Lpl2870#	Lpp3012#	Lpa_04304#
		LPC_3252#	Lpg2941#	Lpl2871#	Lpp3013#	Lpa_04305#
CORE REGION BIOSYNTHESIS						
WaaQ	Heptosyl transferase	LPC_0441	lpg2695	Lpl2622	Lpp2749	Lpa_03933
RmlA (RfbA)	Glucose-1-phosphate thymidylyltransferase	LPC_2532	Lpg0760	Lpl0797	Lpp0826	Lpa_01168
RmlB (RfbB)	dTDP–glucose 4,6-dehydratase RmlB	LPC_2534	Lpg0758	Lpl0795	Lpp0824	Lpa_01166
RmlC	dTDP–4-dehydrorhamnose 3,5-epimerase	LPC_2536	Lpg0756	Lpl0793	Lpp0822	Lpa_01164
RmlD	dTDP–6-deoxy-l-mannose dehydrogenase	LPC_2535	Lpg0757	Lpl0793	Lpp0823	Lpa_01165
	Glycosyltransferase	LPC_2515	Lpg0779	Lpl0818	Lpp0843	Lpa_01190
	Glycosyltransferase	LPC_2516	Lpg0778	Lpl0817	Lpp0842	Lpa_01189
O-CHAIN BIOSYNTHESIS						
KdsA (NeuB)	3-Deoxy-d-manno-octulosonic acid (KDO) 8-phosphate synthase	LPC_0649	Lpg1182	Lpl1191	Lpp1185	Lpa_01838
	HAD superfamily transporter hydrolase	LPC_2456	Lpg0839	Lpl0870	Lpp0901	Lpa_01272
KdsB	3-Deoxy-manno-octulosonate	LPC_1373	Lpg1919	Lpl1883	Lpp1894	Lpa_02777
GmhA	Phosphoheptose isomerase	LPC_3308	Lpg2993	Lpl2921	Lpp3064	Lpa_04384
HisB	d,d-heptose 1,7-bisphosphate phosphatase	LPC_1283	Lpg1838	Lpl1803	Lpp1802	Lpa_02656
WecE	Aminotransferase, predicted pyridoxal phosphate-dependent enzyme	LPC_0840	Lpg1424	Lpl1375	Lpp1379	Lpa_02088
Lag-1	*O*-Acetyltransferase, acetylation of the *O*-polysaccharide	LPC_2517	Lpg0777	Lpl0816	Lpp0841	Lpa_01188
NeuC (NnaA)	*N*-Acylglucosamine 2-epimerase	LPC_2539	Lpg0753	Lpl0790	Lpp0819	Lpa_01161
NeuB	*N*-Acetylneuraminic acid synthetase	LPC_2540	Lpg0752	Lpl0789	Lpp0818	Lpa_01160
		LPC_2524	Lpg0768	Lpg0809	Lpp0833	Lpa_01177
NeuA	CMP–*N*-acetylneuraminic acid synthetase	LPC_2541	Lpg0751	Lpl0788	Lpp0817	Lpa_01159
WecA	O-Antigen initiating glycosyl transferase	LPC_2530	Lpg0762	Lpl0799	Lpp0828	Lpa_01171
LPS TRANSLOCATION						
MsbA	Lipid A export ATP-binding/permease protein MsbA	LPC_1263*	Lpg1819*	Lpl1783*	Lpp1782*	Lpa_02631*
Wzt**	LPS O-antigen ABC transporter Wzt	LPC_2519	Lpg0773	Lpl0814	Lpp0838	Lpa_01186
Wzm**	LPS O-antigen ABC transporter Wzm	LPC_2520	Lpg0772	Lpl0813	Lpp0837	Lpa_01184

The protein paralogs share a high level of homology. In general they have 96–100% of identity and 97–100% of positivity.

#Indicates the proteins with lower homology (73–90%).

**The lpxK–msbA cluster exists in many Gram-negative bacteria. MsbA is known as a specific transporter, which exports core–lipid A from the cytoplasmic to the periplasmic face of the inner membrane, while LpxK phosphorylates the 4′-position of lipid A.*

***The genes wzm and wzt are specific for the Sg1 LPS gene cluster and can be used for rapid detection of L. pneumophila Sg1 in clinical and environmental isolates (Cazalet et al., 2008).*

this interaction promotes the localization of *L. pneumophila* to an acidic compartment, i.e., lysosomes, and intracellular growth of the bacteria is subsequently inhibited (Sawada et al., 2010).

Interestingly, the LPS pattern of *L. pneumophila* grown in broth has been found to be different from the pattern of bacteria grown intracellulary in *Acanthamoeba polyphaga* (Barker et al., 1993). Moreover, during exponential growth, *L. pneumophila* LPS is much more hydrophobic than in post-exponential cultures (Seeger et al., 2010).

In general, *L. pneumophila* LPS plays a crucial role in interaction with host cells and modulation of intracellular trafficking, independently of the Dot/Icm secretion system. The unusual structure of lipid A might help the bacteria to avoid recognition by the innate immune system.

FLAGELLA AND PILI

The first evidence of the presence of flagella and pili structures on the *L. pneumophila* surface was provided by Rodgers et al. (1980). The authors were also the first to observe that pili of *L. pneumophila* vary in length. Later the pili were divided into long (0.8–1.5 μm) and short (0.1–0.6 μm) forms (Stone and Abu Kwaik, 1998). The PilE protein is the constituent of long type IV pili. It is involved in attachment and adherence to host cells as well as natural competence of *L. pneumophila*. At the same time a mutation in the *pilE* gene does not affect the intracellular survival and replication of bacteria (Stone and Abu Kwaik, 1998; Stone and Kwaik, 1999).

Another protein responsible for type IV pili production is the prepilin peptidase PilD. Unlike PilE, PilD is important for successful intracellular proliferation. This protein is also involved in type II secretion activity (Aragon et al., 2000). Both mentioned pili proteins facilitate the formation of biofilms of *L. pneumophila* (Lucas et al., 2006). Interestingly, despite microscopic evidence for the presence of the pili in liquid culture, some fimbrial synthesis genes are induced only in host cells (Bruggemann et al., 2006).

Additionally to pili, *L. pneumophila* exhibits a single monopolar flagellum, which is anchored within both membranes (OM, IM) and peptidoglycan by the basal body. This organelle plays an important role in cell motility, adhesion, and host invasion. It has also been described to be involved in biofilm formation (Heuner and Albert-Weissenberger, 2008). The expression of flagella is influenced by many environmental factors and is controlled by a hierarchical cascade of regulators (Albert-Weissenberger et al., 2010). The transition of the bacteria to the transmissive phase is co-regulated with the expression of flagella. Regulators that control flagellation also control important virulence traits such as lysosome avoidance and cytotoxicity (Gabay et al., 1986; Byrne and Swanson, 1998; Molofsky et al., 2005). On the other hand cytosolic flagellin is described to trigger the macrophage response to a *L. pneumophila* infection. This mechanism is mediated by Naip5/Birc1e, a member of the NLR family. It activates the caspase-1-dependent cell-death pathway that restricts bacterial growth (Molofsky et al., 2006; Ren et al., 2006). Information on the *L. pneumophila* flagellum is excellently reviewed in a recent publication (Heuner and Albert-Weissenberger, 2008).

OUTER MEMBRANE VESICLES

Outer membrane vesicles are shed from the outer membrane by *L. pneumophila* and most other Gram-negative bacteria. They are between 100 and 250 nm in diameter and consist of components from the outer membrane, including LPS, and the periplasm (**Figure 3**). *L. pneumophila* OMVs contain a disproportionately high number of virulence-associated proteins and display lipolytic and proteolytic activities (Galka et al., 2008).

In general, OMVs from other bacteria can mediate interbacterial contact and also the contact to eukaryotic cells. They can kill other bacteria by the delivery of harmful factors. Macromolecule-degrading enzymes in association with OMVs can promote nutrient acquisition, i.e., by cleaving proteins into amino acids which are then taken up by the bacterium. Modulations of biofilm formation and quorum sensing functions have also been assigned to OMVs. In the interaction with eukaryotic host cells, OMVs can deliver toxins and other virulence factors and have been shown to adhere to cell surfaces. In addition, the immune response – cytokine profiles, inflammation, innate immunity – is modified by contact to bacterial OMVs (Ellis and Kuehn, 2010).

So far, OMVs of *L. pneumophila* have been studied to a lower extent. A proteomic analysis revealed 74 different proteins. The export of 33 of these proteins occurs only via OMVs, but not individually via type II, III, or IV secretion systems. Of these OMV-specific proteins, 18 are reported or predicted to contribute to

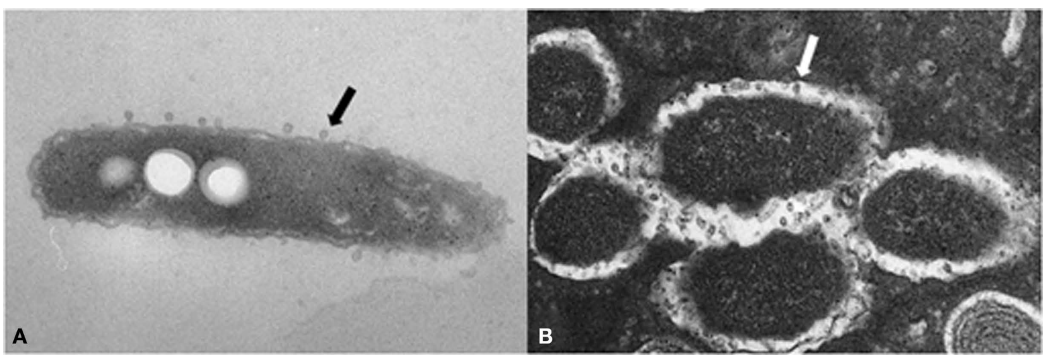

FIGURE 3 | Electron micrographs of *L. pneumophila* and outer membrane vesicles. *L. pneumophila* sheds OMVs (arrows) from its surface during growth in liquid media (A) and within phagosomes of *Dictyostelium discoideum* (B).

pathogenesis and virulence (Galka et al., 2008). This finding led to the conclusion that *L. pneumophila* employs OMVs as vehicles for the transport of virulence factors toward the environment.

The exact mode of interaction of *L. pneumophila* OMVs and host cell surfaces remains to be elucidated. However, an association of OMVs to the cytoplasmic membrane of human alveolar epithelial cells has been shown. The contact between OMVs and the cells resulted in a change in cell morphology, leading to round cells (Galka et al., 2008).

Outer membrane vesicles can also elicit a specific cytokine response from alveolar epithelial cells, resulting in the release of interleukins-6, -7, -8, and -13 as well as G-CSF, IFN-γ, and MCP-1. IL-7 and IL-8 are secreted only after stimulation with OMVs, but not after stimulation with individually secreted proteins.

Legionella pneumophila OMVs increase the growth of *Acanthamoeba castellanii* over the course of 72 h, rather than damaging the host cells (Galka et al., 2008). As *A. castellanii* usually feeds on bacteria, membrane vesicles are thought to serve as a source of nutrients, possibly to attract amoeba toward bacteria, which then infect them.

Latex beads which have been coated with *L. pneumophila* OMVs can inhibit the fusion of *Legionella*-containing phagosomes to lysosomes, thereby preventing death of the bacteria (Fernandez-Moreira et al., 2006). This key feature of *Legionella* infections seems to be mediated by OMVs, at least to a certain degree. The LPS on the surface of OMVs is regulated similarly to LPS on the outer membrane. The phagolysosomal arrest is evoked more strongly by soluble LPS shed into the bacterial surrounding. The arrest efficiency seems to decrease over time (Seeger et al., 2010).

The inhibition of the fusion between phagosomes and lysosomes is only one of the functions of *L. pneumophila* OMVs. They also display proteolytic and lipolytic activities, though the fraction of individually secreted proteins features stronger degradative enzyme activities (Galka et al., 2008). In this way, OMVs might contribute to the dissemination of the infection across tissue barriers such as the alveolar epithelium.

In conclusion, OMVs are believed to be a vehicle for the transport of virulence factors to distant cells or host tissues. Their precise contribution to *L. pneumophila* infections has not been determined yet, but is under investigation.

CONCLUSION

Legionella pneumophila inhabits fresh waters and biofilms. Moreover, this pathogen parasitizes phylogenetically distant hosts such as protozoa and human cells, a process which requires adhesion, invasion, and interactions within the phagosome. Under all these conditions the bacterial cell envelope is the prime structure through which *L. pneumophila* interacts with these fundamentally different environments. Although the potential properties of the cell envelope are ultimately determined by the information stored within the genome, it becomes increasingly evident that molecular identities, spatial distributions, and biochemical activities of many envelope constituents are highly dynamic and vary with *L. pneumophila* growth phases, developmental differentiation processes as well as during the pathogen–host interaction. Therefore, the investigation of phenotypic changes, which take place as the bacteria adapt to different conditions, holds great promise for the understanding of this pathogen. Proteins, carbohydrates, and lipids in the bacterial cell envelope serve both structural and signaling roles, but until recently the main focus of biomedical research was on identification and analysis of proteins. Hereby we have learned that already characterized proteins can have unexpected functions, suggesting the need for more thorough investigations. Based on the current body of information there is also increased awareness that lipids, both of host and bacterial origin, choreograph pathogen stability and host susceptibility to infection. The renewed interest in these historically neglected effector molecules is currently fueled by the advances in lipidomics and glycomics technologies. Thus, identification of unique lipid entities and their biological activities represent an enormously promising new frontier in the infection biology of *L. pneumophila*.

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The *Legionella pneumophila* chaperonin – an unusual multifunctional protein in unusual locations

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The *Legionella pneumophila* chaperonin, high temperature protein B (HtpB), was discovered as a highly immunogenic antigen, only a few years after the identification of *L. pneumophila* as the causative agent of Legionnaires' disease. As its counterparts in other bacterial pathogens, HtpB did not initially receive further attention, particularly because research was focused on a few model chaperonins that were used to demonstrate that chaperonins are essential stress proteins, present in all cellular forms of life and involved in helping other proteins to fold. However, chaperonins have recently attracted increasing interest, particularly after several reports confirmed their multifunctional nature and the presence of multiple chaperonin genes in numerous bacterial species. It is now accepted that bacterial chaperonins are capable of playing a variety of protein folding-independent roles. HtpB is clearly a multifunctional chaperonin that according to its location in the bacterial cell, or in the *L. pneumophila*-infected cell, plays different roles. HtpB exposed on the bacterial cell surface can act as an invasion factor for non-phagocytic cells, whereas the HtpB released in the host cell can act as an effector capable of altering organelle trafficking, the organization of actin microfilaments and cell signaling pathways. The road to discover the multifunctional nature of HtpB has been exciting and here we provide a historical perspective of the key findings linked to such discovery, as well as a summary of the experimental work (old and new) performed in our laboratory. Our current understanding has led us to propose that HtpB is an ancient protein that *L. pneumophila* uses as a key molecular tool important to the intracellular establishment of this fascinating pathogen.

Keywords: HtpB, Hsp60, GroEL, pathogenesis, mitochondria, microfilaments, polyamines

BACKGROUND

CHAPERONINS AND THEIR ESSENTIAL PROTEIN FOLDING FUNCTION

Chaperonins are a family of structurally and functionally conserved, essential proteins, present in virtually all prokaryotic and eukaryotic forms of life. Intuitively, then, contemporary chaperonins must be related to one of the first proteins present in the common ancestor of all organisms currently known. The striking amino acid sequence and structural conservation of the chaperonin groups clearly suggests that these proteins must be very important. The primary function of chaperonins, recognized to be important enough to explain their essential nature, is in helping other proteins to fold properly and reach their native (functional) state.

Because this review is focused on the protein folding-independent functions of the *Legionella* chaperonin, a discussion on the protein folding ability of chaperonins is not forthcoming. Therefore, we provide the following key references for the benefit of those with further interests in this topic (Braig et al., 1994; Lund, 1995; Houry et al., 1999; Kerner et al., 2005; Sigler et al., 1998). In particular, recent comprehensive reviews that cover various aspects of the fascinating structure, biochemistry, and physiology of these formidable protein folding molecular machines (or nanoboxes in which proteins can fold) are those of England et al. (2008), Horwich et al. (2007), Lin and Rye (2006), and Lund (2011).

CLASSIFICATION OF CHAPERONINS

It seems that Hemmingsen et al. (1988) were the first to coin the term "chaperonins" to describe a small group of related proteins involved in "post-translational assembly of oligomeric protein structures." Since then, investigators have recognized the existence of different chaperonin types, which are currently classified into two groups based on their structure and evolutionary origin. Group I chaperonins are found in bacteria and in endosymbiotic organelles of eukaryotes (e.g., mitochondria and chloroplasts), have a mass of ~60-kDa and are typically induced under stress, e.g., heat shock. Therefore, group I chaperonins are also known as heat shock proteins 60 (Hsp60s; Zeilstra-Ryalls et al., 1991). These proteins form homo-oligomeric rings that consist of seven chaperonin subunits (Braig et al., 1994). Two of these 7-mer rings come together to form the 14-mer barrel complex that mediates protein folding in association with a third homo-oligomeric ring, comprised of seven subunits of

co-chaperonin, a protein of ∼10-kDa also known as Hsp10. Association with the 10-kDa co-chaperonins is an exclusive feature of Group I chaperonins. Other designations for Hsp10/Hsp60, are GroES/GroEL, Cpn10/Cpn60, and HtpA/HtpB. The intensively investigated *Escherichia coli* GroEL chaperonin constitutes the paradigm of Group I chaperonins.

Group II chaperonins, also known as TriC (TCP-1 ring complex) or CCT (chaperonin-containing TCP-1), are found in archaea, and the cytoplasm of eukaryotes (Lund, 1995). Group II chaperonins form eight- or nine-membered hetero-oligomeric rings with subunits that may have different masses (Kim et al., 1994; Klumpp and Baumeister, 1998). CCTs mediate the specialized folding of proteins (many of which are linked to the cytoskeleton), but do not team with 10 kDa co-chaperonins, although the protein prefoldin (Ohtaki et al., 2010) has been identified as a co-chaperone for CCTs. Group II chaperonins have an extended apical domain thought to cap the central cavity of the double-ringed complex, which replaces the need for the 7-mer co-chaperonin ring of Group I chaperonins (Fenton et al., 1996; Gutsche et al., 1999; Horwich and Saibil, 1998). Group II chaperonins are heterogeneous and are thought to have evolved by gene duplication and subsequent mutation (Archibald et al., 2000). While conserved within their respective groups, Group I and Group II chaperonins are only distantly related, but thought to share a common protein ancestor (Gupta, 1995).

A third chaperonin group has been recently reported in bacteria (Techtmann and Robb, 2010). Its representative chaperonin is that of the bacterium *Carboxydothermus hydrogenoformans*, which forms a 16-mer structure capable of refolding denatured proteins in an ATP-dependent manner. Group III chaperonins are distantly related to both Group I and Group II chaperonins, and thus they might represent an ancient horizontal transfer event from archaea to bacteria.

PROTEIN FOLDING-INDEPENDENT FUNCTIONS OF GROUP I CHAPERONINS

The Hsp60 of the bacterial endosymbiont *Buchnera aphidicola* (also called symbionin) acts as a histidine kinase (Morioka et al., 1994), whereas the GroEL of symbiotic *Enterobacter aerogenes* is a potent insect toxin (Yoshida et al., 2001), and the chaperonin of *Mycobacterium leprae*, is a protease (Portaro et al., 2002). Two views could be advanced to explain this functional diversity. In the first view, functional diversity is a preserved characteristic of chaperonins. That is, Group I chaperonins started as jacks-of-all-trades and gradually evolved toward specialization in protein folding. Thus, the contemporary examples of diversity mentioned above, represent evolutionary remnants of original functions preserved after specialization. In the second view, functional diversity is a newly emerged characteristic. That is, ancient chaperonins started as specialized proteins that gradually evolved toward functional diversity.

Two cases of functional chaperonin diversity resulting from few amino acid changes seem to favor the second view of "newly emerged functions." Only 11 amino acids are different between the toxic chaperonin from endosymbiotic *E. aerogenes*, and the non-toxic chaperonin of *E. coli*, of which four amino acid positions are critical for toxicity. When the non-toxic *E. coli* chaperonin was engineered at the four critical residues to resemble the *E. aerogenes* chaperonin, it too became a potent insect toxin (Yoshida et al., 2001). In the case of the Hsp65 chaperonin of *M. leprae*, only three amino acids (Thr-375, Lys-409, and Ser-502) comprise the threonine catalytic group responsible for protease activity (Portaro et al., 2002).

In a recent article based on the analysis of 669 complete bacterial genomes, Lund proposed that one of the mechanisms responsible for functional diversity in Group I chaperonins relies on gene duplication followed by unconstrained mutation of the duplicated gene sequences (Lund, 2009). The analysis showed that 467/669 genomes contained a single chaperonin gene, 183/669 genomes contained multiple chaperonin genes (from 2 to a maximum of 7), and 13 *Mycoplasma* genomes contained no discernable chaperonin genes. Lund (2009) thus argued that the essential protein folding needs of a bacterial cell are met by a single chaperonin (whose gene would be constrained for change), while the other chaperonins would be free to mutate and acquire functional specializations. At least in the case of *Mycobacterium tuberculosis*, this notion has been experimentally substantiated. *M. tuberculosis* has two chaperonin genes encoding the chaperonins Cpn60.1 and Cpn60.2, where *cpn60.2* is essential whereas *cpn60.1* can be deleted from the genome (Hu et al., 2008). These two chaperonins are functionally different (Cehovin et al., 2010) supporting the idea of functional diversity afforded by gene duplication.

However, there are other cases in which functional diversity rests on a single chaperonin. As it will be discussed below in detail, one of these cases is the chaperonin of *Legionella pneumophila*. Other examples include those bacterial pathogens that typically use their chaperonins as adherence factors, or immune-modulators. In this capacity, chaperonins have been recently added to the list of "moonlighting" proteins (Jeffery, 2009). The term moonlighting is defined in the Webster's Dictionary of the English Language as "working at a job in addition to one's regular one," and was introduced in the biochemical field to describe those proteins that perform a well-recognized function by day (regular job in a given environment or cellular location), and a not so obvious yet important function by night (other jobs in a different environment or cellular location).

Actinobacillus actinomycetemcomitans (Goulhen et al., 1998; Paju et al., 2000), *Borrelia burgdorferi* (Scorpio et al., 1994), *Chlamydia* spp. (Lund, 2009), *Clostridium difficile* (Hennequin et al., 2001), *Helicobacter pylori* (Huesca et al., 1996), *Haemophilus ducreyi* (Frisk et al., 1998), *Listeria monocytogenes* (Trost et al., 2005), and *Salmonella enterica* sv. Typhimurium (Ensgraber and Loos, 1992), are but some examples of bacterial pathogens that display their chaperonin in extracytoplasmic locations, and where the surface-associated, periplasmic, or released/secreted chaperonin seems to play alternate functional roles. For instance, the chaperonin of some of the aforementioned pathogens acts as an adhesion factor, but there are many that interact with mammalian cell surface receptors to initiate signaling events that result in cytokine production (reviewed by Ranford et al., 2000), phosphorylation of signaling molecules (Zhang et al., 2001, 2004), or other physiological outputs (Galdiero et al., 1997).

Group I chaperonins of endosymbiotic organelles are also functionally diverse, but given the nature of our review and its focus

on a bacterial pathogen, we will not discuss here organellar chaperonins. Therefore, readers interested in the prominent role of chaperonins in immunity and autoimmunity are referred to a recent scholar review (Henderson, 2010) that includes details on the immune-modulatory ability of these proteins. In summary, chaperonins are ancient proteins, essential for the life of eukaryotic and prokaryotic cells. Their essential nature seemingly rests on their protein folding ability, but in several cases chaperonins appear to be multifunctional.

THE CHAPERONIN OF *LEGIONELLA PNEUMOPHILA*, HtpB

The remaining portion of this review will be devoted to a discussion of the *L. pneumophila* chaperonin as a multifunctional ("moonlighting") protein (**Figure 1**), including a presentation of our recent experimental findings. To facilitate the distinction between the chaperonins that we will be discussing, and to respect current nomenclature, the *L. pneumophila* chaperonin will be subsequently referred to as high temperature protein B (for HtpB). The designation HtpA is used for the *L. pneumophila* co-chaperonin, which is encoded by the first gene in the *L. pneumophila htpAB* operon. The chaperonin/co-chaperonin system of *E. coli* will be referred to as GroEL/GroES. Other chaperonins will be referred to as Hsp60 or Cpn60.

HISTORICAL PERSPECTIVE OF HtpB RESEARCH BEFORE 1998

Discovery and initial characterization

Between the mid-1980s and early 1990s, a number of publications reported the existence of a common antigen of about 60-kDa in many bacterial species. Sompolinsky et al. (1980a,b) referred to it as the "common antigen," and Yamaguchi et al. (1989) used the term "cross-reacting protein antigen." These antigens were eventually identified as chaperonins. Similarly, HtpB was first spotted as a 58-kDa common antigen cross-reactive with 60-kDa antigens from several *Legionella* species and other bacteria (Sampson et al., 1986; Plikaytis et al., 1987). This antigen prominently reacted with sera from patients diagnosed with Legionnaires' disease (LD) and was used to confirm, by serology, culture-positive cases of LD (Sampson et al., 1986). This study also showed that when a rabbit serum raised against *L. pneumophila* serogroup 1 was pre-absorbed with whole *L. pneumophila* Philadelphia-1 cells, the 58-kDa antigen was no longer recognized by immunoblot. This is an interesting result because implies that the common antigen was surface exposed on the whole *L. pneumophila* cells used for cross-absorption. Plikaytis et al. (1987) were the first to purify HtpB and raise a rabbit hyperimmune serum against the purified protein, and shortly after, Pau et al. (1988) reported an optimized method for the purification of HtpB. A modification of this optimized method, which involves a combination of ammonium sulfate precipitation, size-exclusion, and ion-exchange chromatography, is the one used in the Garduño lab for the purification of HtpB.

We will close this section by mentioning that Gabay and Horwitz (1985) characterized HtpB as the major cytoplasmic membrane protein of *L. pneumophila*. Their studies are important because they established the ability of HtpB to interact with the bacterial cytoplasmic membrane, a trait that we believe is important in both the translocation of HtpB into the *L. pneumophila* periplasm (see HtpB is Found in Extracytoplasmic Locations below), and across the *Legionella*-containing vacuole (LCV) membrane into the host cell cytosol (see HtpB is Found in Extracytoplasmic Locations and Intracellularly Released HtpB Alters the Actin Cytoskeleton of Host Cells).

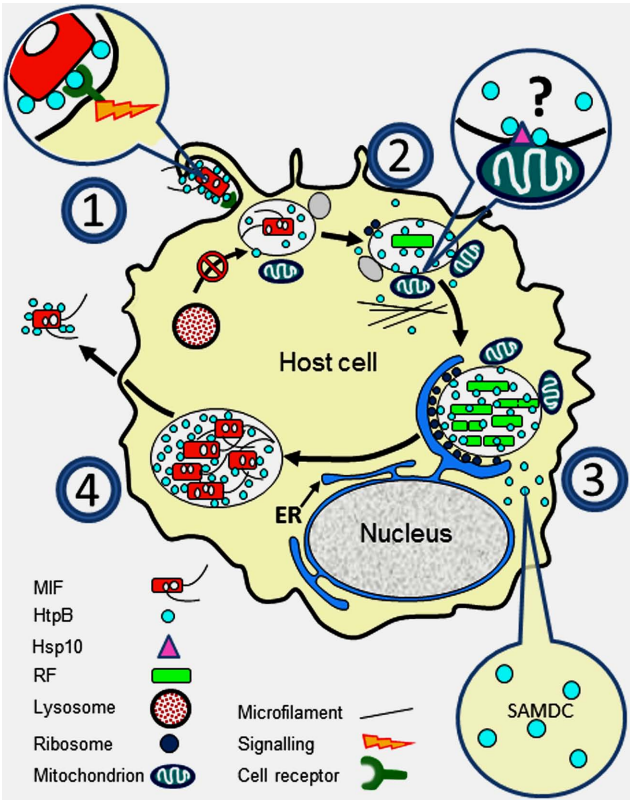

FIGURE 1 | Surface exposed or released HtpB accompanies *L. pneumophila* along its growth cycle in host cells. (1) Extracellular *L. pneumophila* upregulates expression of HtpB in the presence of host cells (see Links Between HtpB and *L. pneumophila* Virulence), and the interaction of surface-exposed HtpB with cell receptors (Inset 1) triggers a signal leading to internalization (see Surface-Exposed HtpB Acts as an Invasion Factor). (2) Internalized legionellae associate with ER-derived vesicles, attracts mitochondria, and inhibit fusion with lysosomes. HtpB bound to beads is sufficient to mimic the last two events (see Surface-Exposed HtpB Alters Organelle Traffic). HtpB reaches the cytoplasm of the host cell where it could alter the actin cytoskeleton (Inset 2). The mechanism by which HtpB attracts mitochondria is unknown, but alteration of actin fibers and tethering via mitochondrial Hsp10 could be involved (see HtpB in the Eukaryotic Cytoplasm has Several Protein Targets). (3) During replication, released HtpB accumulates in the LCV from which it could reach the host cell cytoplasm (see Links Between HtpB and *L. pneumophila* Virulence and HtpB is Found in Extracytoplasmic Locations). Inset 3: HtpB in the cytoplasm of host cells (mammalian and amebal) interacts with SAMDC to potentially increase the intracellular pool of polyamines (see HtpB in the eukaryotic cytoplasm has several protein targets). (4) As *L. pneumophila* differentiates into MIFs, the amount of HtpB associated with the cell envelope and bacterial cell surface increases (see HtpB is Found in Extracytoplasmic Locations). As the LCV ruptures, large amounts of HtpB are likely released together with MIFs. Immunomodulatory effects (see Immunological Studies with HtpB) can be triggered by HtpB at any stage of the cycle. Key: ER, endoplasmic reticulum; RF, replicative form; MIF, mature infectious form; SAMDC, *S*-adenosyl methionine decarboxylase.

Monoclonal antibodies and the unique epitopes of HtpB

Several monoclonal antibodies were raised against HtpB once it was available as a purified protein (Helsel et al., 1988; Sampson et al., 1991; Steinmetz et al., 1991). These early monoclonal antibodies demonstrated that HtpB possesses epitopes cross-reactive with many other Group I chaperonins, as well as HtpB-specific epitopes. Monoclonal antibody GW2X4B8B2H6 (Helsel et al., 1988) does not cross-react with many Group I chaperonins (except for a few, including the *Bordetella* Cpn60), and recognizes the C-terminus of HtpB (Hoffman et al., 1989). We have widely used this antibody to monitor expression of recombinant HtpB. Monoclonal antibody 2125 (Steinmetz et al., 1991) is highly specific for HtpB and does not cross-react with any other bacterial chaperonin tested. Therefore, 2125 has been used as a tool for the rapid identification of *Legionella* spp. (Steinmetz et al., 1992). But our interest here is focused on the screening method used by Steinmetz et al. (1991) to identify their monoclonal antibodies, because they used whole, live, non-permeabilized, non-fixed cells attached to wells of 96-well ELISA plates, which, again, implied that HtpB was surface exposed in (or easily released by) *Legionella*. However, these investigators could not detect *Legionella* whole cells by immunofluorescence microscopy. Another interesting finding of Steinmetz et al. (1991) is that not all the *L. pneumophila* strains tested had surface-exposed HtpB, in spite of showing abundant HtpB after sonication. In conclusion, experimentation with monoclonal antibodies against HtpB has clearly shown that HtpB has unique structural regions not found in other Group I chaperonins, and also suggested that HtpB is surface exposed in some strains of *L. pneumophila*.

Early molecular biology experiments with HtpB

Paul S. Hoffman's lab was the first to clone and express the *L. pneumophila htpAB* operon in *E. coli* (Hoffman et al., 1989) and a year later, the nucleotide sequence of *htpB* was published almost simultaneously by Sampson et al. (1990) and Hoffman et al. (1990). There was good agreement between the two published DNA sequences of *htpB*, but only Hoffman et al. (1990) reported the sequence and gene organization of the *htpAB* operon. The expression of ectopic HtpB in *E. coli* also allowed Hoffman et al. (1989) to determine that HtpB could not complement a temperature-sensitive GroEL defect in *E. coli* strain CG218 [*groEL100*(Ts)]. This is an important experimental result because it indicated, at the molecular level, that GroEL and HtpB are not functionally equivalent.

Links between HtpB and L. pneumophila virulence

Hoffman et al. (1990) showed by immunofluorescence microscopy that HtpB is detectable on the surface of the virulent *L. pneumophila* Philadelphia-1 strain SVir suspended in Dulbecco-modified Eagle's medium (DMEM). In contrast, surface-exposed HtpB was only weakly detectable on the salt-tolerant avirulent derivative AVir suspended in DMEM. Clearly, only virulent legionellae suspended in DMEM had the ability to display HtpB on their cell surface, an observation that provided the first link between HtpB and virulence. These investigators also showed that HtpB is abundantly expressed (and released) in *L. pneumophila*-infected HeLa cells, which were immuno-labeled with an intense diffuse pattern (rather than a particulate one), suggesting that HtpB was free in the LCV where this bacterium replicates (Hoffman et al., 1990). Its abundant release in the LCV also suggested that HtpB might play a role in the intracellular establishment of *L. pneumophila*.

An early response of *L. pneumophila* strain 2064 to the presence of host cells involves *de novo* synthesis of increasing amounts of HtpB (Fernandez et al., 1996; see Induction of HtpB Expression by Heat Shock and Presence of Host Cells below). However, an isogenic, salt-tolerant, avirulent derivative of 2064 was unable to respond, and showed no *de novo* synthesis of HtpB in the same experimental conditions used for 2064 (Fernandez et al., 1996). This observation provided an additional link between HtpB and *L. pneumophila* virulence, and suggested that HtpB might be required at an early stage of the infection process, even before *L. pneumophila* is internalized. In conclusion, the abilities to produce new HtpB in response to host mammalian cells, and display HtpB on the bacterial cell surface, are lost in avirulent legionellae.

Induction of HtpB expression by heat shock and presence of host cells

High temperature protein B is induced by heat shock. Increased levels of HtpB were detected in *L. pneumophila* (Lema et al., 1988) and in *L. pneumophila* and *E. coli* (Hoffman et al., 1989) upon temperature increases. However, the maximum increase in HtpB expression upon heat shock was ~twofold, and at all temperatures tested HtpB remained as one of the most abundant proteins in *L. pneumophila*. This constitutes a pattern of heat shock that is different from the pattern typically seen in other bacteria (e.g., *E. coli* as shown in Hoffman et al., 1989), where the basal levels of chaperonin are low and a sharp increase is observed at high temperatures. Clearly, HtpB is not a typical Hsp in *L. pneumophila*. In addition, HtpB seems to be induced in virulent *L. pneumophila* by the presence of mammalian host cells (monocytes and L929 cells), as demonstrated by Fernandez et al. (1996) using pulse radiolabeling in cycloheximide-treated, *Legionella*-infected cells. The induced synthesis of new HtpB did not require bacterial internalization (inhibited with cytochalasin D), suggesting that contact with host cells was sufficient to trigger the response. Finally, Fernandez et al. (1996) determined by immunoelectron microscopy that HtpB epitopes were present on the phagosomal membrane and the cytoplasm of the infected cell.

Immunological studies with HtpB

From its very discovery, HtpB was regarded as strongly antigenic. Thus, investigators focused on establishing whether HtpB was a protective antigen, potentially applicable for vaccination against LD. Immunization with HtpB protected guinea pigs from a lethal aerosol challenge with *L. pneumophila*, and the protection was mediated by a strong cellular response (Blander and Horwitz, 1993). These authors wondered how HtpB is released intracellularly to elicit a cellular response, and performed immunoelectron microscopy localization studies (reported as unpublished data) indicating that HtpB was abundantly released into phagosomes of infected human monocytes. Finally these authors also mentioned that HtpB is released into the supernatant of liquid *L. pneumophila* cultures, suggesting it could be a secreted protein.

Weeratna et al. (1994) also immunized guinea pigs with HtpB, but contrary to the results of Blander and Horwitz (1993), they did not record a strong protective effect. The response to HtpB immunization was mainly humoral. However, guinea pigs that recovered from a *L. pneumophila* infection showed strong cutaneous delayed-type hypersensitivity, as well as strong lymphocyte proliferative responses to HtpB, suggesting that the presentation of HtpB during infection differs from the presentation of soluble HtpB during vaccination. To date, the experimental differences observed in the protective abilities of HtpB between these two immunization studies have not been resolved.

Purified bacterial chaperonins, including HtpB, are capable of triggering the secretion of interleukin (IL)-1 and the transcription of several cytokine genes in antigen presenting cells (Retzlaff et al., 1994), an effect demonstrated to be LPS-independent. In particular, HtpB was shown to interact with macrophage cell receptors and trigger a signaling cascade that involved PKC (Retzlaff et al., 1996). The IL-1β response was greatly reduced by heat inactivation of HtpB, a treatment that would not affect LPS-induced effects (Retzlaff et al., 1996).

In summary, HtpB is highly immunogenic, capable of interacting with cell surface receptors on macrophages, and able to elicit immunological responses via activation of signaling cascades. These early studies with HtpB resonate with those that recognized chaperonins as an important danger signal easily recognized by antigen presenting cells (Bethke et al., 2002), as part of an immune surveillance mechanism (Zügel and Kaufmann, 1999).

Are there multiple copies of HtpB in L. pneumophila?

We would like to end this historical perspective with a brief discussion of the puzzling notion advanced by Lema and Brown (1995) that *L. pneumophila* has two HtpB chaperonins, encoded by two copies of the *htpB* gene. By SDS-PAGE, these authors showed that *L. pneumophila* has two HtpB species of different mass and protease-digestion patterns. Southern blot analysis of DNA hybridized with an *htpAB* probe showed two distinct bands. These results are in sharp conflict with those of Hoffman et al. (1989), who by Southern blot showed only one *htpAB* locus. In addition, the completed genome sequences of five different *L. pneumophila* strains (D'Auria et al., 2010), indicate that there is only one copy of the *htpAB* operon in the common lab strains of *L. pneumophila*. Our own results (see HtpB Exists in Different Forms and HtpB is Essential for *L. pneumophila* Viability below) also confirm the presence of only one *htpAB* locus in two *L. pneumophila* strains.

HtpB RESEARCH – 1998 TO DATE

The evidence presented above, reveals HtpB as an intriguing *L. pneumophila* protein that potentially moonlights as a virulence factor. There is only one copy of the *htpAB* operon in the *L. pneumophila* chromosome, which shows the typical gene organization of Group 1 chaperonins (**Figure 2**), where a single regulatory region with one σ^{32} stress promoter (recognized by RpoH) and a housekeeping σ^{70} promoter, is present upstream of the co-chaperonin gene *htpA*. The putative *htpAB* transcripts produced from each of the promoters are bicistronic. Dr. K. Brassinga (currently at the University of Manitoba, Canada) mapped three integration host factor (IHF) binding sites in the regulatory region of the *htpAB* operon. One of these IHF binding sites overlaps an UP element immediately upstream of the σ^{32} stress promoter, and has been hypothesized to be responsible for the high basal level of HtpB expression in *L. pneumophila* (unpublished results). Interestingly, the expression of *L. pneumophila* IHF is developmentally regulated (Morash et al., 2009), with the highest levels being present in the differentiated mature infectious forms that emerge from host cells. What follows is an account of the HtpB research performed in our lab, which has confirmed the virulence functions of this intriguing chaperonin.

HtpB is found in extracytoplasmic locations

To substantiate previous (mostly anecdotal) suggestions that HtpB is found on the cell surface of *L. pneumophila* (see Discovery and Initial Characterization, Monoclonal Antibodies and the Unique Epitopes of HtpB, and Links Between HtpB and *L. pneumophila* Virulence), Garduño et al. (1998a) undertook a detailed ultrastructural study based on immunoelectron microscopy, to define the localization of HtpB in *L. pneumophila*. Using a polyclonal antibody raised against the purified ectopic HtpB expressed in *E. coli*, and the monoclonal antibody GW2X4B8B2H6 (Helsel et al., 1988), it was found that ∼58% of the HtpB epitopes detected by immunoelectron microscopy were extracytoplasmic. An additional ∼16% of the epitopes were found in the cytoplasmic membrane. Among the extracytoplasmic HtpB epitopes, ∼30–40% were associated with the outer membrane or on the bacterial cell surface. In addition, the polyclonal antibody labeled the surface of whole, unfixed *L. pneumophila* cells, confirming the presence of surface-exposed HtpB. To date, similar results have been obtained with the Philadelphia-1 strains Svir, Lp02, and JR32, and the Olda clinical isolate 2064. Garduño et al. (1998a) also demonstrated that *L. pneumophila* abundantly releases HtpB in the LCV while replicating in HeLa cells, confirming the previous suggestion of Blander and Horwitz (1993) that HtpB accumulates in phagosomes, and explaining the diffuse labeling pattern observed in *L. pneumophila*-infected HeLa cells by Hoffman et al. (1990).

This immunolocalization study also showed that in *E. coli* the GroEL and HtpB chaperonins largely reside in the cytoplasm. Thus, we hypothesized that *L. pneumophila* must have

FIGURE 2 | Upstream regulatory region and gene organization of the *L. pneumophila htpAB* operon. Diagram (not at scale) showing the known regulatory elements in the promoter region and the putative bicistronic transcripts (thin, right angle arrows) produced from the σ^{32} stress promoter and the housekeeping σ^{70} promoter. The dotted thick line represents an UP element, and the solid thick lines represent integration host factor binding sites. SD, Shine–Dalgarno sequence. The regulatory mechanism that controls the expression of the *htpAB* operon is not well understood.

a translocation mechanism, not present in *E. coli*, which allows the mobilization of HtpB to extracytoplasmic locations, including the bacterial cell surface. Using a combined experimental approach involving immunoelectron microscopy, protease-sensitivity, osmotic shock, and immunoblotting we have determined that ∼1% of the total cell-associated HtpB is present in the periplasm of *L. pneumophila*, and that a functional Dot/Icm type IV secretion system is required for the surface localization of HtpB (Chong et al., 2006). That is, loss-of-function *dot* mutations led to absence of surface-exposed HtpB and its accumulation in the periplasm of *L. pneumophila*. In particular, an Lp02 Δ*dotB* mutant accumulated up to fourfold more HtpB in the periplasmic space than the parent strain Lp02 (Chong et al., 2006). We still do not know how HtpB reaches the periplasm of *L. pneumophila*, but from the periplasm it reaches the bacterial cell surface in a Dot/Icm-dependent manner (unpublished results). It is possible that the strong association of HtpB with the inner membrane of *L. pneumophila* (Gabay and Horwitz, 1985) results in its passage to the periplasm, by a mechanism similar to that described for the cell-penetrating peptides (Zorko and Langel, 2005). A similar mechanism could be invoked for the passage of HtpB across the LCV membrane (see Intracellularly Released HtpB Alters the Actin Cytoskeleton of Host Cells).

Structural changes of the bacterial cell envelope during the morphological differentiation of *L. pneumophila*, correlate with an increased level of periplasmic HtpB and its association with the outer membrane, as detected by immunogold electron microscopy (Garduño et al., 1998b) and cell fractionation (Garduño et al., 2002). Finally, Galka et al. (2008) found small amounts of HtpB among the secreted proteins of *L. pneumophila*, and a larger amount in outer membrane vesicles (OMVs). We were also able to detect HtpB in purified OMVs by immunoblot, but detection had to rely on our polyclonal HtpB-specific antibody, because monoclonal antibody GW2X4B8B2H6 was not reactive with this material, suggesting that in OMVs the C-terminus of HtpB is hidden.

Collectively, the experimental results presented in this section suggest that HtpB is clearly present in extracytoplasmic locations, and that extracytoplasmic HtpB appears to be important for *L. pneumophila* biology, including its morphological differentiation.

HtpB exists in different forms

The notion advanced by Lema and Brown (1995) that *L. pneumophila* has two HtpB chaperonins is appealing, not at the gene level, but at the protein level, mainly because in our own investigations we have often seen in SDS-PAGE gels two distinct protein bands clearly labeled with HtpB-specific antibodies. In addition, under non-reducing conditions, an additional species of HtpB with an apparent mass of 80-kDa is shown (unpublished data). This 80-kDa band is only labeled with polyclonal antibody and is not recognized by monoclonal antibody GW2X4B8B2H6, suggesting that the C-terminus of this form of HtpB is not accessible. However, when this band is excised from the non-reducing gel and then re-run in a reducing SDS-PAGE gel, a single 60-kDa HtpB band is observed, which can now be labeled with monoclonal antibody GW2X4B8B2H6. Additional evidence for the existence of post-translational modifications in HtpB, comes from the analysis of our various preparations of purified HtpB. When HtpB is purified as a recombinant protein from *E. coli*, it runs in 2-D protein gels as a series of clustered spots of slightly different isoelectric points (pI). This pattern is common in bacterial chaperonins, particularly GroEL, where the differences in pI are likely due to different levels of phosphorylation (Sherman and Goldberg, 1992). It should be considered here that, inevitably, this preparation of recombinant HtpB is mixed with GroEL, which would increase the heterogeneity of the sample. However, 2-D protein gels of the highly purified HtpB from *L. pneumophila* show a series of scattered spots of different mass and pI, all of which yield identity to HtpB by mass spectrometry (unpublished data). Thus, it is clear that HtpB experiences post-translational modifications in *L. pneumophila*, which might involve crosslinking via disulfide bonds, phosphorylation, cleavage, and(or) altered binding abilities. Some of these modifications have been documented in other bacterial chaperonins. For instance, the phosphorylated chaperonins of *E. coli* (Sherman and Goldberg, 1994), *M. tuberculosis* (Kumar et al., 2009), and *Streptomyces granaticolor* (Bobek et al., 2004) have altered binding properties, and the secreted chaperonin of *M. tuberculosis* Cpn60.2 is cleaved by the surface anchored protease Rv2224c (Rengarajan et al., 2008). We currently do not know whether the differentially processed HtpB forms are meant to have different locations or perform particular functions, but homologs of Rv2224c are not found in *L. pneumophila*.

We discovered that overexpression of HtpB in *L. pneumophila* correlates with filamentation (unpublished results). That *L. pneumophila* forms long filaments is a widely known fact, and filamentation has been previously linked to the ability of *L. pneumophila* to survive in the environment and form biofilms (Piao et al., 2006). Thus, we have identified *htpB* as the first *L. pneumophila* gene implicated in filamentation. Furthermore, HtpB expressed alone from an IPTG-induced promoter, or in combination with HtpA from its own promoter, is sufficient to induce filamentation in *E. coli*. Expression of HtpA alone from its own promoter does not induce filamentation in *E. coli* (unpublished results) The molecular mechanism that links HtpB and filamentation remains to be elucidated, but we hypothesize that it is mediated by one of the HtpB forms present in the bacterial cytoplasm (simply because in *E. coli* HtpB is confined to the cytoplasm). That is, excess HtpB could result in either sequestration or misfolding of a protein involved in cell division (Kerner et al., 2005), or stabilization/activation of a cell division inhibitor, e.g., MinD (Houry et al., 1999). Alternatively, excess *htpB* transcript could interact with other transcripts or with RNA-binding factors, modifying the expression of components of the cell division machinery. Interestingly, impairment of the *E. coli* GroEL function by temperature-sensitive mutations (Horwich et al., 1993), and severe heat shock in some bacterial species, e.g., *Aeromonas salmonicida* (Garduño et al., 1992) results in filamentation, but the mechanism involved is unknown. Since HtpB is upregulated during the interaction of *L. pneumophila* with mammalian cells (refer to Links Between HtpB and *L. pneumophila* Virulence and Induction of HtpB Expression by Heat Shock and Presence of Host Cells above, and Fernandez et al., 1996) it would be expected that the interacting legionellae would become filamentous, a phenomenon that we have observed in human macrophage lines.

HtpB is essential for L. pneumophila viability

Attempts to replace *htpB* with a kanamycin- or a gentamicin-resistance cassette repeatedly yielded negative results (Chong et al., 2009). We recovered numerous putative post-allelic replacement clones with the correct antibiotic selection phenotype, but in all clones tested we still detected HtpB by immunoblot and *htpAB* by PCR. This is not surprising because chaperonins are essential and bacteria harboring a single chaperonin gene cannot afford to lose it. However, in bacteria with multiple chaperonin genes, usually one of the genes can be deleted (refer to Protein Folding-Independent Functions of Group I Chaperonins above, and Hu et al., 2008). Therefore, we attempted to delete *htpAB* in a *L. pneumophila* mutant carrying the *groELS* operon of *E. coli* in its chromosome. Immunoblot confirmed that recombinant GroEL was expressed in the mutant at levels comparable to those of HtpB. Nonetheless, allelic exchange of *htpAB* with a gentamicin-resistance cassette was still unsuccessful, suggesting that *groELS* could not genetically complement the *htpAB* operon. Interestingly, Southern blot analysis of putative post-allelic replacement clones showing the correct antibiotic-resistance phenotype (from *L. pneumophila* carrying or not a chromosomal *groELS* operon) indicated the presence of two *htpAB* loci, one apparently intact and another with the integrated gentamicin-resistance cassette.

In summary, the *htpAB* locus is essential for the viability of *L. pneumophila*, cannot be genetically complemented by the *groELS* operon of *E. coli*, and attempts to delete it result in genetic rearrangements that seem to involve gene duplication. Not being able to obtain a Δ*htpB* mutant, and being convinced that the use of temperature-sensitive *htpB* mutants is not useful to study the protein folding-independent functions of HtpB (mainly because chaperonins fold so many important proteins in bacterial cells (Fujiwara et al., 2010) and would thus be impossible to ascribe phenotypes to either HtpB or its obligate folding substrates), we have relied on functional tests, which involve purified or recombinant HtpB, to determine whether HtpB is a *bona fide* moonlighting protein.

HtpB meets the defining characteristics of a moonlighting protein

As explained in Section "Protein Folding-Independent Functions of Group I Chaperonins" above, a moonlighting protein performs two different roles when it is in different cellular locations or in different molecular environments. If HtpB is found in cytoplasmic and extracytoplasmic locations, as well as associated with the cytoplasmic membrane of *L. pneumophila*, we wondered whether it would play different functional roles according to its location. In the following subsections we will describe HtpB as a multifunctional protein that according to its location and molecular environment plays different roles.

Surface-exposed HtpB acts as an invasion factor. The HtpB found on the legionellae surface (as confirmed by its susceptibility to trypsin and neutralization by antibodies) turned out to play the role of an invasion factor, mediating the internalization of *L. pneumophila* by HeLa cells (Garduño et al., 1998b). Five different lines of experimental evidence collectively indicated that surface-exposed HtpB interacts with specific receptors on HeLa cells promoting both attachment and internalization of *L. pneumophila* (or inert HtpB-coated latex microbeads). We attempted to identify the HeLa cell receptor for HtpB, and focused upon an ∼70-kDa HeLa cell membrane protein pulled down by HtpB-coated beads. In addition, a protein band of the same molecular size was labeled in an overlay membrane assay where HeLa cell membrane proteins separated by SDS-PAGE were transferred to nitrocellulose, incubated with purified HtpB, and subsequently washed and labeled with an HtpB-specific antibody (unpublished data). Although we were not able to unequivocally identify this protein, others have reported a number of receptors for Group I chaperonins, which include Toll-like receptor (TLR)-4 (Ohashi et al., 2000), TLR-2 (Nussbaum et al., 2006; Vabulas et al., 2001), the β2 integrin CD18 (Long et al., 2003), and cellular prion protein (Watarai et al., 2003). Regardless of the identity of the HeLa cell receptor for HtpB, a signaling event was clearly involved in the phagocytosis of HtpB-coated beads into a tight phagosome (Garduño et al., 1998b).

Surface-exposed HtpB alters organelle traffic. In HeLa cells, the internalized HtpB-coated beads appeared to traffic differently than bovine serum albumin (BSA)-coated beads, so we engaged in the characterization of trafficking events that followed the internalization of HtpB-coated beads. It took several years to complete a series of experiments that substantiated the notion that HtpB-coated beads indeed have a unique trafficking in relation to beads coated with GroEL or BSA. These experiments showed that internalized HtpB-coated beads attract mitochondria in CHO cells and macrophages, delay the fusion of phagosomes with Texas red-ovalbumin-labeled lysosomes in CHO cells and bone marrow-derived mouse macrophages, and induce a transient disappearance of stress fibers in CHO cells (Chong et al., 2009). Therefore, the purified HtpB attached to inert microbeads is capable of mimicking 3 post-internalization events that typify the early trafficking of *L. pneumophila*, and constitutes the first *L. pneumophila* protein that alone is sufficient to recruit mitochondria.

Outer membrane vesicles purified from *L. pneumophila* cultures and attached to microbeads via antibodies that recognize the *L. pneumophila* lipopolysaccharide, were able to transiently inhibit phagosome–lysosome fusion (Fernandez-Moreira et al., 2006). Since HtpB is present in OMVs in a unique form (see HtpB is Found in Extracytoplasmic Locations above), and HtpB-coated beads also transiently inhibit phagosome–lysosome fusion, we are tempted to speculate here that the HtpB present in OMVs might moonlight as a factor that delays fusion with lysosomes.

Intracellularly released HtpB alters the actin cytoskeleton of host cells. Since our intention was to conduct a direct comparison between the effects of HtpB from without (as it would be presented by extracellular *L. pneumophila*) and its effects from within (as it would be presented by intracellular *L. pneumophila* during infection), we needed a host cell type that would interact well with, and internalize, exogenously added protein-coated beads while being also amenable for genetic manipulation to express ectopic HtpB in their cytoplasm. CHO cells met these requirements, and therefore our experiments were focused on the stably transfected CHO-AA8 Tet-Off cells (Clontech-BD, Palo Alto, CA, USA) carrying an integrated vector (pTRE2*hyg*) containing the *htpB* gene.

These cells are subsequently referred to as CHO-*htpB* cells (Chong et al., 2009). The aforementioned HtpB effects from without (see Surface-Exposed HtpB Alters Organelle Traffic above), were investigated in CHO-*htpB* cells not expressing ectopic HtpB to which we added beads coated with HtpB, or the control proteins BSA and GroEL.

The first experiment conducted with CHO-*htpB* cells to address effects from within, was to determine whether or not HtpB is indeed presented from within as a protein that reaches the infected cell's cytoplasm. Using fusions with the translocation reporter gene *cyaA* (encoding the calmodulin-dependent *Bordetella pertussis* adenylate cyclase subunit) we were able to determine that during infection of CHO-*htpB* cells with *L. pneumophila* strains Lp02 and JR-32, HtpB reaches the cytoplasm of the infected cell. These results were confirmed in U937-derived macrophages (unpublished data). Therefore, we confidently proceeded to investigate the effects of HtpB from within, which required induction of ectopic HtpB in CHO-*htpB* cells in the absence of doxycycline.

The ectopically expressed HtpB in CHO-*htpB* cells (presented from within as the HtpB released from the LCV during infection) induced the disappearance of stress fibers and the relocalization of polymerized actin at the periphery of the cell. The same effect (but transiently) was produced by HtpB presented from without (see Surface-Exposed HtpB Alters Organelle Traffic), indicating the ability of HtpB to trigger the same effect from opposite sides of a membrane. The most convincing explanation for this observation is that HtpB is capable of triggering a signaling pathway by interacting with membrane receptors, and that this interaction involves the integration of HtpB in the membrane. Alternatively, it is possible that the HtpB present in the eukaryotic cytoplasm acts as a foreign protein folding machine that could trigger conformational changes in specific host factors and initiate signaling cascades. In this respect, it should be recalled that (i) several chaperonin receptors do exist (see Surface-Exposed HtpB Acts as an Invasion Factor), (ii) chaperonins, in general, have demonstrated their ability to act as signaling molecules (Ranford et al., 2000), (iii) chaperonins can integrate into membranes (Török et al., 1997), and (iv) chaperonins can interact with small GTP-binding proteins like Ras (Ikawa and Weinberg, 1992). We have hypothesized that the alteration of actin microfilaments could be involved in the altered trafficking of mitochondria in *L. pneumophila*-infected cells, and in cells with internalized HtpB-coated beads (Chong et al., 2009).

HtpB in the eukaryotic cytoplasm has several protein targets. To search for eukaryotic proteins that could potentially interact with the intracellularly released HtpB, we expressed HtpB in the genetically tractable eukaryote *Saccharomyces cerevisiae*, and also conducted a series of yeast two-hybrid assays.

In *S. cerevisiae*, HtpB (but not GroEL nor the yeast Hsp60) induced pseudohyphal growth, a yeast phenotype assumed during sexual reproduction that is tightly regulated by a Ras2-controlled signaling cascade (Chong et al., 2006). That HtpB uses this signaling cascade was demonstrated by showing that a *S. cerevisiae* Δ*ras2* mutant does not filament upon expression of ectopic HtpB. These observations were followed by a series of yeast 2-hybrid assays against a yeast genomic library and a HeLa cell cDNA library, where HtpB (bait) was shown to interact with yeast *S*-adenosyl methionine decarboxylase (SAMDC), mammalian merlin-associated protein, and mitochondrial Hsp10 (Chong et al., 2006, and unpublished results). The hit with SAMDC was particularly meaningful in relation to pseudohyphal growth, mainly because alterations in intracellular levels of polyamines had been previously correlated with fungal filamentation (Herrero et al., 1999). We cloned *SPE.2*, the yeast gene that encodes SAMDC, and determined that its overexpression in *S. cerevisiae* also induced pseudohyphal growth, a result that validated SAMDC as a target of HtpB, and linked polyamines to HtpB and pseudohyphal growth signaling in *S. cerevisiae*. It was puzzling, however, that SAMDC was not identified in the yeast 2-hybrid screening of the HeLa cDNA library, but we have recently obtained evidence for the interaction of HtpB with mammalian and amebal SAMDC, by far western and dot blot (unpublished results). The fact that SAMDC is part of the mechanism by which HtpB effects intracellular signaling and filamentation in yeast, clearly established a link between HtpB and polyamines. Therefore, we wondered whether polyamines have a physiological impact on *L. pneumophila*.

It turns out that polyamines enhance the intracellular growth of *L. pneumophila*, whereas the inhibition of their synthesis impairs such growth. In addition, according to our bioinformatics analysis of the *L. pneumophila* genomes, *L. pneumophila* lacks 10 of the 12 enzymes described so far that are involved in the biosynthesis of polyamines in bacteria. This was a striking finding suggesting that *L. pneumophila* is incapable of synthesizing all polyamines, and that it might acquire them directly from its hosts. Therefore, we have hypothesized that one of the functions performed by the HtpB released into the cytoplasm of host cells could be to (through its interaction with SAMDC) increase the intracellular pool of polyamines, which *L. pneumophila* subsequently takes up. We are currently testing this hypothesis by: (i) measuring the levels of polyamines in CHO-*htpB* cells expressing and not expressing HtpB, as well as in *L. pneumophila*-infected cells, and (ii) determining whether HtpB extends the half-life of mammalian or amebal SAMDC, protecting it from early natural degradation. For now the role of polyamines on the physiology of *L. pneumophila*, and the hypothetical role of HtpB in the process constitutes an unfolding story.

As for the interactions with merlin-associated protein and Hsp10, future investigation awaits to elucidate their meaning. However, both interactions could have potential implications for the already identified effects of HtpB in mammalian cells. That is, merlin-associated protein is a member of the band 4.1 superfamily (Takeuchi et al., 1994) considered microfilament reorganizers. The protein Merlin itself is closely related to ezrin, radixin, and moesin, which are involved in the organization of cortical actin (McClatchey and Fehon, 2009). An HtpB interaction with these proteins is certainly relevant to the redistribution of actin filaments in CHO cells exposed to HtpB-coated beads and in CHO-*htpB* cells expressing HtpB (see Intracellularly Released HtpB Alters the Actin Cytoskeleton of Host Cells above). However, any specific involvement is yet to be demonstrated. On the other hand, an interaction with mitochondrial Hsp10 could be relevant to the recruitment of mitochondria by HtpB-coated beads (see Surface-Exposed HtpB Alters Organelle Traffic above) simply because Hsp10 has been

Table 1 | Identified functions of the *L. pneumophila* chaperonin, HtpB, according to its location in the bacterial cell and in the host cell.

HtpB location	Identified functions (confirmed or hypothetical)	Reference(s)
Bacterial cytoplasm	Protein folding (hypothetical based on essentiality)	Chong et al. (2009)/UR
	Filamentation factor (confirmed)	
Bacterial inner membrane	Lipochaperonin (hypothetical)	Török et al. (1997)
Bacterial outer membrane and bacterial surface	Invasion factor (confirmed)	Chong et al. (2009)/Garduño et al. (1998b)/
	Signaling molecule (confirmed)	Retzlaff et al. (1994)
	Immunomodulator (confirmed)	
Bacterial OMVs	Inhibition of phagosome-lysosome fusion (hypothetical)	UR
Microbead surface (as a purified protein)	Recruitment of mitochondria (confirmed)	Chong et al. (2009)
	Alteration of actin cytoskeleton (confirmed)	
LCV membrane	Recruitment of mitochondria (hypothetical)	Chong et al. (2009)
	Alteration of actin cytoskeleton (hypothetical)	
Host cell cytoplasm	Alteration of actin cytoskeleton (confirmed)	Chong et al. (2009)/UR
	Modulation of polyamine levels (hypothetical)	
	Intracellular signaling (hypothetical)	

UR, unpublished results.

detected on the surface of mitochondria, as well as in other extra-mitochondrial locations where Hsp10 moonlights as the early pregnancy factor (Sadacharan et al., 2001). This is not entirely surprising since Hsp10 is a mitochondrial protein whose encoding gene resides in the cell nucleus, and it is synthesized in the eukaryotic cytosol, from where Hsp10 needs to be imported into the mitochondria (Ryan et al., 1997). While mitochondrial protein import is mostly co-translational, it is entirely possible that some Hsp10 molecules could stay on the mitochondrial surface (bound to the import apparatus) after translation, and therefore be available to interact with HtpB.

AN INTEGRATED FUNCTIONAL MODEL FOR HtpB

The identified functions of HtpB (both confirmed and hypothetical) are summarized in **Table 1**. Based on these functions we have envisioned the following model to explain how HtpB moonlighting activities might impact the biology and pathogenesis of *L. pneumophila* (**Figure 1**): HtpB in the bacterial cytoplasm meets the essential protein folding needs of *L. pneumophila* helping in adaptation to stress and mounting responses to potential hosts. At the same time, elevated levels of HtpB in the bacterial cytoplasm correlate with filamentation, a phenotype that seems to favor the survival of *L. pneumophila* in the aquatic environment. As the major cytoplasmic membrane protein of *L. pneumophila*, HtpB could fulfill a lipochaperonin function (Török et al., 1997). Surface-exposed HtpB, which increases in the presence of mammalian host cells, as well as during the morphological differentiation of *L. pneumophila* into mature infectious forms, interacts with eukaryotic cell receptors and mediates attachment to and invasion of host cells. The abundantly released HtpB in the lumen of early phagosomes and LCV has no identified functions, as yet, but possibly it is from this compartment that HtpB reaches the cytoplasm of host cells, either via OMVs (see HtpB is Found in Extracytoplasmic Locations), or by direct passage through the LCV membrane (Zorko and Langel, 2005). It is in the cytoplasm of host cells (either free in the cytosol, or bound to the LCV membrane) that HtpB mediates recruitment of mitochondria, alters the actin cytoskeleton of the host cell, and putatively increases the intracellular pool of polyamines.

The study of HtpB functions, which are not seemingly shared by other Group 1 chaperonins, promises to increase our general understanding of chaperonin biology and the evolution of intracellular pathogens that have adapted to the human host by using an ancient protein tool.

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Type IVB secretion systems of *Legionella* and other Gram-negative bacteria

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Type IV secretion systems (T4SSs) play a central role in the pathogenicity of many important pathogens, including *Agrobacterium tumefaciens*, *Helicobacter pylori*, and *Legionella pneumophila*. The T4SSs are related to bacterial conjugation systems, and are classified into two subgroups, type IVA (T4ASS) and type IVB (T4BSS). The T4BSS, which is closely related to conjugation systems of IncI plasmids, was originally found in human pathogen *L. pneumophila*; pathogenesis by *L. pneumophila* infection requires functional Dot/Icm T4BSS. A zoonotic pathogen, *Coxiella burnetii*, and an arthropod pathogen, *Rickettsiella grylli* – both of which carry T4BSSs highly similar to the *Legionella* Dot/Icm system – are evolutionarily closely related and comprise a monophyletic group. A growing body of bacterial genomic information now suggests that T4BSSs are not limited to *Legionella* and related bacteria and IncI plasmids. Here, we review the current knowledge on T4BSS apparatus and component proteins, gained mainly from studies on *L. pneumophila* Dot/Icm T4BSS. Recent structural studies, along with previous findings, suggest that the Dot/Icm T4BSS contains components with primary or higher-order structures similar to those in other types of secretion systems – types II, III, IVA, and VI, thus highlighting the mosaic nature of T4BSS architecture.

Keywords: *Legionella, Coxiella, Rickettsiella,* Dot/Icm, type IVB secretion, type IV secretion, protein secretion, conjugation

INTRODUCTION

Legionella pneumophila is the causative agent of the acute pneumonia known as legionellosis or Legionnaires' disease. The genus *Legionella* was established in 1979 after a large outbreak at the American Legion convention in Philadelphia 3 years earlier (Brenner et al., 1979). *L. pneumophila* enters eukaryotic host cells using the host cells' own mechanisms: phagocytosis or macropinocytosis (Horwitz, 1984; Watarai et al., 2001b; Peracino et al., 2010). Early studies by Marcus Horwitz and his colleagues revealed that the *Legionella*-containing vacuoles (LCVs) escape from endocytic maturation processes, including the acidification of LCVs and LCV–lysosome fusion (Horwitz, 1983; Horwitz and Maxfield, 1984). The LCVs acquire endoplasmic reticulum (ER)-like properties over time and *L. pneumophila* multiply within the resulting replicative niche (Horwitz and Silverstein, 1980; Swanson and Isberg, 1995; Tilney et al., 2001).

By 1998, taking advantage of the available forward genetic approach, Ralph Isberg's and Howard Shuman's laboratories independently discovered ~20 *L. pneumophila* genes that are required for the establishment of the replicative niche, intracellular replication, or macrophage killing (Berger et al., 1994; Brand et al., 1994; Segal and Shuman, 1997; Andrews et al., 1998; Purcell and Shuman, 1998; Segal et al., 1998; Vogel et al., 1998). These genes have been named independently by the two groups: *dot* (for defect in organelle trafficking) or *icm* (for intracellular multiplication). The *dot/icm* genes were believed to encode a type IV secretion system (T4SSs) – defined as bacterial macromolecular transport systems closely related to conjugation systems – because (a) several Dot/Icm proteins have limited sequence-level similarity to components of conjugation systems; and (b) *L. pneumophila* has the Dot/Icm-dependent ability to mediate the conjugal transfer of IncQ plasmids (Segal and Shuman, 1997; Segal et al., 1998; Vogel et al., 1998). When the nucleotide sequences of IncI plasmids *col*Ib-P9 and R64 became available to the public in 1999–2000, it became obvious that the *dot/icm* genes are closely related to the *tra/trb* genes of these IncI plasmids (Segal and Shuman, 1999b; Komano et al., 2000; Wilkins and Thomas, 2000). It had been known that T4SSs play central roles as DNA or protein transporters in the pathogenicity of many important pathogens, including *Agrobacterium tumefaciens*, *Bordetella pertussis*, *Brucella* species, and *Helicobacter pylori*. In 2002, *L. pneumophila* was shown to deliver a protein substrate RalF to the host cell cytosol using the Dot/Icm system; this established that the Dot/Icm system can translocate effector proteins (Nagai et al., 2002). Since then, over a hundred *L. pneumophila* proteins have been experimentally shown to be translocated via the Dot/Icm system (Hubber and Roy, 2010 as a review). Together, the *dot/icm* genes encode a T4SS classified as type IVB, which is closely related to the I-type conjugation systems (Lawley et al., 2003), but is distinct from the conventional T4SSs now classified as type IVA (Christie and Vogel, 2000; Sexton and Vogel, 2002). Hereafter in this article, we use the term "type IVB secretion system (T4BSS)" to mean the secretion/conjugation system family closely related to the *Legionella* Dot/Icm system and the I-type

conjugation system of IncI conjugal plasmids, unless otherwise indicated.

Transport substrate proteins, including VirE2 and VirF of the *Agrobacterium* VirB/D type IVA secretion system (T4ASS), have C-terminal translocation signals (Vergunst et al., 2000, 2005). Likewise, RalF carries a C-terminal disordered region necessary for translocation via the Dot/Icm system (Amor et al., 2005; Nagai et al., 2005). The C-terminal signal hypothesis has been confirmed by studies on a number of *L. pneumophila* effector proteins.

Coxiella burnetii is a zoonotic pathogen and the causative agent of human Q-fever. Like *L. pneumophila*, *C. burnetii* establishes a specialized replicative compartment within host cells; the properties of lysosome-derived *Coxiella*-containing vacuoles are distinct from those of ER-derived LCVs. *C. burnetii* carries genes closely related to the *L. pneumophila dot/icm* genes (Segal and Shuman, 1999b; Sexton and Vogel, 2002; Seshadri et al., 2003). The *C. burnetii* Dot/Icm proteins, including DotB, IcmW, and IcmS, have been shown to be able to functionally substitute for their *L. pneumophila* counterparts in intracellular replication; this implies that the *Coxiella* Dot/Icm system is functional and plays a critical role in interactions with its host cells (Zamboni et al., 2003; Zusman et al., 2003). Until recently, *C. burnetii* was believed to be an obligate pathogen (Omsland et al., 2009). *L. pneumophila* has been successfully employed as a surrogate host to identify and analyze *C. burnetii* effector proteins (Pan et al., 2008; Voth et al., 2009; Chen et al., 2010).

Bacteria of the genus *Rickettsiella* are obligate intracellular pathogens of a wide variety of arthropods. A phylogenetic study of *Rickettsiella popilliae* and *Rickettsiella grylli* demonstrated that these bacteria carry genes orthologous to *dotB*, *dotO/icmB*, and *icmQ* (Leclerque and Kleespies, 2008). Analysis of the published draft genome sequence of *R. grylli* (GenBank accession no. NZ_AAQJ02000001) demonstrated that *R. grylli* encodes a nearly full set of the *dot/icm* genes on its chromosome (**Figure 1**). A phylogenetic analysis using 16S rRNA sequences placed *R. grylli* as the nearest neighbor of *C. burnetii*, under the family Coxiellaceae (Roux et al., 1997). The families Legionellaceae and Coxiellaceae are the only members of the order Legionellales, showing the intracellular pathogens *Legionella*, *Coxiella*, and *Rickettsiella* to be evolutionarily closely related bacteria, and comprise a monophyletic group.

GENETIC ORGANIZATIONS OF T4BSSs

We noticed that the current release of the BLAST non-redundant protein database (nr) contains quite a few proteins from various pathogenic and environmental bacteria that have significant similarity to *L. pneumophila* Dot/Icm proteins. This tempted us to conduct phylogenetic analyses of these Dot/Icm-related proteins.

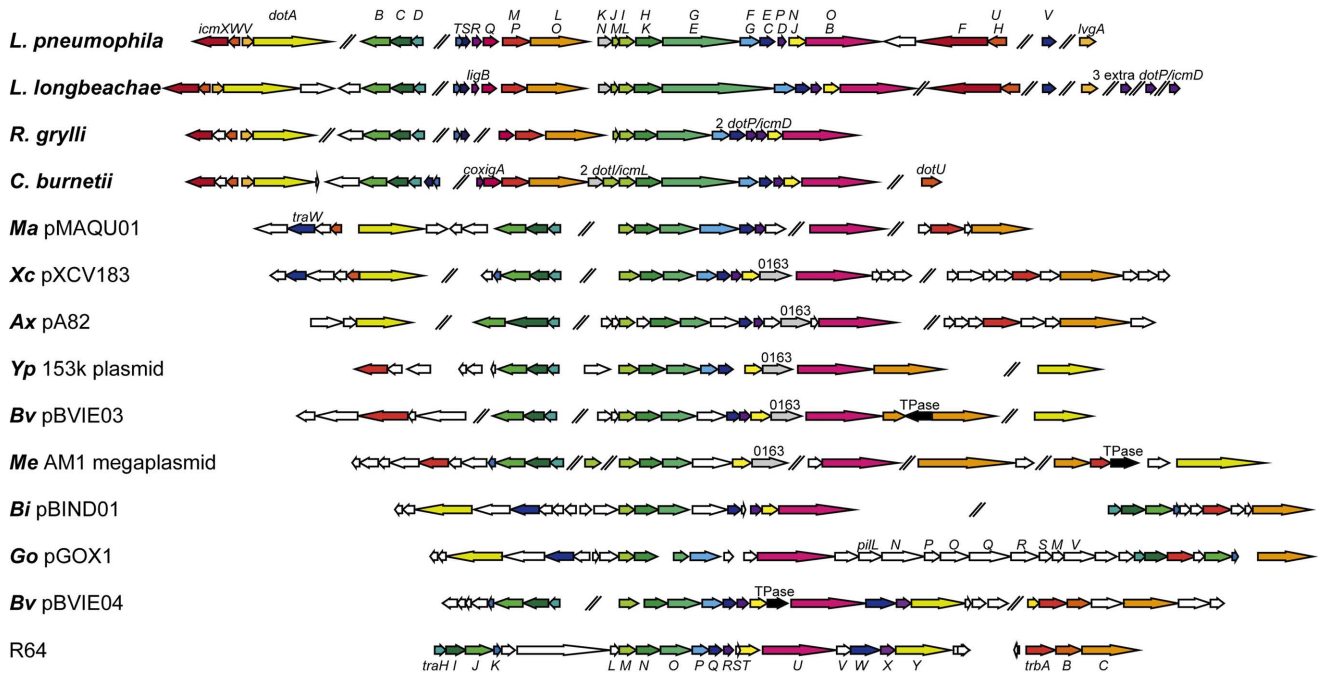

FIGURE 1 | Genetic organizations of selected T4BSSs. Genetic organizations of T4BSSs from the following bacteria or plasmids are illustrated. *Legionella pneumophila* strain Philadelphia 1 (GenBank accession no. NC_002942); *Legionella longbeachae* NSW150 (NC_013861); *Rickettsiella grylli* (NZ_AAQJ02000001); *Coxiella burnetii* RSA 493 (NC_002971); *Marinobacter aquaeolei* VT8 pMAQU01 (NC_008738); *Xanthomonas campestris* pv. *vesicatoria* str. 85-10 pXCV183 (NC_007507); *Achromobacter xylosoxidans* A8 pA82 (NC_014642); *Yersinia pseudotuberculosis* IP 31758 153 kbp plasmid (NC_009705); *Burkholderia vietnamiensis* G4 pBVIE03 (NC_009229); *Methylobacterium extorquens* AM1 megaplasmid (NC_012811); *Beijerinckia indica* subsp. *indica* ATCC 9039 pBIND01 (NC_012811); *Gluconobacter oxydans* 621H pGOX1 (NC_006672); *Burkholderia vietnamiensis* G4 pBVIE04 (NC_009228), and IncI plasmid R64 (NC_005014). ORFs designated as "0163" are conserved in several T4BSSs but not in *Legionella*, *Coxiella*, *Rickettsiella*, or R64. ORFs designated as "TPase" are putative transposase derivatives. Notably, *B. vietnamiensis* G4 harbors multiple plasmids that carry distinct T4BSSs.

We constructed a phylogenetic tree of a C-terminal domain of DotG/IcmE (862–1046; **Figure 2**), because DotG is supposed to be a central core component of the T4BSS, and the DotG domain is conserved in T4BSSs and T4ASSs (see below for details). *Legionella* species and their plasmids have been shown to encode T4ASSs (Lvh and Trb), genomic-island associated T4SSs (GI or LGI) which may function as integrative and conjugative elements (ICEs), and/or F-type conjugation systems (Segal et al., 1999; Cazalet et al., 2004, 2010; Chien et al., 2004; Glockner et al., 2008; D'Auria et al., 2010; Kozak et al., 2010; Schroeder et al., 2010). The proteins of these distinct systems are distantly homologous to DotG and are suitable as outgroups in the phylogenetic analysis. DotG of *Legionella*, *Coxiella*, and *Rickettsiella*, as well as DotG-homolog TraO of various plasmids, form distinct monophyletic clades. In addition to these, a dozen proteins from a wide variety of proteobacteria are placed in the major clade of DotG/TraO, distinct from outgroups. Similar results were obtained from a phylogenetic analysis using secretion ATPase DotB (data not shown). We looked closely at the genomic sequences of bacteria that encode DotG- and DotB-like proteins and whose genome projects have been completed, to identify other T4BSS components; in **Figure 1**, we have illustrated their genetic organizations.

Interestingly, nearly all the T4BSSs found in the analysis are encoded on plasmids. Notable exceptions include *Legionella*, *Coxiella*, and *Rickettsiella* Dot/Icm systems. It is most likely that a common ancestor of these closely related bacteria acquired a T4BSS on its chromosome, and that the T4BSS played a critical role in survival of the ancestor. The T4BSS acquisition on chromosome might be related to the alteration of life style as intracellular bacterium. Genes encoding T4BSS tend to gather in several conserved gene clusters; it appears that there is little pressure to keep them in a single locus. The conserved gene clusters include (a) *dotD–dotC–dotB (traH–traI–traJ* in I-type conjugation systems), (b) *dotM/icmP–dotL/icmO (trbA–trbC)*, and (c) *dotI/icmL–dotH/icmK–dotG/icmE (traM–traN–traO)*. Together with other genes found in all T4BSSs, including *dotA (traY)* and *dotO/icmB (traU)*, these conserved genes are expected to encode core components that play fundamental roles in transport activity. On the other hand, the genes found only in the Dot/Icm systems of *Legionella* and related bacteria may encode components that are important for life as intracellular pathogens. In the following section, we discuss T4BSS component proteins in detail, taking advantages of developing genomic information and structural insights. We do not intend to thoroughly review the type IVB effector proteins and the regulation of T4BSSs here. Please refer to excellent reviews recently published on these subjects (Segal et al., 2005; Ninio and Roy, 2007; Shin and Roy, 2008; Franco et al., 2009; Isberg et al., 2009; Hubber and Roy, 2010).

CORE COMPONENTS OF T4BSSs

THE PUTATIVE CORE COMPLEX OF THE *L. PNEUMOPHILA* Dot/Icm SYSTEM

In 2006, Joseph Vogel and his colleague reported an excellent systematic study on component proteins of the Dot/Icm system (Vincent et al., 2006b). Notably, they demonstrated that DotC, DotD, DotF/IcmG, DotG/IcmE, and DotH/IcmK were fractionated into outer membrane fractions. DotC and DotD are lipoproteins and sorted to outer membranes even in the absence of other Dot/Icm components. DotH is localized to the outer membranes, which requires the lipoproteins DotC and DotD. Thus, DotC and DotD appear to behave as a pilotin for DotH. DotF and DotG are intrinsically inner membrane proteins with single transmembrane helices. In wild-type *L. pneumophila*, DotF and DotG are fractionated into both inner and outer membrane fractions. The outer membrane fractionation of DotF and DotG requires the DotC, DotD, and DotH. These results suggest the presence of a complex spanning both inner and outer membranes that contains these five proteins, DotC, DotD, DotF, DotG, and DotH (**Figure 3**).

DotG/IcmE: THE CENTRAL COMPONENT OF THE CORE COMPLEX?

In 2009, major advances in structural research on T4ASS were achieved by Gabriel Waksman and his colleagues. They biochemically isolated the core complex spanning both inner and outer membranes from the conjugation system of an IncN plasmid pKM101 (**Figure 4A**; Fronzes et al., 2009). Furthermore, they isolated the outer membrane complex from the core complex treated with trypsin, and solved the crystal structure of the outer membrane complex (**Figure 4B**; Chandran et al., 2009). The core complex has 14-fold rotation symmetry and contains three proteins, VirB7, VirB9, and VirB10, at a 1:1:1 molar ratio. In the outer membrane complex, VirB10 faces to the central cavity and two alpha helices from each monomer are inserted into the outer membrane. VirB9 constitutes the outer lobe. VirB7 takes an extended form and wraps around the complex.

DotG is an integral membrane protein with single transmembrane helix in an N-terminal region. As described above, the C-terminal region of DotG (862–1046) is well conserved in T4BSSs including I-type conjugation systems (TraO). However, the sizes of DotG family proteins significantly vary: *Legionella*, *Coxiella,* and *Rickettsiella* DotG proteins are significantly larger than other siblings because of a variable region, which often contains penta-peptide repeats (Segal et al., 1998), between the N-terminal transmembrane and the C-terminal conserved regions. As previously suggested (Segal et al., 1998; Vogel et al., 1998), the C-terminal conserved region of DotG is significantly similar to the TrbI domain (Pfam PF03743) found in VirB10 family proteins of T4ASSs. Interestingly, the VirB10 region in the outer membrane complex of the pKM101 conjugation system corresponds nicely to the Pfam TrbI domain. The size variation of VirB10 family proteins is documented as well; for instance, Cag7/CagY of the *Helicobacter cag*PAI-associated T4ASS is far larger than the *Agrobacterium* VirB10 (Liu et al., 1999). These suggest that T4BSS has an outer membrane complex similar to that of T4ASS, and DotG is the counterpart of VirB10 in the outer membrane complex.

DotC, DotD, AND DotH/IcmK

Besides VirB10, the core complex of T4ASS also contains VirB7 and VirB9. *Agrobacterium* VirB7 is an outer membrane lipoprotein; it forms a heterodimer with VirB9 and stabilizes several VirB proteins, including VirB9 (Fernandez et al., 1996). None of T4BSS proteins has detectable sequence-level similarity to T4ASS VirB7 and VirB9. Possible candidates for T4BSS counterparts of these VirB proteins include DotC, DotD, and DotH. Like DotG, DotC, DotD, and DotH are well conserved in T4BSSs, including

FIGURE 2 | A phylogenetic tree of DotG/IcmE$_{862–1046}$. Proteins that have regions homologous to DotG/IcmE$_{862–1046}$ were selected by multiple rounds of PSIBLAST (Altschul et al., 1997) using the non-redundant protein database (nr), as of November 30, 2010. *Legionella* proteins homologous to Ti plasmid VirB10, RP4 plasmid TrbI and F plasmid TraB were incorporated in the analysis as outgroups. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA4 (Tamura et al., 2007).

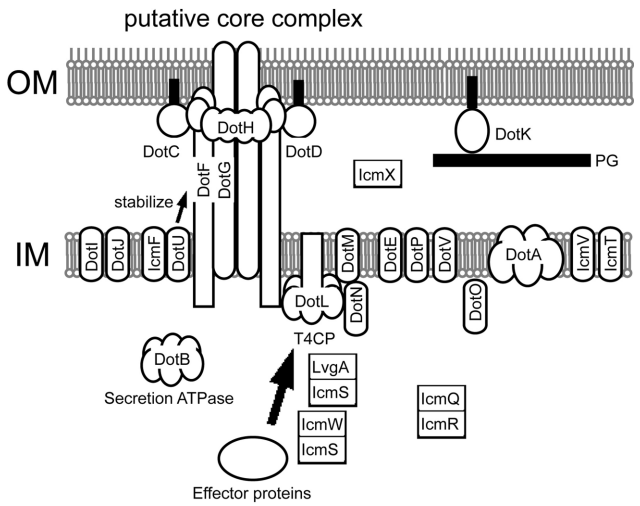

FIGURE 3 | *Legionella pneumophila* Dot/Icm T4BSS. The putative core complex containing DotC, DotD, DotH, DotG, and DotF was suggested by Vincent et al. (2006b). A possible scenario of its assembly is as follows: (1) outer membrane lipoproteins DotC and DotD recruit intrinsic periplasmic protein DotH to the outer membrane, thus forming a DotC–DotD–DotH outer membrane complex; (2) the C-terminal domain of DotG participates in the outer membrane complex, resulting in a complex spanning both inner and outer membranes; and (3) DotF participates in the core complex by binding to DotG and/or the DotC–DotD–DotH complex. Subcellular localization of Dot/Icm proteins are depicted based on lines of experimental evidence (Roy and Isberg, 1997; Zuckman et al., 1999; Coers et al., 2000; Matthews and Roy, 2000; Sexton et al., 2004a,2004b; Vincent et al., 2006b), or prediction from amino acid sequences (DotE, DotJ, DotV, and IcmT).

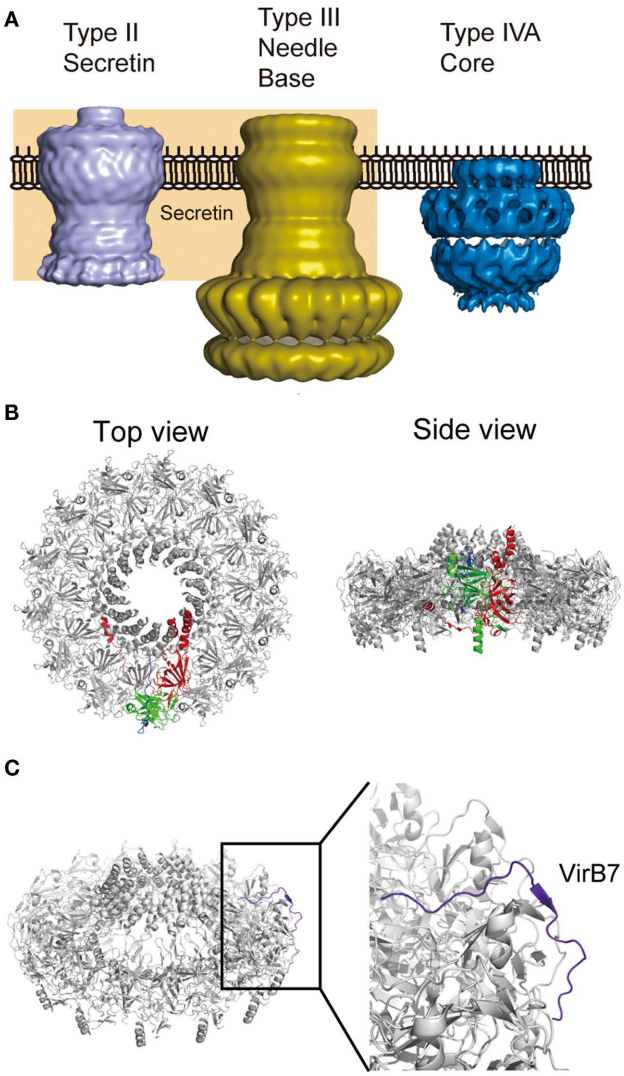

FIGURE 4 | Core complex of pKM101 T4ASS. (A) Comparison of electron micrographic structures of *Vibrio cholera* secretin GspD (type II secretin; EMDB accession EMD-1763; Reichow et al., 2010), type III injectisome isolated from Δ*invJ Salmonella typhimurium* (type III needle base; EMD-1224; Marlovits et al., 2006), and T4ASS core complex of pKM101 conjugal plasmid (type IVA core; EMD-5031; Fronzes et al., 2009). **(B)** Top and side views of pKM101 outer membrane complex (PDB accession 3JQO; Chandran et al., 2009). One of each protomer in the complex is shown in color: VirB7 (blue), VirB9 (green), and VirB10 (red). **(C)** VirB7 takes an extended form in the complex. Figures are generated using PyMol (Schrodinger, 2010) and resources deposited to indicated databases.

I-type conjugation systems (TraI, TraH, and TraN, respectively). However, the genes encoding DotH and DotG, and the genes encoding DotC and DotD are often found in separate gene clusters. In contrast, the genes encoding VirB7, VirB9, and VirB10 are typically found in single gene clusters of T4ASSs.

DotC and DotD are outer membrane lipoproteins required for the outer membrane targeting of DotH (Vincent et al., 2006b). *L. pneumophila* strains that produce lipidation-site cysteine mutant of DotC or DotD are partially defective in intracellular growth (Yerushalmi et al., 2005). The defects due to these mutations are additive, which suggest a genetic interaction between DotC and DotD. DotD consists of a disordered N-terminal domain and a globular C-terminal domain (Nakano et al., 2010). The crystal structure of the C-terminal domain is remarkably similar to the N-terminal subdomain of secretins, whereas these domain/subdomains are poorly related to each other at the amino acid sequence-level (Nakano et al., 2010; **Figure 5**). Secretins form a protein family that participates in several macromolecule translocation processes across bacterial outer membranes, notably type II and type III secretion (Genin and Boucher, 1994; Hardie et al., 1996). Secretins are integral outer membrane proteins that form substrate conduits. The protease-resistant C-terminal domain of secretins forms rings with 12- or 14-fold rotation symmetry (Opalka et al., 2003; Collins et al., 2004; Chami et al., 2005), and is embedded into the outer membrane. The N-terminal region of secretins extends into the periplasm and may interact with inner membrane partners as well as substrates. The periplasmic domain of secretins contains a most N-terminal DotD-like subdomain, followed by often-repeated Secretin_N domain(s) (Pfam 03958). Crystal structures of periplasmic domains of enterotoxigenic *Escherichia coli* (ETEC) GspD and enteropathogenic *E. coli* (EPEC) EscC secretins, from type II and type III secretion systems, respectively, containing the N-terminal DotD-like subdomains, were not captured as multimers of cylindrical shape (Korotkov et al., 2009; Spreter et al., 2009). Recently cryo-EM structure of

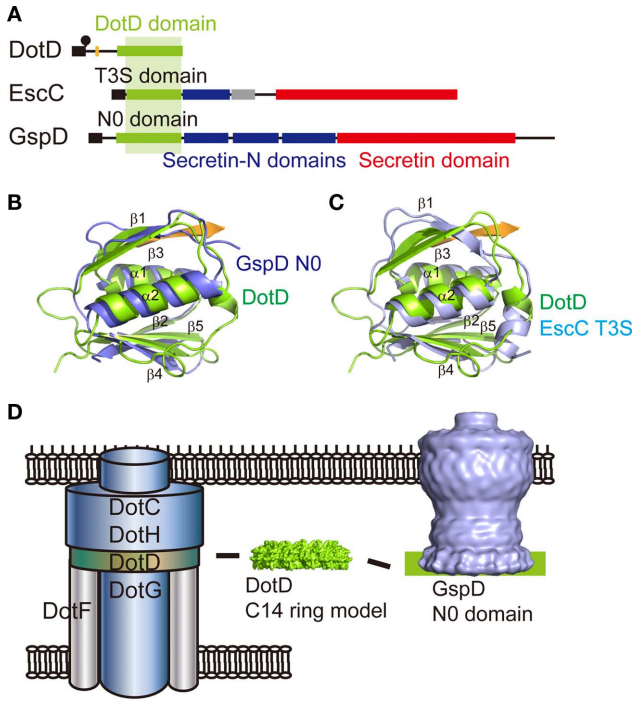

FIGURE 5 | Comparison of the C-terminal domain of DotD with secretin periplasmic subdomains. (A) Domain organizations of *L. pneumophila* DotD (Nakano et al., 2010), EPEC secretin EscC (Spreter et al., 2009), and ETEC secretin GspD (Korotkov et al., 2009). **(B)** DotD (green, PDB accession 3ADY) superimposed onto the N0 domain of ETEC secretin GspD (blue, PDB 3EZJ). **(C)** DotD (green) superimposed onto the T3S domain of EPEC secretin EscC (light blue, PDB 3GR5). **(D)** A model of T4BSS core complex. DotD may form a periplasmic ring, like the N0 domain of type II secretin.

dodecameric full-length type II secretin GspD from *Vibrio cholera* has been reported (**Figures 4A** and **5D**; Reichow et al., 2010). The periplasmic domain of *V. cholera* GspD forms a vestibule, which binds to the substrate cholera toxin and tip of pseudopilins. These findings imply that the C-terminal domain of DotD may form a periplasmic ring that is a part of the T4BSS core complex (**Figure 5D**). It should be noted that there is no counterpart of the putative DotD ring in the pKM101 core complex.

Assuming that the core complex of T4BSS carries the VirB9 counterpart, DotH is the strongest candidate. Both proteins are recruited to outer membranes, probably through interaction with cognate outer membrane lipoproteins. Structural analyses indicated that VirB9 is rich in beta-strands (Bayliss et al., 2007; Chandran et al., 2009). Similarly, the protease-resistant C-terminal domain, representing about two thirds of DotH, is predicted to be rich in beta-strands, using PHDsec (our unpublished results). Surface exposure of residues Asn-226, Pro-227, and Asp-228 of *Agrobacterium* VirB9 has been reported (Bayliss et al., 2007). Likewise, the surface exposure of DotH in *L. pneumophila* treated in certain conditions has been reported (Watarai et al., 2001a). Along these lines, the N-terminal disordered domain of DotD may serve as the VirB7 counterpart. The VirB7 in the pKM101 outer membrane complex takes an extended conformation and wraps around the complex (**Figure 4C**; Chandran et al., 2009). Mature VirB7 of pKM101 is a small peptide of 33 amino acids long, comparable in size to the N-terminal disordered region of DotD (46 amino acids), which may interact with outer membrane components such as DotH. Notably, some VirB7 family T4ASS proteins including *Helicobacter* CagT are significantly larger than *Agrobacterium* VirB7, having an extra C-terminal region whose function is not known (Alvarez-Martinez and Christie, 2009).

Alternatively, DotC and DotH may structurally correspond to the Secretin_N and Secretin domains, respectively. In this case, DotD, DotC, and DotH form an outer membrane complex functionally equivalent to secretins. The DotG outer membrane complex may associate with the secretin-like DotC–DotD–DotH complex. Clearly, further studies are called to clarify the nature of the T4BSS core complex.

DotF/IcmG

DotF is a ∼30 kDa protein composed of a small cytoplasmic domain, a transmembrane domain, and a large periplasmic domain. The periplasmic domain contains a putative coiled-coil region that is potentially responsible for protein–protein interaction, such as self-association and interaction with DotG (Vincent et al., 2006b). The DotF homologs can be found in T4BSSs, including I-type conjugation systems (TraP), whereas some T4BSSs have no protein significantly similar to DotF. Furthermore, even among homologous proteins, the region of similarity is limited mostly to C-terminal regions. Consistently, unlike other components of the putative core complex, DotF appears to be not essential for full activities of the Dot/Icm system. An internal deletion/kanamycin-cassette insertion *dotF* mutant (*icmG635*::Kan) was shown to be partially cytotoxic to HL-60 cells (Purcell and Shuman, 1998). This *dotF* mutant is not able to replicate within a protozoan host, *Acanthamoeba castellanii*, but shows only a partial defect in growth within HL-60 cells (Segal and Shuman, 1999a). Similar results were obtained using an in-frame *dotF* deletion strain (our unpublished results). Two-hybrid interactions of DotF and several effector proteins, including RalF, LidA, and Sid proteins, have been reported (Luo and Isberg, 2004). This raises the possibility that DotF is somehow involved in substrate recognition or signal transduction from the transport substrates to the T4BSS machinery.

DotI/IcmL AND DotJ/IcmM

DotI and DotJ are closely related integral inner membrane proteins, essential to Dot/Icm-dependent activities. Both proteins carry single transmembrane helices in N-terminal conserved regions. DotJ consists only of the N-terminal conserved region. DotI has an extra periplasmic domain. DotI is conserved in all T4BSSs including I-type conjugation systems (TraM). *Legionella* species encode multiple additional DotI-related proteins whose functions are not known. *C. burnetii* appears to have only the DotI-type protein but in two copies. *R. grylli* has both DotI- and DotJ-types of proteins in terms of domain organization, but the N-terminal conserved regions of both proteins are more closely related to that of DotI than that of DotJ. Other T4BSSs have only the DotI-type proteins. These suggest that the gene duplication of *dotI* occurred in a common ancestor of *Legionella*, *Coxiella*, and

Rickettsiella, and the DotJ-type protein was evolved after species differentiation.

The gene encoding DotI is associated with the genes encoding core components DotH and DotG, and the gene order *dotI–dotH–dotG* appears to be well conserved in T4BSSs. These suggest pivotal roles of the DotI in T4BSS activities. DotI (and DotJ) may form an inner membrane complex that associates with the core complex.

DotE/IcmC, DotV, AND DotP/IcmD

DotE and DotV are closely related small integral inner membrane proteins having four transmembrane helices. TraQ, of the I-type conjugation system, has the same domain organization as DotE and DotV, but the sequence-level similarity, if any, between them is difficult to detect by homology search programs (BLAST, e.g.). Proteins with domain organization similar to DotE/DotV are found in most T4BSSs; the genes are typically located immediately downstream of *dotF*. DotV is only found in *Legionella* species.

DotP, which has sequence-level similarity to DotE/DotV, appears to be a shorter version of DotE/DotV, having two transmembrane helices. As with TraQ, TraR of the I-type conjugation system has the same domain organization as DotP, but poor sequence-level similarity. Proteins having similar domain organization to DotP are found in most T4BSSs; the genes are typically located immediately downstream of *dotE*. In addition to the two transmembrane helices, DotP is predicted to have a cleavable signal sequence at the N-terminus (Purcell and Shuman, 1998). Interestingly, multiplication of the gene encoding DotP is found in *L. longbeachae* and *R. grylli* genomes.

DotO/IcmB

DotO is a large protein associated with all T4BSSs, including I-like conjugation systems (TraU). The DotO family protein is distantly homologous to VirB4 of T4ASS. Like VirB4, DotO has conserved Walker motifs for nucleotide binding (Purcell and Shuman, 1998). A cellular localization study showed *L. pneumophila* DotO to be targeted to the inner membrane (Vincent et al., 2006b). The surface exposure of DotO was reported in *L. pneumophila* that had been treated under the same conditions as those in which DotH was surface-exposed (Watarai et al., 2001a). The specific function of DotO, however, remains unclarified.

DotL/IcmO, DotM/IcmP, AND DotN/IcmJ: T4CP AND ITS PARTNERS

DotL is a member of the type IV coupling protein family (T4CP). T4CPs are associated with nearly all type IV secretion and conjugation systems, and are related to the FtsK/SpoIIIJ family DNA motor proteins (Errington et al., 2001; Aussel et al., 2002; Massey et al., 2006). The typical T4CP has transmembrane helices at its N-terminus, followed by a large cytoplasmic domain. The cytoplasmic domain carries conserved Walker motifs and forms a hexamer ring, which is anchored to the inner membranes via the N-terminal transmembrane helices (Gomis-Ruth et al., 2001, 2002). Studies on conjugation systems indicate that T4CP interacts with a nucleoprotein complex called relaxosome (de la Cruz et al., 2010). The relaxosome contains a protein called relaxase, which is a bona fide protein substrate; relaxase is translocated into recipient cells even in the absence of trailing DNA (Draper et al., 2005; Lang et al., 2010). These suggest that T4CP links protein substrates as well as DNA substrates to membrane-embedded transport apparatus of T4SSs.

T4BSSs are also associated with T4CPs DotL (TrbC in I-type conjugation systems). The genes encoding DotL are often coupled with the genes encoding DotM (TrbA), whereas the homolog of TrbB of I-type conjugation systems is missing in the Dot/Icm systems of *Legionella* and related bacteria. DotL is essential for viability of *L. pneumophila* strain Lp02 (Buscher et al., 2005). This phenomenon is strain-dependent: DotL is not essential for the viability of *L. pneumophila* strain JR32, a derivative of strain Philadelphia 1, as Lp02 is (Buscher et al., 2005). Transposon-inserted suppressor mutations of lethality through DotL disruption were mapped in several *dot/icm* genes, suggesting that lethality requires a functional Dot/Icm system (Buscher et al., 2005). Insertion mutants of DjlA (DnaJ-like protein) were also identified as suppressors for the lethality phenotype (Vincent et al., 2006a). DjlA mutants of *Legionella* species have been shown to be severely defective in intracellular growth (Ohnishi et al., 2004; Vincent et al., 2006a). These suggest involvement of the DnaK chaperone system in the assembly/quality control of the Dot/Icm apparatus. Interestingly, DotM and a cytoplasmic/inner membrane protein, DotN, showed the same essentiality as DotL, suggesting genetic interactions between these proteins (Buscher et al., 2005). Furthermore, DotL and DotM proteins are destabilized in Lp02-derived strains lacking DotL, DotM, or DotN, suggesting biochemical interactions between these proteins (Vincent et al., 2006b).

DotB: SECRETION ATPase POSSIBLY ORIGINATED FROM THE TYPE IV PILUS BIOGENESIS SYSTEM

DotB is another protein in T4BSS that carries conserved Walker motifs. Purified DotB is a hexametric ATPase in a ring shape, the activity of which is essential to Dot/Icm-dependent activities (Sexton et al., 2004b, 2005). The formation of the ring structure does not require ATP binding/hydrolysis. In *L. pneumophila* cells the majority of DotB was found to be cytoplasmic, while small amounts of DotB were recovered in inner membrane fractions.

Secretion ATPases from type II and type IV secretion systems are well conserved at the sequence-level. DotB is no exception; DotB orthologs are well conserved in T4BSSs, including I-type conjugation systems (TraJ). Interestingly, phylogenetic analyses clearly indicate that DotB has a closer relationship to PilT, the ATPase involved in the retraction of type IV pili, than to VirB11 of T4ASS (Planet et al., 2001). Type IV pilus biogenesis systems are closely related to the type II secretion system, and DotB is found in the major clade to which ATPases from type II secretion and type IV pilus biogenesis systems belong. In this connection, it is notable that many plasmids harboring T4BSSs carry type IV pilus biogenesis systems as well. Of particular interest, the gene encoding DotO and the *pil* genes encoding a type IV pilus biogenesis system of *Gluconobacter oxydans* pGOX1 comprise a single transcription unit (**Figure 1**). The common ancestor of the genes encoding DotB might originate from the co-existing type IV pilus biogenesis system. In summary, phylogenetic analyses clearly indicate that at least one component of T4BSS has a distinct origin from T4ASS, which highlights the mosaic nature of T4BSS architecture.

IcmT

IcmT is a small integral inner membrane protein. IcmT orthologs can be found in most T4BSSs, including I-type conjugation systems (TraK). The gene encoding IcmT is often associated with the gene cluster *dotD–dotC–dotB*. IcmT is essential for Dot/Icm-dependent activities, but its specific function remains to be clarified.

DotA

DotA is one of the most mysterious components of T4BSSs. DotA is required for Dot/Icm-dependent activities; historically, defective DotA mutants have been frequently used in studies of the Dot/Icm system with regard to pathogenicity of *L. pneumophila*. The DotA in *L. pneumophila* is an integral inner membrane protein composed of a cleavable signal sequence, seven transmembrane helices, a large periplasmic domain and a small cytoplasmic C-terminal domain (Roy and Isberg, 1997). DotA is well conserved at sequence-level in T4BSSs, including I-like conjugation systems (TraY), but the large periplasmic domain appears to be specific to DotA orthologs in *Legionella* species. It should be emphasized that cleavable signal sequences of integral inner membrane proteins is rarely found in prokaryotes. More surprisingly, DotA is somehow secreted into the extracellular milieu from culture-grown *L. pneumophila* in a Dot/Icm-dependent fashion (Nagai and Roy, 2001). The extracellular DotA forms ring-like oligomers with unknown 46 kDa protein. The elucidation of the specific function of DotA awaits future studies.

COMPONENTS ONLY FOUND IN Dot/Icm SYSTEMS OF *LEGIONELLA* AND CLOSELY RELATED BACTERIA

IcmF AND DotU/IcmH

IcmF and DotU were originally identified as components of the *L. pneumophila* Dot/Icm system; most T4BSSs lack them, with the notable exception of those in *Legionella* species. Homologous proteins to IcmF and DotU are prevalent in a wide variety of proteobacteria, while genes encoding them are associated with gene clusters encoding the conserved IcmF-associated homologous proteins (IAHPs). Now it has been well established that these IcmF and DotU homologs are components of the type VI secretion system (Cascales, 2008). *L. pneumophila* IcmF and DotU are partially required for Dot/Icm-dependent activities (Sexton et al., 2004a; VanRheenen et al., 2004; Zusman et al., 2004). The loss of IcmF or DotU results in decreased amounts of core components, most notably DotH and DotG (Sexton et al., 2004a). Moreover, the overexpression of DotH was shown to suppress defects in intracellular replication of the double-deletion mutant of IcmF and DotU. This suggests that IcmF and DotU work together to stabilize the core complex of the Dot/Icm system (Sexton et al., 2004a).

IcmW, IcmS, AND LvgA

IcmW, IcmS, and LvgA are small acidic cytoplasmic proteins partially required for Dot/Icm-dependent activities (Zuckman et al., 1999; Coers et al., 2000; Vincent and Vogel, 2006; Vincent et al., 2006b). These proteins are destabilized in *L. pneumophila* mutant strains lacking IcmW, IcmS, or LvgA, suggesting interactions between them (Vincent and Vogel, 2006; Vincent et al., 2006b). Binary complexes of IcmW–IcmS and of IcmS–LvgA have been consistently reported (Ninio et al., 2005; Vincent and Vogel, 2006; Cambronne and Roy, 2007). It is not clear if the ternary complex exists. The physical properties of these proteins mimic those of transport chaperones of type III secretion systems, which are often associated with cognate type III effector proteins and required for their translocation and/or stability in bacterial cells. This prompted speculation that these proteins may interact with effector proteins like the type III chaperones; several effector proteins, including WipA, SdeA, SidH, and SidG, were identified as proteins that interact with IcmW and/or IcmS (Bardill et al., 2005; Ninio et al., 2005; Cambronne and Roy, 2007). A detailed study on the effector protein SidG showed that the IcmW–IcmS complex binds to a distinct region of SidG from its C-terminal translocation signal, which probably results in a conformational change that facilitates recognition of the C-terminal translocation signal by the Dot/Icm machinery (Cambronne and Roy, 2007). Decreased levels of DotL and DotM have been seen in IcmW or IcmS mutants, which suggest potential interactions between DotL–DotM and IcmW–IcmS (Vincent et al., 2006b). The IcmW–IcmS complex may therefore play a role in recruitment of effector proteins to the transport apparatus (**Figure 3**).

Interestingly, IcmS is only found in *Legionella, Coxiella, and Rickettsiella* Dot/Icm systems, whereas IcmW is distributed beyond this scope; IcmW orthologs are found in *Marinobacter aquaeolei* VT8 pMAQU01 and *Xanthomonas campestris* pv. *vesicatoria* str. 85-10 pXCV183 as well. LvgA orthologs are found only in *Legionella* species. Taken into account their possible function facilitating substrate translocation, and the large numbers of effector proteins that *L. pneumophila* are believed to translocate, it is tempting to speculate that these proteins evolved to meet the increasing demands to translocate a wide variety of effector proteins as intracellular pathogens.

IcmQ AND IcmR

IcmQ and IcmR are cytoplasmic proteins required for Dot/Icm-dependent activities (Coers et al., 2000). IcmQ is composed of an N-terminal domain, a short linker domain, and a large C-terminal domain. Purified IcmQ tends to aggregate, which can be prevented by the addition of purified IcmR (Dumenil and Isberg, 2001). This suggests a chaperone–substrate kind of relationship between IcmR–IcmQ. Purified IcmQ associates with synthetic lipid vesicles, leading to vesicle disruption, as evidenced by the release of preloaded calcein dye (Dumenil et al., 2004). The C-terminal domain plays a primary role in membrane targeting mediated by electrostatic interactions, while the N-terminal domain may be inserted into lipid bilayers and disrupts membranes (Dumenil et al., 2004; Raychaudhury et al., 2009). The N-terminal domain also binds to IcmR, which prevents IcmQ from the stable association with lipid vesicles. Consistently in *L. pneumophila* lacking IcmR, significant amounts of IcmQ are localized to membrane fractions (Dumenil et al., 2004). Thus IcmR may have a regulatory function on IcmQ. The site of function as well as the specific function of IcmQ is unclear.

While IcmQ orthologs are found in *Legionella*, *Coxiella*, and *Rickettsiella* Dot/Icm systems, the situation regarding IcmR is more complicated. Proteins homologous to IcmR are found only in *L. pneumophila*. In other bacteria closely related to *L. pneumophila*,

the genes located at immediately upstream of the genes encoding IcmQ encode proteins functionally equivalent, but poorly related, to IcmR (so-called FIRs). It has been reported that various *Legionella* species (other than *L. pneumophila*) and *C. burnetii* carry FIRs which bind to cognate IcmQs (Feldman and Segal, 2004; Feldman et al., 2005). Structural analysis of the complex of the N-terminal domain of IcmQ (Qn) and IcmR provides insights into the molecular basis of the IcmQ–FIR interaction (Raychaudhury et al., 2009). The Qn–IcmR complex forms a four-helix bundle – two helices each from IcmQ and IcmR. Two alpha helices of IcmQ are amphipathic and the formation of the Qn–IcmR complex is mediated by hydrophobic interactions. The hydrophobic nature of residues participating in the interaction with IcmQ is conserved among IcmR and FIRs, although they are poorly related at sequence-level in general. It still remains unclear why IcmR/FIRs are so divergent compared to other Dot/Icm components.

IcmX

IcmX is a primarily periplasmic protein conserved in *Legionella*, *Coxiella*, and *Rickettsiella* Dot/Icm systems (Matthews and Roy, 2000). It has been suggested that IcmX is a distant homolog of TraW of I-type conjugation systems (Segal et al., 2005), but we are unable to follow the similarity, if any, between IcmX and TraW by homology search and phylogenetic analyses. A truncated form of IcmX was found in culture supernatant in a Dot/Icm-dependent fashion (Matthews and Roy, 2000). Its physiological meaning, as well as the specific function of IcmX, remains unclear.

IcmV

IcmV is an integral inner membrane protein conserved in *Legionella*, *Coxiella*, and *Rickettsiella* Dot/Icm systems, whose specific function remains unknown.

DotK/IcmN

DotK is an outer membrane lipoprotein found in *Legionella* species and *C. burnetii* Dot/Icm systems. Two *L. pneumophila* mutants, which carry a transposon insertion in the coding region of DotK (*icmN*3007::Kan) or a transposon insertion accompanying a partial deletion of the promoter and the coding regions of DotK (*icmN*3006::Kan), were reported to be partially defective in growth within a protozoan host, *A. castellanii* (Segal et al., 1998; Segal and Shuman, 1999a). However, the defect was not complemented with plasmids carrying DotK or DotKJIHG, which makes it difficult to interpret the data on these mutations. DotK carries the OmpA family domain (Pfam PF00691; Morozova et al., 2004), which is conserved in bacterial peptidoglycan-binding proteins – notably in an outer membrane porin OmpA, a flagellar stator MotB and peptidoglycan-associated lipoproteins (PALs). The OmpA family domain functions as a peptidoglycan-binding domain, suggesting that DotK anchors the Dot/Icm apparatus to the peptidoglycan layer.

PERSPECTIVES

Genomic and metagenomic analyses have revealed that T4BSSs are widely prevalent in bacterial realm (**Figure 1**). It is well recognized that *Legionella* and *Coxiella* Dot/Icm T4BSSs play pivotal roles in infection, while most of other T4BSSs may represent conjugation systems. *Rickettsiella* is a facultative arthropod pathogen and phylogenetically closely related to *Legionella* and *Coxiella*. Recently some pea aphids were reported to carry *Rickettsiella* species as endosymbionts which modify insect body color (Tsuchida et al., 2010). *Rickettsiella* infection appears to up-regulate production of green pigments by host insects. Although the molecular mechanisms underlying the phenomenon have not been clarified, it is tempting to guess that *Rickettsiella* T4BSS may play a role in endosymbiosis. Future studies on T4BSSs of *Legionella* and related bacteria might shed lights not only on the molecular basis of bacterial pathogenesis but also on evolutionary history from intracellular pathogens to mutualistic endosymbionts.

Structural studies on secretion systems of pathogenic bacteria including T4SSs are rapidly advancing in recent years. Together with pioneer works on characterizations of *L. pneumophila* Dot/Icm proteins, we are getting a grip on the T4BSS core complex containing DotC, DotD, DotH, DotG, and DotF (**Figures 3** and **5D**). Intriguingly, the T4BSS core complex appears to be considerably different from the core complex of pKM101 T4ASS. Sequence-level similarity is only found between C-terminal domains of DotG and VirB10. T4BSS appears to be more complicated than T4ASS; T4BSSs contain roughly twice the number of component proteins than T4ASSs. Lines of evidence now suggest the mosaic nature of T4BSS architecture: (a) secretion ATPase DotB is phylogenetically related to ATPases from type II secretion and related systems; (b) *L. pneumophila* Dot/Icm T4BSS contains the genes encoding *icmF* and *dotU*, which are now recognized to encode components of the type VI secretion system; and (c) The C-terminal domain of DotD is structurally similar to a N-terminal subdomain of secretins of type II and type III secretion systems. The nature of the T4BSS core complex as well as the roles of component proteins in type IVB secretion and I-type conjugation remain as major challenges of future studies.

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Anchors for effectors: subversion of phosphoinositide lipids by *Legionella*

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The facultative intracellular pathogen *Legionella pneumophila* replicates in free-living amoebae and macrophages within a distinct compartment, the "*Legionella*-containing vacuole" (LCV). LCV formation involves phosphoinositide (PI) glycerolipids, which are key factors controlling vesicle trafficking pathways and membrane dynamics of eukaryotic cells. To govern the interactions with host cells, *L. pneumophila* employs the Icm/Dot type IV secretion system and more than 250 translocated "effector proteins" that presumably subvert host signaling and vesicle trafficking pathways. Some of the effector proteins anchor through distinct PIs to the cytosolic face of LCVs and promote the interaction with host vesicles and organelles, catalyze guanine nucleotide exchange of small GTPases, or bind to PI-metabolizing enzymes, such as OCRL1. The PI 5-phosphatase OCRL1 and its *Dictyostelium* homologue Dd5P4 restrict intracellular growth of *L. pneumophila*. Moreover, OCRL1/Dd5P4, PI 3-kinases (PI3Ks), and PI4KIIIβ regulate LCV formation and localization of the effector protein SidC, which selectively decorates the LCV membrane. SidC and its 20-kDa "P4C" fragment are robust and specific probes for phosphatidylinositol-4-phosphate, and SidC can be targeted to purify intact LCVs by immuno-magnetic separation. Taken together, bacterial PI-binding effectors as well as host PIs and PI-modulating enzymes play a pivotal role for intracellular replication of *L. pneumophila*, and the PI-binding effectors are valuable tools for the analysis of eukaryotic PI lipids.

Keywords: amoeba, *Dictyostelium*, *Legionella*, macrophage, phosphoinositides, pathogen vacuole, type IV secretion

INTRODUCTION

The causative agent of Legionnaires´ pneumonia, *Legionella pneumophila*, replicates intracellularly in free-living amoebae and macrophages of the innate immune system. Within these phagocytic host cells, the bacteria employ a conserved mechanism to form a unique replication-permissive compartment, the "*Legionella*-containing vacuole" (LCV). Thus, amoeba and in particular the genetically tractable social soil amoeba *Dictyostelium discoideum*, are valuable models to study the mechanism of LCV formation on a molecular and cellular level (Solomon and Isberg, 2000; Steinert and Heuner, 2005; Hilbi et al., 2007; Cosson and Soldati, 2008). Within macrophages and amoebae, LCVs avoid fusion with lysosomes, but associate with mitochondria and smooth vesicles and eventually fuse with the endoplasmic reticulum (ER; Horwitz, 1983; Lu and Clarke, 2005; Robinson and Roy, 2006). To accommodate the transfer between host cells and environmental niches, *L. pneumophila* switches from a replicative to a transmissive growth phase, which involves a complex gene regulation network, including an apparent quorum sensing system (Molofsky and Swanson, 2004; Tiaden et al., 2010; Hilbi et al., 2011).

Intact LCVs from infected *D. discoideum* amoebae can be isolated and purified using a simple two-step protocol (Urwyler et al., 2010). To this end, *D. discoideum* producing the LCV and ER marker calnexin-GFP were infected with *L. pneumophila* fluorescently labeled with DsRed. Subsequently, LCVs in cell-free homogenates were isolated by immuno-magnetic separation using a primary antibody against the *L. pneumophila* "effector protein" SidC (Substrate of Icm/Dot transporter), which specifically decorates the LCV membrane, and a secondary antibody coupled to magnetic beads. The enriched LCVs were further separated by density gradient centrifugation. The proteome of purified LCVs analyzed by liquid chromatography coupled to tandem mass spectrometry (MS/MS) revealed more than 560 host proteins, including small GTPases, as well as protein or lipid kinases and phosphatases (Urwyler et al., 2009b).

Components of the LCV host cell proteome include several small GTPases of the secretory (Arf1, Rab1, Rab8) or endosomal (Rab7, Rab14) vesicle trafficking pathways (Urwyler et al., 2009b). Using GFP fusion proteins, the recruitment of the Rab GTPases to the LCV membrane was verified. While Rab8 and Rab14 have not been previously identified on LCVs, the proteome data confirmed earlier findings on LCV localization of Arf1 (Kagan and Roy, 2002), Rab1 (Derre and Isberg, 2004; Kagan et al., 2004), and Rab7 (Clemens et al., 2000). The proteome of isolated LCVs was also analyzed in another study that led to the identification of more than 150 host proteins. These include markers of the ER as well as the early and the late endosomal pathways, which are represented by the coatomer or the vacuolar H^+-ATPase, respectively (Shevchuk et al., 2009). In agreement with the notion that *L. pneumophila* modulates phagosome maturation in a sophisticated manner, the effector protein SidK has been shown to inhibit the vacuolar H^+-ATPase, thereby preventing acidification of the LCV (Xu et al., 2010). Together, these studies indicate that LCVs communicate extensively not only with the early and late secretory pathway, but also with early and late steps of the endosomal vesicle trafficking pathway (**Figure 1**).

The formation of LCVs is a robust and complex process that requires the bacterial Icm/Dot (Intracellular multiplication/Defective for organelle trafficking) type IV secretion system (T4SS; Segal et al., 2005). More than 250 different effector proteins are translocated by the Icm/Dot T4SS into the host cell, where they subvert signal transduction and vesicle trafficking pathways by targeting phosphoinositide (PI) metabolism, small GTPases, ubiquitination, microtubuli-dependent trafficking or apoptotic pathways (Brüggemann et al., 2006; Isberg et al., 2009; Urwyler et al., 2009a; Weber et al., 2009b; Hubber and Roy, 2010). While some of the effector proteins target host factors or organelles in a distance from LCVs, many effectors decorate the LCV membrane, thereby directly modulating interactions of this compartment with host vesicles or organelles. In this review, we summarize the current knowledge about how *L. pneumophila* subverts the host cell's PI metabolism to form LCVs and replicate intracellularly.

EUKARYOTIC PI METABOLISM AND ITS SUBVERSION BY INTRACELLULAR PATHOGENS

Phosphoinositide glycerolipids play a pivotal role in the regulation of eukaryotic membrane dynamics, cytoskeleton architecture, and signal transduction (De Matteis and Godi, 2004; Di Paolo and De Camilli, 2006; Michell, 2008). The phosphatidylinositol (PtdIns) moiety of these lipids contains glycerol, which is esterified with two fatty acids (usually arachidonic acid and stearic acid) and a *myo*-inositol 1-phosphate head group. The inositol carbohydrate head group of PI lipids is oriented to the cytoplasmic side of membranes and can be hydrolyzed by PI-specific phospholipase C or reversibly phosphorylated/dephosphorylated at the 3, 4, and/or 5 positions by PI kinases or phosphatases, respectively. The resulting mono- or poly-phosphorylated PIs, jointly with activated small GTPases, recruit distinct effector proteins to specific organelles and thereby co-define the identity and integrity of subcellular compartments as well as cellular membrane dynamics (Shin and Nakayama, 2004; Behnia and Munro, 2005). PtdIns-3-phosphate (PtdIns(3)*P*) or PtdIns-4-phosphate (PtdIns(4)*P*), e.g., represent "signposts" of endosomal and secretory trafficking pathways, respectively, and recruit specific effector proteins to membranes involved in these trafficking routes.

In accordance with the importance of PIs for membrane trafficking of eukaryotes, several intracellular bacteria, such as *Listeria*, *Shigella*, *Salmonella*, *Brucella*, and *Mycobacterium* spp., exploit PI metabolism to infect host cells, establish a replicative niche and subvert host cell signaling (Pizarro-Cerda and Cossart, 2004; Hilbi, 2006; Weber et al., 2009b). The subversion of PI metabolism by vacuolar pathogens has been studied in some detail in *Mycobacterium tuberculosis*, the causative agent of the chronic pulmonary disease tuberculosis. The pathogen grows in "*Mycobacterium*-containing vacuoles" (MCVs), which accumulate the small GTPase Rab5 but not Rab7 and exclude the acidifying vacuolar H^+-ATPase as well as lysosomal hydrolases (Russell et al., 2002).

Mycobacterium tuberculosis adopts a dual strategy involving lipid toxins and PI-metabolizing enzymes to keep the levels of PtdIns(3)*P* on MCVs low, thus arresting the bactericidal endo-

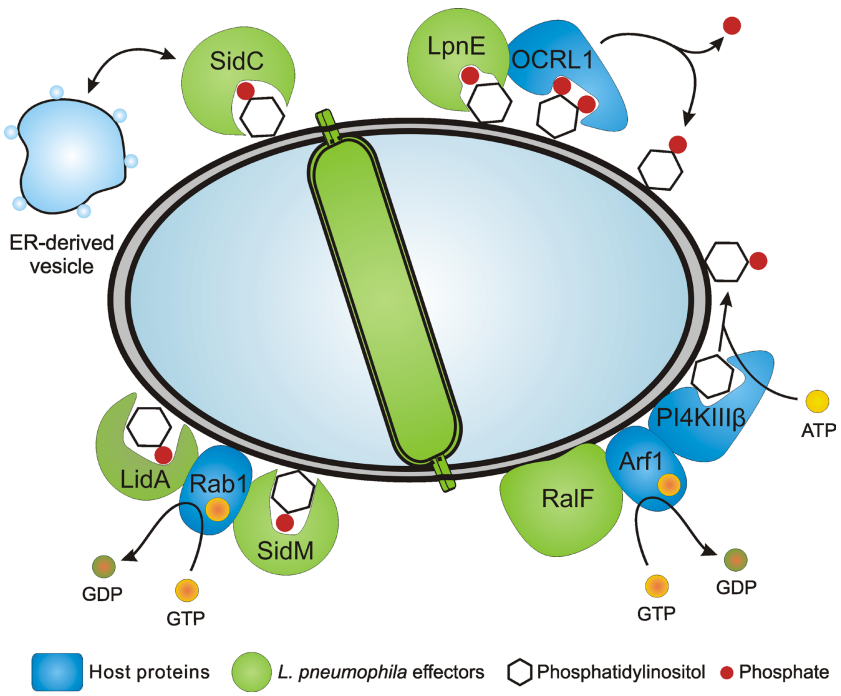

FIGURE 1 | *Legionella pneumophila* PI-binding effector proteins and LCV formation. *L. pneumophila* employs the Icm/Dot T4SS to form a replication-permissive LCV that communicates with secretory as well as with endocytic vesicle trafficking pathways and eventually fuses with the ER. Several effector proteins anchor to the LCV membrane through PtdIns(4)*P* or PtdIns(3)*P* and promote the interaction with the ER and ER-derived vesicles (SidC), catalyze GEF activity of the small GTPase Rab1 (SidM, LidA), or bind PI-metabolizing enzymes such as the 5-phosphatase OCRL1 (LpnE). The Icm/Dot substrate RalF is an Arf1 GEF that might indirectly recruit PI4KIIIβ.

cytic pathway. The glycosylated PI analogue lipoarabinomannan (LAM) and its precursor PtdIns mannoside (PIM) are trafficking toxins (Chua et al., 2004). LAM inhibits a calmodulin kinase II-dependent activation of the class III PtdIns 3-kinase (PI3K) hVps34 (Vergne et al., 2003) and thereby reduces PtdIns(3)*P* on MCVs and prevents the delivery of the vacuolar H^+-ATPase as well as acidic hydrolases (Fratti et al., 2003). Conversely, PIM promotes the homotypic fusion of phagosomes with early endosomes in a PI3K-independent manner, thus allowing continuous communication between MCVs and endosomes, despite the trafficking block imposed by the depletion of PtdIns(3)*P* (Chua et al., 2004; Vergne et al., 2004). To further reduce PtdIns(3)*P* on MCVs, *M. tuberculosis* secretes the PI phosphatases SapM (Vergne et al., 2005) and MptpB (Beresford et al., 2007).

The role of bacterial PI-binding effector proteins, LCV PIs and host PI-modulating enzymes for LCV formation and intracellular replication of *L. pneumophila* will be discussed in the following sections.

THE Icm/Dot SUBSTRATE SidC BINDS PtdIns(4)*P* AND PROMOTES LCV-ER FUSION

The *L. pneumophila* protein SidC was identified as an Icm/Dot substrate by a Cre/*loxP*-based protein translocation assay, using Icm/Dot-mediated conjugative transport between a donor and a recipient bacterium (Luo and Isberg, 2004). Immuno-fluorescence studies further showed that SidC is translocated to the cytoplasmic side of the vacuole, where the protein decorates the LCV membrane.

The amount of SidC bound to the LCV membrane depends on the presence or absence of PI3Ks in *D. discoideum* (Weber et al., 2006b), and therefore, we tested *in vitro*, whether purified GST-SidC fusion protein directly binds PIs. Indeed, purified SidC and its paralogue SdcA (72% identity) were found to selectively bind PtdIns(4)*P* in protein–lipid overlay assays (**Figure 2**), as well as in phospholipid vesicle pull down experiments (Weber et al., 2006b). In contrast, the Icm/Dot substrate SidD did not bind to any PIs or other lipids. Further analysis of the PtdIns(4)*P*-binding domain of SidC revealed that a 20-kDa fragment near the C-terminus was sufficient to selectively bind the PI (Ragaz et al., 2008; **Figure 2**). The PtdIns(4)*P*-binding domain was termed "P4C" (PtdIns(4)*P*-binding domain of SidC) and shows no homology with eukaryotic PI recognition folds, such as the PH (pleckstrin homology), PX (phagocyte oxidase homology), FYVE (Fab1-YotB-Vac1-EEA1), ENTH/ANTH (epsin/AP180 N-terminal homology), FERM (band 4.1-ezrin-radixin-moesin) or KR (lysine/arginine) domains (Downes et al., 2005; Varnai and Balla, 2006; Lemmon, 2008).

Deletion of *sidC* and the adjacent gene *sdcA* from the *L. pneumophila* genome does not impair intracellular replication of the bacteria (Luo and Isberg, 2004; Ragaz et al., 2008). Furthermore, in absence of *sidC* and *sdcA* the acquisition of Rab1 or the endosomal marker p80 is not altered; yet, only 20% of LCVs acquire the ER markers calnexin-GFP and GFP-HDEL, indicating that the interaction of LCVs with the ER is severely impaired upon deletion of these genes (Ragaz et al., 2008). The finding that reduced ER acquisition does not impair intracellular replication of the Δ*sidC-sdcA* strain was unexpected, since defective ER acquisition of LCVs due to a dominant negative form of the small GTPase Sar1 (Kagan and Roy, 2002) or due to the lack of the Icm/Dot substrate SidJ (Liu and Luo, 2007) did inhibit intracellular growth of *L. pneumophila*.

The ER acquisition phenotype of *L. pneumophila* lacking *sidC* and *sdcA* is complemented by either *sidC* or *sdcA*, and the amount of calnexin-GFP and SidC on LCVs is directly proportional. Biochemical experiments revealed that SidC and a 70-kDa N-terminal fragment incubated with lysates of macrophages or *D. discoideum* bind ER and secretory vesicles (containing calnexin, protein disulfide isomerase and Rab1), but neither lysosomes (containing LAMP-1or "common antigen-1") nor Golgi fragments (containing giantin). Thus, the N-terminal part of SidC promotes the communication of LCVs with ER-derived vesicles, while the C-terminal part harbors the Icm/Dot translocation determinant and the PtdIns(4)*P*-binding domain P4C (**Figure 1**).

SidC and in particular the 20-kDa PtdIns(4)*P*-binding fragment P4C are stable and can be produced with high yields in *E. coli* as GST fusion proteins (Weber et al., 2006b; Ragaz et al., 2008). Moreover, P4C can be ectopically produced in *D. discoideum*, and the probe labels the PtdIns(4)*P*-positive LCV membrane in amoebae infected with *L. pneumophila*. Similarly, P4C might be a

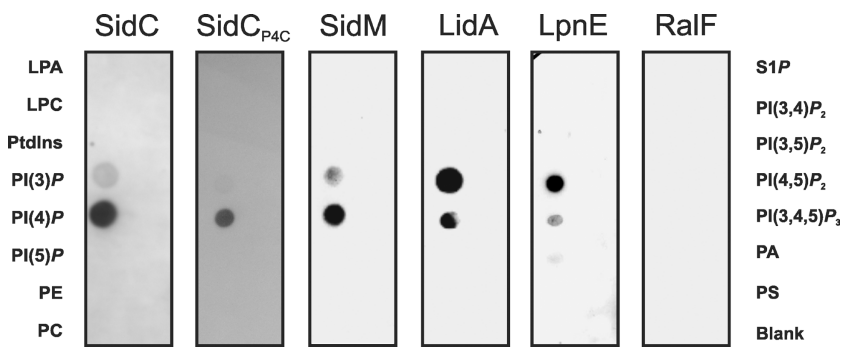

FIGURE 2 | Protein-lipid overlay of *L. pneumophila* PI-binding effector proteins. GST fusion proteins (200 nM) of SidC, SidC$_{P4C}$, SidM, LidA, LpnE, and RalF were affinity purified, and binding to different synthetic di-hexadecanoyl-PI lipids (100 pmol) immobilized on nitrocellulose membranes was analyzed by a protein–lipid overlay assay using an anti GST antibody. Left lanes: lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylinositol (PtdIns), PtdIns phosphate (PI(n)*P*), phosphatidylethanolamine (PE), phosphatidylcholine (PC). Right lanes: sphingosine-1-phosphate (S1*P*), PtdIns phosphate (PI(n)*P*), phosphatidic acid (PA), phosphatidylserine (PS).

suitable PtdIns(4)*P* probe in other eukaryotic cells, including yeast, *Drosophila melanogaster* and mammalian cells. Taken together, the purified or heterologously produced SidC and P4C proteins are useful as prokaryotic PtdIns(4)*P*-binding probes in biochemical and cell biological experiments.

THE Rab1 GEF SidM IS A MAJOR PtdIns(4)*P*-BINDING EFFECTOR PROTEIN

To address the question, whether *L. pneumophila* proteins other than SidC also bind to PIs, bacterial lysates were incubated with agarose beads coated either with one of the seven naturally occurring mono- or poly-phosphorylated PIs or with PtdIns. The eluate from washed beads was separated by SDS-PAGE, and a single protein binding predominantly and specifically to PtdIns(4)*P* was identified by MS as the effector protein SidM (also termed DrrA; Brombacher et al., 2009). Further analysis showed that SidM is indeed a major *L. pneumophila* PtdIns(4)*P*-binding protein (**Figure 2**), which competes with SidC for binding to this PI on LCVs. The PtdIns(4)*P*-binding domain of SidM comprises a 12-kDa fragment that was termed "P4M" (PtdIns(4)*P*-binding of SidM) and is not related to other prokaryotic or eukaryotic PI-binding domains. The high-resolution structure of a SidM fragment including the P4M domain revealed that the effector protein employs a novel fold to bind PtdIns(4)*P* with an unprecedented high affinity in the nanomolar range (Schoebel et al., 2010). Compared to full length SidM, the affinity of the 12-kDa P4M domain for PtdIns(4)*P* is reduced (Brombacher et al., 2009), and therefore, the 20-kDa P4C domain, which retains its PI-binding affinity, appears to be the superior PtdIns(4)*P* probe.

SidM is an Icm/Dot substrate and shows activity as a Rab1 guanine nucleotide exchange factor (GEF), thus activating and recruiting this small GTPase to LCVs (Machner and Isberg, 2006; Murata et al., 2006). The finding that the GEF SidM binds to PtdIns(4)*P* represents a novel link between the modulation of host GTPases and the exploitation of PIs by pathogenic bacteria (**Figure 1**). SidM has been suggested to also have activity as a Rab GDP dissociation inhibitor (GDI) displacement factor (GDF), which removes GDI from Rab1-GDP, thus allowing access of the GEF domain to the small GTPase (Ingmundson et al., 2007; Machner and Isberg, 2007). However, the GDF activity turned out to be intrinsic to the GEF activity, rather than a distinct activity (Schoebel et al., 2009).

The membrane cycle of Rab1 is closed by the Icm/Dot substrate LepB, which is a Rab1 GTPase activating protein (GAP) that inactivates and removes Rab1 from membranes (Ingmundson et al., 2007). LepB and another *L. pneumophila* protein with weak homology to SNAREs and tethering proteins termed LepA have originally been proposed to promote the non-lytic egress of the bacteria from amoebae via a novel pathway, leading to bacteria-filled respirable vesicles (Chen et al., 2004, 2007).

Interestingly, SidM also catalyzes the "AMPylation" (adenosine mono-phosphorylation) of Rab1 at an N-terminal tyrosine residue (Müller et al., 2010). AMPylation of Rab1 "constitutively activates" the small GTPase, since the covalent modification barely affects the GEF activity of SidM but impairs the GAP activity of LepB. In addition to Rab1, SidM AMPylates several other Rab GTPases, including Rab8 and Rab14 (Müller et al., 2010), which are also recruited to LCVs (Urwyler et al., 2009b). Thus, SidM might prolong the activation of several Rab GTPases on the LCV membrane. Rab1-AMP does not bind eukaryotic Rab1 effectors anymore, but still interacts with a SidM auxiliary protein: the *L. pneumophila* Rab1 effector LidA (Müller et al., 2010).

LidA (Lowered viability in the presence of *dotA*) is an Icm/Dot substrate that decorates LCVs and promotes the recruitment of early secretory vesicles to LCVs (Conover et al., 2003; Derre and Isberg, 2005). In biochemical experiments LidA was found to interact with several small Rab GTPases (Rab1, Rab6, Rab8) and to support the GEF activity of SidM by binding to GDI-free Rab1 (Machner and Isberg, 2006). In turn, wild-type and constitutively active Rab8 also interact with LidA in *L. pneumophila* lysates (Urwyler et al., 2009a). LidA represents another effector that localizes to the LCV membrane by interacting with distinct PIs, since it binds to PtdIns(4)*P* and, with an apparently slightly lower affinity, also to PtdIns(3)*P* (Brombacher et al., 2009; **Figure 2**).

Lastly, the Icm/Dot-translocated effector protein RalF does not bind to any PIs or other lipids (Brombacher et al., 2009; **Figure 2**). RalF (Recruitment of Arf1 to the *Legionella* phagosome) was the first Icm/Dot substrate identified and characterized as an Arf1-specific GEF, which is required to recruit Arf1 to the LCV membrane (Nagai et al., 2002; **Figure 1**). Upon deletion of *ralF* from the *L. pneumophila* chromosome the small GTPase Arf1 does not localize to LCVs anymore, yet intracellular replication of the bacteria is not impaired. Taken together, these results indicate that two classes of Icm/Dot-translocated *L. pneumophila* GEFs localize to LCV membranes, one of which by binding to PIs.

THE VIRULENCE FACTOR LpnE BINDS PtdIns(3)*P* AND OCRL1

LpnE (*Legionella pneumophila* entry) is a Sel1 repeat protein of the tetratricopeptide-repeat family (Newton et al., 2006). The *lpnE* gene was found to be specific for *L. pneumophila* in a subtractive genomic hybridization screen by comparing *L. pneumophila* to non-virulent *L. micdadei*. In agreement with a function for LpnE as a virulence factor, an *L. pneumophila lpnE* deletion mutant strain is impaired for infection of *Acanthamoeba castellanii* amoebae, entry into human macrophage-like cells, intracellular trafficking, and virulence in the A/J mouse strain (Newton et al., 2007). While LpnE is secreted into *L. pneumophila* culture supernatants, the mechanism remains unclear, since neither the Icm/Dot T4SS nor the Lsp T2SS seems to be involved.

Purified recombinant LpnE selectively binds PtdIns(3)*P* (Weber et al., 2009a; **Figure 2**), indicating that the Sel1 repeat protein represents another PI-binding virulence factor of *L. pneumophila*. Moreover, LpnE interacts with the N-terminus of the human enzyme OCRL1 ($OCRL1_{1-236}$) heterologously produced in *D. discoideum*, and conversely, purified GST-$OCRL1_{1-236}$ binds LpnE in *L. pneumophila* lysates. OCRL1 (Oculocerebrorenal syndrome of Lowe 1) and its *Dictyostelium* homologue Dd5P4 (*D. discoideum* 5-phosphatase 4) are PI-metabolizing enzymes implicated in intracellular replication of *L. pneumophila* (see below).

In summary, the studies discussed above indicate that *L. pneumophila* exploits the mono-phosphorylated host PIs PtdIns(4)*P* and PtdIns(3)*P* to anchor the effector proteins SidC, SidM, LidA, and LpnE to the LCV membrane. The PI-binding effectors then interfere with host vesicle trafficking and signal transduction (**Figure 1**).

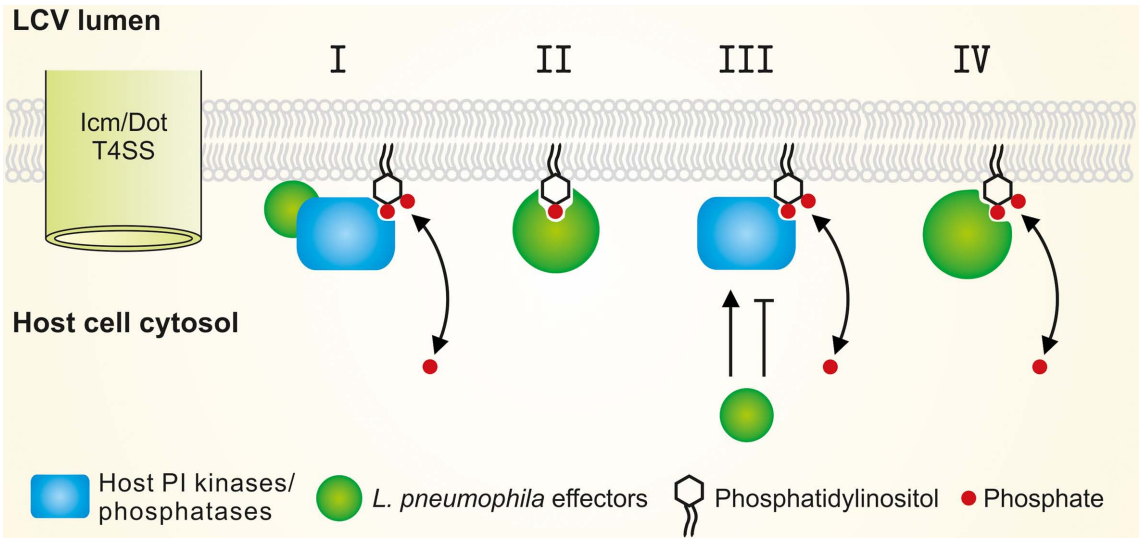

FIGURE 3 | Modulation of the LCV PI pattern by *L. pneumophila* effectors. *L. pneumophila* modulates the LCV host PI pattern through the Icm/Dot T4SS. Translocated factors might (I) activate small GTPases and thus recruit host PI-metabolizing enzymes, (II) titrate (mask) PIs, (III) directly activate or inhibit host PI-metabolizing enzymes, and/or (IV) represent bacterial PI phosphatases or kinases.

HOST PIs AND PI-METABOLIZING ENZYMES INVOLVED IN LCV FORMATION

Since the Icm/Dot substrates SidC, SidM, and LidA are present on the LCV membrane and bind to PtdIns(4)*P in vitro*, this PI is expected to be a lipid component of LCVs and enriched on this compartment. Indeed, PtdIns(4)*P* was identified on LCV membranes by using either an anti-PtdIns(4)*P* antibody, or purified GST fusion proteins of the eukaryotic PH_{FAPP1} domain (specifically binding PtdIns(4)*P*) or prokaryotic SidC as a probe (Weber et al., 2006b).

The mammalian enzyme OCRL1 and *D. discoideum* Dd5P4 are homologous inositol-polyphosphate 5-phosphatases, which hydrolyze PtdIns(4,5)P_2 to yield PtdIns(4)*P*. GFP fusion proteins of OCRL1 or Dd5P4 localize to LCVs via their N-termini ($OCRL1_{1–236}$, $Dd5P4_{1–132}$) in *D. discoideum*, and OCRL1 accumulates on LCVs in RAW 264.7 macrophages (Weber et al., 2009a; **Figure 1**). In *D. discoideum* lacking Dd5P4 the amount of SidC on LCVs is reduced, suggesting that in absence of Dd5P4 less PtdIns(4)*P* is produced on the LCV membrane, and consequently, less SidC binds to this compartment. Interestingly, two to three orders of magnitude more *L. pneumophila* are released from *D. discoideum* lacking Dd5P4, and therefore, the bacteria grow intracellularly much more efficiently in absence of this inositol-polyphosphate 5-phosphatase. It is currently unknown, how Dd5P4 restricts intracellular growth of *L. pneumophila*. However, since the mammalian homologue OCRL1 promotes retrograde trafficking from endosomes to the *trans*-Golgi network (TGN; Johannes and Popoff, 2008), a functional retrograde vesicle trafficking pathway might play a role.

A host PI-metabolizing enzyme termed PtdIns 4-kinase IIIβ (PI4KIIIβ) is likely also involved in the production of PtdIns(4)*P* on LCVs. Depletion of PI4KIIIβ by RNA interference in *Drosophila melanogaster* Kc167 phagocytes, which are permissive for intracellular growth of *L. pneumophila*, significantly reduced the amount of SidC on LCV membranes (Brombacher et al., 2009). The effect was specific for PI4KIIIβ, since depletion of the isoenzymes PI4KIIIα or PI4KIIα did not reduce the amount of LCV-bound SidC. PI4KIIIβ forms PtdIns(4)*P* in the TGN upon recruitment by the activated small GTPase Arf1 (Godi et al., 1999). Yet, it is currently unknown, whether PI4KIIIβ also localizes to LCVs. Together, these findings suggest that OCRL1/Dd5P4 as well as PI4KIIIβ are implicated in LCV formation and likely catalyze the production of PtdIns(4)*P* on the LCV membrane (**Figure 1**).

PI3Ks represent another class of PI-metabolizing enzymes that play important roles for vesicle trafficking and signal transduction in eukaryotic cells. *D. discoideum* deletion mutants and pharmacological inhibitors revealed that the uptake of *icm/dot* mutant *L. pneumophila* depends on PI3Ks. In contrast, the efficient uptake of wild-type *L. pneumophila* by *D. discoideum* (Hilbi et al., 2001; Weber et al., 2006a,b) or by human macrophage-like HL-60 cells (Khelef et al., 2001) is barely affected by PI3Ks, indicating that Icm/Dot-proficient bacteria might bypass PI3Ks during entry. In another study, the uptake of *L. pneumophila* was reported to require PI3Ks (Tachado et al., 2008). Yet, this work used murine J774A.1 macrophage-like cells, which do not support intracellular growth of *L. pneumophila*. Thus, the formation of a replication-permissive vacuole by *L. pneumophila* might depend on bypassing PI3K signaling.

Upon deletion or inhibition of PI3Ks in *D. discoideum*, *L. pneumophila* replicates more efficiently within the amoebae, and *icm/dot* mutant bacteria are killed less effectively, in agreement with the well-established role for PI3Ks in the bactericidal endocytic pathway (Weber et al., 2006b). Recent studies confirmed that *L. pneumophila* interferes with the PI-sensitive fusion of LCVs with acidic vacuoles and indicated that the inhibition of intracellular replication by PI3Ks is restricted to early steps in the infection (Peracino et al., 2010). Notably, the stimulation of intracellular replication of *L. pneumophila* by pharmacological "PI3K inhibitors"

might also be due to an effect on PI4KIIIβ (see above). PI4KIIIβ is a type III PI4K that, in contrast to the type II PI4Ks, is also inhibited by wortmannin (Balla and Balla, 2006).

Finally, the 30-kDa inositol monophosphate phosphatase IMPA was identified as a host component of purified intact LCVs, suggesting that this PI phosphatase might also play a role in LCV formation and intracellular replication of *L. pneumophila* (Urwyler et al., 2009b). In summary, the PI lipid PtdIns(4)*P* as well as the host PI-metabolizing enzymes OCRL1/Dd5P4, PI4KIIIβ, PI3Ks, and IMPA are likely constituents of LCVs and implicated in its formation.

MODULATION OF THE PI PATTERN ON LCVs BY *L. PNEUMOPHILA*

PtdIns(4)*P* accumulates on LCVs harboring wild-type but not *icm/dot* mutant *L. pneumophila* (Weber et al., 2006b). Since these vacuoles are vastly different from one another, comparing the two compartments does not indicate, whether the PI pattern on LCVs is modulated directly by the Icm/Dot T4SS. However, upon infection of phagocytes approximately 20% of wild-type *L. pneumophila* do not reside in a calnexin-positive ER-derived compartment, but rather in a lysosomal compartment, which is positive for the vacuolar H^+-ATPase component VatM in *D. discoideum* and for LAMP-1 in macrophages. The quantification of PtdIns(4)*P* on VatM-positive vacuoles containing either wild-type or *icm/dot* mutant *L. pneumophila* revealed that 42% of the former, but only 6% of the latter accumulated PtdIns(4)P (Weber et al., 2006b). This finding indicates that wild-type *L. pneumophila* survive in a VatM-positive compartment and interfere with the LCV PI metabolism in an Icm/Dot-dependent manner. Furthermore, modulation of the LCV PI pattern (increasing the amount of PtdIns(4)*P*) is not sufficient to generate a replication-permissive ER-derived compartment.

To modulate the LCV PI pattern *L. pneumophila* effectors might (i) activate small GTPases and thus recruit host PI-metabolizing enzymes, (ii) titrate (mask) PIs, (iii) produce factors directly activating or inhibiting host PI-metabolizing enzymes, and/or (iv) translocate bacterial PI-metabolizing enzymes, i.e., PI phosphatases or kinases (**Figure 3**). Currently, no *L. pneumophila* factors are known, which directly modify the activity of host PI-metabolizing enzymes or phosphorylate/dephosphorylate PIs.

Legionella pneumophila produces and translocates two GEFs that recruit and activate small GTPases and thus might indirectly modulate the LCV PI pattern: RalF (Nagai et al., 2002) and SidM (Machner and Isberg, 2006; Murata et al., 2006) recruit the small GTPases Arf1 and Rab1to LCVs (**Figure 1**). Arf1 has been shown to recruit PI4KIIIβ at the TGN (Godi et al., 1999), and a number of small GTPases including Arf1 (Lichter-Konecki et al., 2006) and Rab1 (Hyvola et al., 2006) target OCRL1 to endosomal membranes. Both PI4KIIIβ and OCRL1 produce PtdIns(4)*P* and are likely enzymatically active on LCVs (Brombacher et al., 2009; Weber et al., 2009a). If *L. pneumophila* modulates the PI levels on LCVs via the translocation of GEFs, the concentration of PtdIns(4)*P* on LCVs should decrease in absence of RalF or SidM, and consequently, less SidC should bind to LCVs. However, in absence of RalF the amount of SidC on LCVs remained constant, and in absence of SidM even significantly more SidC bound to LCVs, accounting also for the fact that SidM itself binds to PtdIns(4)*P* (Brombacher et al., 2009). In light of these results, *L. pneumophila* likely does not (or at least not exclusively) modulate the LCV PI pattern by the indirect recruitment of host PI-metabolizing enzymes. Thus, while it is clear that *L. pneumophila* modulates the LCV PI pattern in an Icm/Dot-dependent manner, the mechanistic aspects of this process have not been elucidated yet.

CONCLUSIONS AND PERSPECTIVES

Legionella pneumophila employs the Icm/Dot T4SS to form in a complex and robust process a replication-permissive LCV. Several Icm/Dot-translocated effector proteins anchor to the LCV membrane through PtdIns(4)*P* or PtdIns(3)*P* and promote the interaction of LCVs with host vesicles and organelles, small GTPases or PI-metabolizing enzymes. The PI-binding domains of these effectors bind PIs with high affinity and are useful as specific lipid probes. Further research should address the identification and characterization of other *L. pneumophila* PI-binding effector proteins. Of particular interest is the mechanism, by which *L. pneumophila* actively modulates the LCV PI pattern in an Icm/Dot-dependent manner. A comprehensive understanding of how *L. pneumophila* exploits the PI metabolism of host cells will continue to provide mechanistic insights into basic biological processes and shed light on the virulence of this important opportunistic human pathogen.

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Effector glycosyltransferases in *Legionella*

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Legionella causes severe pneumonia in humans. The pathogen produces an array of effectors, which interfere with host cell functions. Among them are the glucosyltransferases Lgt1, Lgt2 and Lgt3 from *L. pneumophila*. Lgt1 and Lgt2 are produced predominately in the post-exponential phase of bacterial growth, while synthesis of Lgt3 is induced mainly in the lag-phase before intracellular replication of bacteria starts. Lgt glucosyltransferases are structurally similar to clostridial glucosylating toxins. The enzymes use UDP–glucose as a donor substrate and modify eukaryotic elongation factor eEF1A at serine-53. This modification results in inhibition of protein synthesis and death of target cells. In addition to Lgts, *Legionella* genomes disclose several genes, coding for effector proteins likely to possess glycosyltransferase activities, including SetA (subversion of eukaryotic vesicle trafficking A), which influences vesicular trafficking in the yeast model system and displays tropism for late endosomal/lysosomal compartments of mammalian cells. This review mainly discusses recent results on the structure–function relationship of Lgt glucosyltransferases.

Keywords: *Legionella*, virulence factor, glycosyltransferase, eEF1A, protein synthesis

MULTIFACETED MECHANISMS IN *LEGIONELLA* – HOST CELL INTERACTION

Legionella is a fastidious Gram-negative bacterium, causing severe pneumonia in humans named Legionnaires' disease. Among known species of *Legionella*, the most important human pathogen is *Legionella pneumophila*, strains of which account for more than 90% of morbidity records due to legionellosis (Diederen, 2008). Despite the description of at least 15 serogroups in this species, *L. pneumophila* serogroup 1 is responsible for over 80% of cases of the disease (Yu et al., 2002). *Legionella longbeachae* and *Legionella bozemanii* are the next most common etiological agents of Legionnaires' disease, accounting for up to 7% of *Legionella* infections in Europe and in the USA (Muder and Yu, 2002). Interestingly, in Australia and New Zealand *L. longbeachae* is responsible for ~30% of Legionnaires' disease cases (Yu et al., 2002). In contrast to well-studied *L. pneumophila*, virulence mechanisms utilized by *L. longbeachae* and *L. bozemanii* are largely unknown.

Legionella pneumophila is able to multiply inside eukaryotic cells – either in free-living unicellular organisms (amebae and ciliated protozoa) or in mammalian cells (macrophages, monocytes, epithelial cells; Jules and Buchrieser, 2007). After uptake by host cells, the *Legionella*-containing phagosome is subjected to specialized biogenesis steps, leading to transformation of this organelle into a niche that supports multiplication of the bacteria (Isberg et al., 2009). A specialized type IV secretion system (T4SS), encoded by *dot* and *icm* gene clusters, translocates numerous bacterial effectors (>300 effectors; Hubber and Roy, 2010) into target cells, which participate in the change of the phagosome into a "replicative vacuole" (Ninio and Roy, 2007; Ensminger and Isberg, 2009). Several *Legionella* effectors target small GTP-binding proteins, participating in regulation of vesicular trafficking of the host cell. These eukaryotic proteins are molecular switches, which are regulated by a GTPase cycle. *Legionella* effectors specifically switch on or switch off these GTP-binding proteins. For example, the mammalian Arf1 protein, which is involved in vesicle formation in the Golgi, is activated by *Legionella* protein RalF, which acts as a GDP/GTP exchange factor for this small GTPase (Nagai et al., 2002). Another example is the Ras-superfamily protein Rab1, which regulates various steps of vesicle trafficking in eukaryotic cells. Rab1 is manipulated by several *Legionella* effectors, including DrrA/SidM, LidA, and LepB (Machner and Isberg, 2006; Murata et al., 2006). DrrA/SidM is a multifunctional protein, containing guanine nucleotide exchange activity and adenylyltransferase activity (Ingmundson et al., 2007; Machner and Isberg, 2007; Muller et al., 2010). *Legionella* effector LidA enhances Rab1 recruitment by DrrA/SidM (Machner and Isberg, 2006) and the effector LepB behaves as a Rab1 GTPase-activating protein, which inactivates the Rab protein (Ingmundson et al., 2007).

However, not only vesicular trafficking is targeted during biogenesis steps of the *Legionella*-containing vacuole. *L. pneumophila* is able to maintain a neutral pH inside its phagosome (Horwitz and Maxfield, 1984). It was shown recently that T4SS effector SidK targets vacuolar ATPase by interacting with VatA (also called VMA1), one of the key components of the vesicular proton pump, which is involved in ATP hydrolysis. *Legionella* effector SidK inhibits ATP hydrolysis, thereby blocking proton translocation and vacuole acidification (Xu et al., 2010). Similar to other pathogens *L. pneumophila* is able to exploit the eukaryotic ubiquitin-conjugating system for establishing successful intracellular infection (Ivanov and Roy, 2009). To

achieve this, the bacterium produces several T4SS effector proteins that function in the eukaryotic ubiquitination pathway (Kubori et al., 2008; Price et al., 2009, 2010; Lomma et al., 2010). *Legionella*-induced modulation of target cell survival is also observed during intracellular proliferation of the bacterium. Several *Legionella* effector proteins have been shown to participate in apoptotic and anti-apoptotic processes either directly or indirectly (Laguna et al., 2006; Abu-Zant et al., 2007; Banga et al., 2007). Moreover, *L. pneumophila* modulates inflammatory responses through NF-κB (Ge et al., 2009; Losick et al., 2010), induces mitochondrial recruitment and microfilament rearrangements (Chong et al., 2009) or regulates MAP kinase response to bacteria (Li et al., 2009). Thus, all these findings indicate an extremely complex *Legionella*-host cell interaction.

LGTs AS A NEW FAMILY OF GLUCOSYLTRANSFERASES IN *L. PNEUMOPHILA*

Recently it was shown that glycosyltransferases (GTs) are highly effective virulence factors of *Legionella*. These enzymes target eukaryotic substrates by covalent attachment of glycosyl moieties to eukaryotic proteins thereby altering their functions (Belyi and Aktories, 2010).

The first glucosyltransferase purified from *L. pneumophila* Philadelphia-1 strain was *Legionella* glucosyltransferase 1 (Lgt1). Lgt1 has a molecular mass of 59.7 kDa and modifies a ~50-kDa component in cytoplasmic fraction of eukaryotic cells (Belyi et al., 2003). The enzymatic activity is sugar-specific, i.e., only UDP–glucose, but not UDP–galactose, UDP–*N*-acetyl-galactosamine, UDP–*N*-acetyl-glucosamine, UDP–glucuronic acid, or GDP-mannose serves as donor substrate in the reaction (Belyi et al., 2006).

The primary amino acid sequence of Lgt1 shares little homology with known proteins. The only notable similarity is found between the central region of Lgt1 and the catalytic core of clostridial glucosylating toxins (CGT; **Figure 1A**; **Table 1**). In this region several groups of conserved amino acid residues could be identified, including the two aspartic amino acids D_{246} and D_{248}, representing the DXD-motif – a known hallmark of GTs (Belyi et al., 2006).

Database searches in the sequenced genomes of six *L. pneumophila* strains (Philadelphia-1, Corby, Lens, Paris, 2300/99 Alcoy, and 130b) disclosed altogether 13 open reading frames with significant sequence homology with Lgt1 (**Table 2**). Based upon the level of identity, these gene products can be grouped into three families: Lgt1 through Lgt3 [in Philadelphia-1 strain the gene IDs (identification labels, used to distinguish coding sequences) are *lpg1368*, *lpg2862*, and *lpg1488*, coding for ~60 kDa Lgt1, ~70 kDa Lgt2, and ~100 kDa Lgt3, respectively]. Only one copy of each gene family member is present in the corresponding genome. Philadelphia-1 strain contains the full set of the genes (i.e., *lgt1*, *lgt2*, and *lgt3*), whereas the other strains possess only *lgt1* and *lgt3*. Representatives within each family are ~90% identical in amino acid sequences whereas homology between the three groups' members are in the range of 15–27%. Lgt1, Lgt2, and Lgt3 are serologically distinct and do not display antigenic cross-reactivity (Belyi et al., 2008).The enzymes are grouped into the glucosyltransferase family GT88 in the carbohydrate modifying enzymes database (http://www.cazy.org/GT88.html; Coutinho et al., 2003).

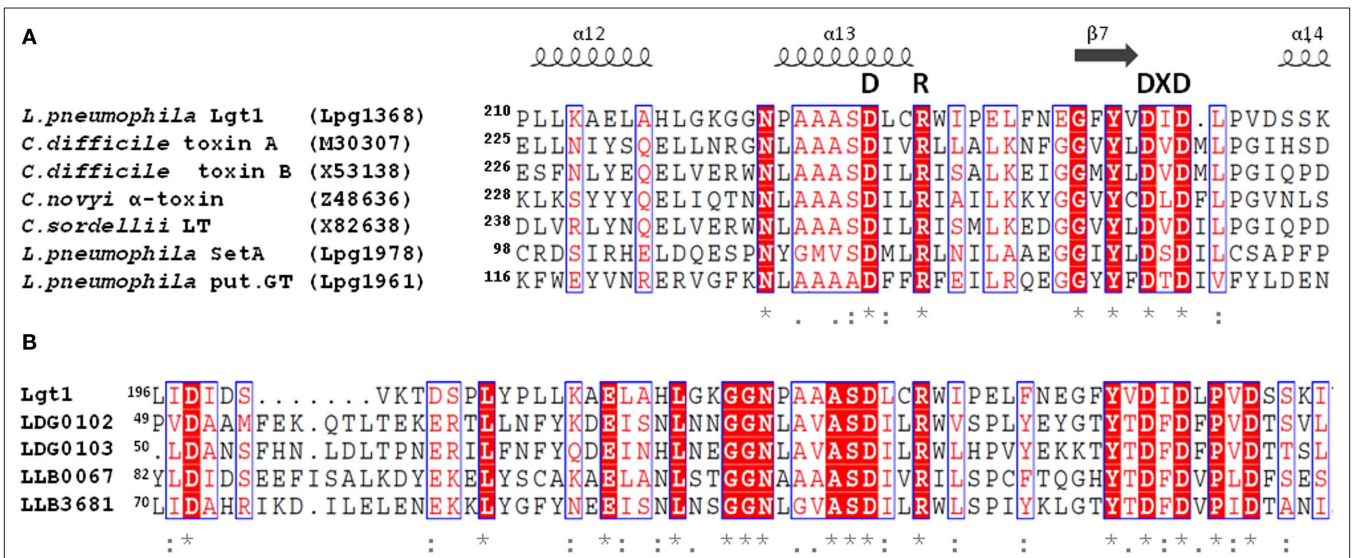

FIGURE 1 | (A) Alignment of partial amino acid sequences of Lgt1, SetA, Lpg1961 from *L. pneumophila* Philadelphia-1 strain with that of proteins from a clostridial glucosylating toxin family: Toxins A and B from *C. difficile*, α-toxin from *C. novyi*, and lethal toxin from *C. sordellii*. Gene bank accession numbers of the corresponding coding sequences are shown in brackets. Essential amino acids mentioned in the text are highlighted (DXD-motif, GT-A triad). **(B)** Alignment of partial amino acid sequences of Lgt1 from *L. pneumophila* Philadelphia-1 with that of putative glycosyltransferases found in translated genomes of *L. drancourtii* LLAP12 and *L. longbeachae* D-4968. Identification codes for Lgt1 and putative glycosyltransferases in strain LLAP12 of *L. drancourtii* and strain D-4968 of *L. longbeachae* (two products in each strain) are Lpg1368, LDG0102/LDG0103, and LLB0067/LLB3681 respectively. Proteins LLO1578 and LLO1721 found in translated sequenced genome of *L. longbeachae* NSW150 were 100% identical to LLB0067 and LLB3681 from *L. longbeachae* D-4968 respectively and are not shown on the figure for simplicity reason. Identical amino acid residues are denoted by asterisks, highly conserved residues by double dots, and modestly conserved residues by dots. The secondary structural elements were deduced from the structure of Lgt1 (pdb 3JSZ). The alignment was prepared using ESPript 2.2 (http://espript.ibcp.fr).

Table 1 | Comparison of glucosyltransferases Lgt from *L. pneumophila* with large clostridial toxins.

Property	*L. pneumophila* Lgts	Large clostridial toxins
Molecular mass	60–100 kD	250–310 kD
Target substrate	Large G-proteins (eEF1A, Hbs1)	Small G-proteins (Rho/Ras proteins)
Co-substrates	UDP–glucose	UDP–glucose, UDP–*N*-acetyl-glucosamine
CAZY classification	GT-A family, GT88	GT-A family, GT44
Target amino acid in substrate	Serine	Threonine
Stereochemical type of glycosylation	Retaining	Retaining
Substrate recognition requirements	Low	High
Intracellular translocation mode	Type IV secretion system	Receptor-mediated endocytosis
Cellular effect	Inhibition of protein synthesis	Destruction of the actin cytoskeleton, inhibition of Rho/Ras signaling

Table 2 | Amino acid sequence identity of proteins of the Lgt-family of *L. pneumophila* (Philadelphia-1, Corby, Lens, Paris, 2300/99 Alcoy, and 130b). Proteins belonging to Lgt1, Lgt2, or Lgt3 groups were cross-aligned pair-wise to determine the degree of identical amino acid residues. The homology is shown as a percentage of identical amino acid residues. Lgt1-group glucosyltransferases are marked by green, Lgt2 – by blue, and Lgt3 – by yellow color.

	Philadelphia-1		Corby		Lens		Paris		2300/99 Alcoy		130b	
	lpg2862	lpg1488	L pc0784	L pc0903	L pl1319	L pl1540	L pp1322	L pp1444	L pa02017	L pa02168	L pw13751	L pw15081
Lpg1368	19.8%	17.0%	98.1%	18.5%	88.6%	17.4%	97.9%	17.0%	98.5%	16.8%	90.1%	16.7%
lpg2862		26.1%	19.5%	29.7%	22.2%	27.5%	20.6%	26.5%	19.6%	27.0%	22.5%	27.0%
lpg1488			16.5%	86.2%	15.7%	89.4%	16.1%	94.5%	16.6%	95.3%	16.2%	93.9%
L pc0784				18.2%	87.8%	16.9%	97.5%	16.2%	99.6%	16.6%	89.3%	16.1%
L pc0903					17.7%	81.0%	18.3%	84.4%	18.4%	88.5%	18.1%	85.6%
L pl1319						17.1%	88.6%	15.8%	88.2%	16.1%	96.2%	15.8%
L pl1540							17.2%	88.2%	17.0%	92.2%	17.3%	94.1%
L pp1322								16.6%	97.9%	16.7%	90.3%	16.7%
L pp1444									16.3%	93.3%	16.1%	92.2%
L pa02017										17.0%	89.7%	16.3%
L pa02168											16.5%	96.6%
L pw13751												16.8%

To accomplish their functions bacterial virulence factors should be translocated into cytoplasm of a target cell. *Legionella* glucosyltransferases apparently miss a specific receptor-binding and translocation domain, which is typical for bacterial AB-type exotoxins. Accordingly, they do not produce toxic effects, when added into mammalian cell culture medium, indicating the requirement of a specialized secretion system. As shown in experiments using adenylate cyclase- or β-lactamase-chimeras, all Lgts are secreted via T4SS (de Felipe et al., 2005, 2008; Hurtado-Guerrero et al., 2010).

Often T4SS effectors are produced during the stationary phase of bacterial growth (Bruggemann et al., 2006; Zusman et al., 2007). At this stage bacterial cells become remarkably virulent and display a transmission phenotype (Byrne and Swanson, 1998). Also the production of Lgt1 and Lgt2 is strongly increased at the stationary phase of bacterial growth in broth; however, Lgt3 is detectable mainly in the pre-logarithmic phase of *in vitro* cultivation. Same results are obtained in *in vivo* experiments using the protozoan *Acanthamoeba castellanii* model as a host for *L. pneumophila*. Levels of mRNA coding for Lgt1 is maximal at late phase of co-infection, while *lgt3* is expressed mainly at the initial stage of bacterium–ameba interaction (Belyi et al., 2008). These experiments suggest differential regulation of glucosyltransferase activity in *L. pneumophila*, which, in turn, indicates specific roles of each enzyme in bacterial virulence. One can speculate that Lgt3 is important for initiation of infection cycle, while Lgt1/Lgt2 is necessary for egress of *Legionella* from the host cell.

TARGETING OF eEF1A BY *LEGIONELLA* GLUCOSYLTRANSFERASES

Legionella glucosyltransferase 1, Lgt2, and Lgt3 glucosylate an ~50-kDa component in mammalian cell extracts, which has been identified as elongation factor 1A (eEF1A). All these *Legionella* glucosyltransferases modify serine-53 of eEF1A (Belyi et al., 2006).

Elongation factor eEF1A, which is one of the most abundant proteins in eukaryotic cells, plays a key role in ribosome-dependent protein synthesis (Ramakrishnan, 2002). It possesses GTP-binding and GTPase activities and is required for the recruitment of aminoacylated tRNA to the A-site of mRNA-charged ribosomes. In addition, eEF1A was shown to be involved in several other cellular processes (Mateyak and Kinzy, 2010), including translational control, assembling/folding of newly synthesized proteins and proteosomal degradation of incorrectly folded peptides (Hotokezaka et al., 2002; Chuang et al., 2005), lipotoxic cell death (Borradaile et al., 2006), apoptosis (Ruest et al., 2002), nuclear export (Khacho et al., 2008), viral propagation (Matsuda et al., 2004), and regulation of actin cytoskeleton and cell morphology (Ejiri, 2002; Gross and Kinzy, 2005).

No structural data for mammalian eEF1A is available; however the very similar yeast elongation factor 1A from *Saccharomyces cerevisiae* has been crystallized and analyzed in detail (Andersen et al., 2000). The obtained structure shows that eEF1A is composed of three domains (**Figure 2**): domain 1 consists of ~240 residues and is characterized by a Ras-like fold (Kjeldgaard et al., 1996). It contains consensus sequences of typical GTP-binding proteins and is termed therefore "G-domain." Key features of this domain are binding and hydrolysis of GTP. Domains 2 and 3, consisting of 89 and 107 residues respectively, have a β-barrel structure and are involved in interaction with different targets like aminoacyl-tRNA and the elongation factor eEF1Bα, which is a GDP/GTP exchange factor of eEF1A (Andersen et al., 2000).

Serine-53 of eEF1A (**Figure 2**, shown in yellow), which is modified by Lgt, is located in the G-domain near the switch-1 region of the GTPase (Belyi et al., 2006, 2008). For the prokaryotic analog EF-Tu, it is known that the switch-1 region undergoes major conformational changes, depending on the nucleotide bound (GDP or GTP; Abel et al., 1996; Vetter and Wittinghofer, 2001). However in eEF1A the switch-1 region is not well defined, because two additional helices (A* and A′) are present, and no nucleotide-dependent structural changes in this region have been reported for eEF1A so far. Noteworthy, bacterial EF-Tu lacks Ser-53 excluding alteration of protein synthesis by glucosylation in *Legionella*.

Surprisingly, fragments of recombinant eEF1A are better substrates for glucosylation than full size eEF1A *in vitro*. Truncation analysis revealed that considerable portions of the elongation factor are dispensable for substrate recognition. Neither domains 2 nor 3 of eEF1A are necessary for glucosylation. Even the G-domain can be reduced to a decapeptide comprised of residues 50-GKGSFKYAWV-59. This peptide represents the loop of the helix–loop–helix region formed by helices A* and A′ of eEF1A and is part of the first turn of helix A′ (**Figure 2**, shown in red). Substitution of Ser-53, Phe-54, Tyr-56, or Trp-58 with alanine prevents or strongly decreases glucosylation. Even more surprising is the finding that modification of the decapeptide by Lgt1 is more efficient than the glucosylation of the isolated full length eEF1A. This suggests that the substrate properties of eEF1A depend on a specific conformation of the full length protein, which allows modification by the *Legionella* enzymes (Belyi et al., 2009).

Hbs1 PROTEIN AS A NOVEL SUBSTRATE OF *LEGIONELLA* GLUCOSYLTRANSFERASE Lgt

In silico screenings with the minimal peptide sequence, which is accepted as substrate for glucosylation by Lgts, retrieved the 70-kDa Hsp70 subfamily B suppressor 1 (Hbs1) as another possible target for Lgt1. Hbs1 shares significant sequence similarities with eEF1A (19% identity) and releasing factor eRF3 (24% identity) all over the protein. Moreover, yeast Hbs1 and human Hbs1-like proteins contain the decapeptides 210-GKSSFKFAWI-219 and 311-GKASFAYAWV-320, respectively, which are modified by Lgts. *In vitro*, all Lgt-family members are capable of glucosylating Hbs1. However, so far it is not known whether Hbs1 is a substrate of *Legionella* glucosyltransferases in intact cells.

The functional role of Hbs1 has been the topic of several investigations. First, it was shown that an increased copy number of Hbs1 suppresses the growth defect of the *S. cerevisiae* double mutant in *ssb1* and *ssb2* genes. Proteins Ssb1/2 are chaperones of the Hsp70 family that are associated with translating ribosomes and may aid in the passage of the nascent polypeptide through the ribosome channel into the cytosol (Nelson et al., 1992). Thus, these first experiments suggest a role of Hbs1 in the translational machinery although its precise function has not been established.

First direct indication toward the role of Hbs1 in eukaryotic cell physiology came from studies on the mechanism of RNA surveillance in yeast. Stalled translational complexes, which halt in elongation due to inhibitory structures or defects of translated mRNA

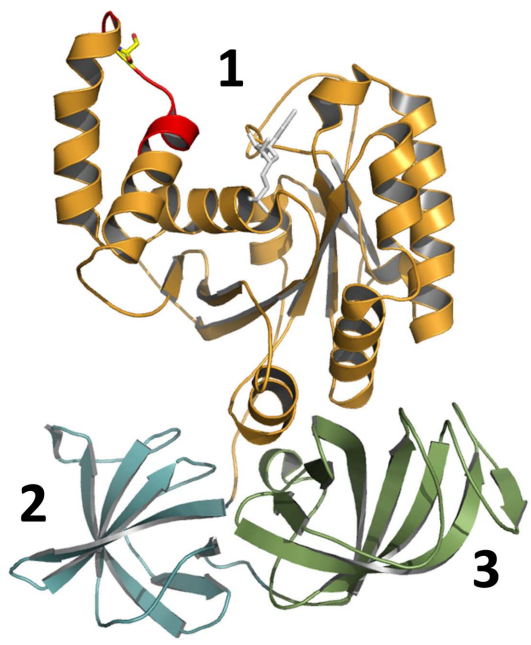

FIGURE 2 | Structural view of yeast elongation factor eEF1A (adapted from pdb 1IJF). Elongation factor eEF1A consists of three main structural parts: domain 1 (G-domain), domain 2, and domain 3 (indicated by numbers). The decapeptide (GKGSFKYAWV), which is a sufficient substrate for glucosylation by Lgt, is shown in red. Serine-53, which is modified by glucosyltransferases Lgt, is shown in yellow. The complexed fragment of eEF1Bα molecule, which is present in the original structure, is omitted.

(e.g., hairpin loops, rare codons, chemical damage), are subjected to specific degradation steps, termed "no-go-decay" (NGD). NGD starts with endonucleolytic cleavage of mRNAs near the site of the stall followed by degradation of produced 5′ and 3′ ribonucleic acid fragments (Doma and Parker, 2006). Such initial cleavage appears to depend on Hbs1 and another protein Dom34. Deletion of Dom34 avoid endonucleolytic cleavage, while deletion of Hbs1 strongly reduced but not prevented NGD. The latter observation suggests that Hbs1 although important is not absolutely required for this type of mRNA surveillance system. Recent studies by using *in vitro* reconstituted yeast translation system shed more light onto the function of Hbs1/Dom34 complex. According to these data, Hbs1/Dom34 directly destabilizes the mRNA:ribosome complex and promotes recycling of its functional components (Shoemaker et al., 2010). So far, however, it completely enigmatic how processes of NGD are related to the infection biology of *Legionella*.

STRUCTURAL AND MECHANISTIC FEATURES OF *L. PNEUMOPHILA* GLUCOSYLTRANSFERASE Lgt1

The crystal structure of Lgt1 was solved recently by two independent research groups almost simultaneously (**Figure 3A**; Hurtado-Guerrero et al., 2010; Lu et al., 2010). In principle the structures resemble two catalytic states. One structure represents the *catalytic competent state* with intact UDP–glucose and the divalent ion preformed for acceptor binding and modification (*Lpl*GT·UDP–glucose·Mg^{2+} pdb 2WZG, 3JSZ). The second structure most likely exhibits the *product state* with the donor substrate hydrolyzed (*Lpp*GT·UDP·glucose·Mn^{2+}, pdb 2WZF).

In general, the structure of Lgt1 shows a mixed α/β-fold, which is grouped into the GT-A family of GTs. Lgt1 can be dissected into three different structural domains. Domain I consists of seven N-terminal α-helices (α1–α7) with yet unknown functions (**Figure 3A**). Domain II constitutes the typical GT GT-A core domain with a twisted continuous central β-sheet surrounded by α-helices presenting the double Rossmann fold-like signature (α8–α15/β1–β10). This nucleotide binding domain harbors the donor substrate-binding site and catalytic important residues. Domain III is a predominant α-helical "protrusion domain" (α16–α30/β11–β12) suggested to be involved in acceptor substrate-binding (Hurtado-Guerrero et al., 2010). As a common structural feature in GTs, Lgts possess a C-terminal flexible loop, which seems to be important for the proper arrangement of the acceptor binding site and the release of the reaction products (**Figure 3A**). Structural BLASTs with Lgt1 show highest similarity with the CGT toxin B from *C. difficile*, lethal toxin from *C. sordellii,* and α-toxin from *C. novyi*. Similarity is restricted to the catalytic core of the GTs, where interestingly several catalytically important residues are structurally very well conserved (**Figure 1A**). The N-terminal helical domains (domain I) of toxin B and Lgts are topologically unrelated and the function as a subcellular sorting signal, as found in CGTs, is not analyzed yet for Lgts (Mesmin et al., 2004; Kamitani et al., 2010). Sequence comparison of Lgt1 with the other family members Lgt2 and Lgt3 shows an overall identity of only 18–28%, due to several additional coiled-coil domains and C-terminal extensions. Nevertheless, remarkably high conservation is found in the nucleotide binding site, the suggested catalytic amino acids, and the acceptor binding groove. The unique function or structural importance of the C-terminal extension of Lgt3 remains elusive. It was shown that Lgt2 and Lgt3 and several other *Legionella* effectors have their type IV secretion signal in the C-terminus of the protein. In contrast Lgt1 seems to have its type IV secretion signal sequence in the N-terminal region (Hurtado-Guerrero et al., 2010). The exact position or motifs for this signal are not known yet. In Lgt1 it was speculated that this region is located within the first 10 amino acids, which were unfortunately disordered in the crystal structures.

UDP–GLUCOSE BINDING POCKET

As depicted in **Figure 3B** the binding of the sugar nucleotide proceeds via loops of the central β-sheet and the protrusion domain in a "curled under" conformation typical in GTs (Gibson et al., 2004; Qasba et al., 2005). In this tense conformation the glucose moiety is tucked underneath the pyrophosphate bridge positioning the anomeric carbon of glucose in such a manner to provide access for the incoming acceptor substrate. The nucleotide portion is bound by three loops (α12–α13, α4–α8, C-terminal loop) mainly via hydrogen bonding to the backbone. The uracil ring of UDP is sandwiched between Trp-139 and Pro-225 by hydrophobic stacking. The distal part of the glucosyl moiety of the donor substrate is bound by a typical triad binding geometry formed by Asp-230, Arg-233, and Asp-246 (**Figure 3B**; Negishi et al., 2003; Jank et al., 2007). This specific hydrogen bonding network might determine the sugar selectivity at the 4′-OH position, thus using glucose instead of galactose. Comparison of UDP–glucose bound to Lgt1 in the intact and cleaved form showed that in both states the nucleotides are bound in the same manner and adopt the same conformation. The main structural divergence is seen in a positional shift of the anomeric carbon of about 1.6 Å. Interestingly the same shift is recognized in the structure of *C. difficile* toxin B. Structural analysis of carbohydrate metabolizing enzymes as glycosidases reveal a similar movement of the anomeric atom of the sugar after hydrolysis, here called "electrophilic migration" (Vocadlo et al., 2001). This conserved movement substantiates the mechanistic importance of global structural rearrangements of the GT leading to a significant distortion of the donor substrate during transition state and hydrolysis.

DXD-MOTIF

The DXD-motif (Asp-246 and Asp-248) upstream of a short hydrophobic patch is the remarkable motif for GTs of the GT-A type and crucial for divalent cation binding (**Figures 1 and 3**). In Lgt1 the cation is coordinated in an octahedral complex where two valences are occupied by the α- and β-phosphates of UDP. As in several other GTs only the second aspartic acid of the DXD-motif is involved in direct cation coordination, the first residue coordinates Mn^{2+} through a water molecule and hydrogen bonds a distal glucose hydroxyl. The remaining two valences are occupied by water molecules. Only mutation of the first aspartic acid lead to dramatic reduction in enzyme activity showing its fundamental importance (Hurtado-Guerrero et al., 2010). The role of the divalent metal ion in Lgts as in other GTs seems to be severalfold. Binding of the metal ion in conjunction with the donor substrate is a prerequisite for the induction of a conformational change in the C-terminal

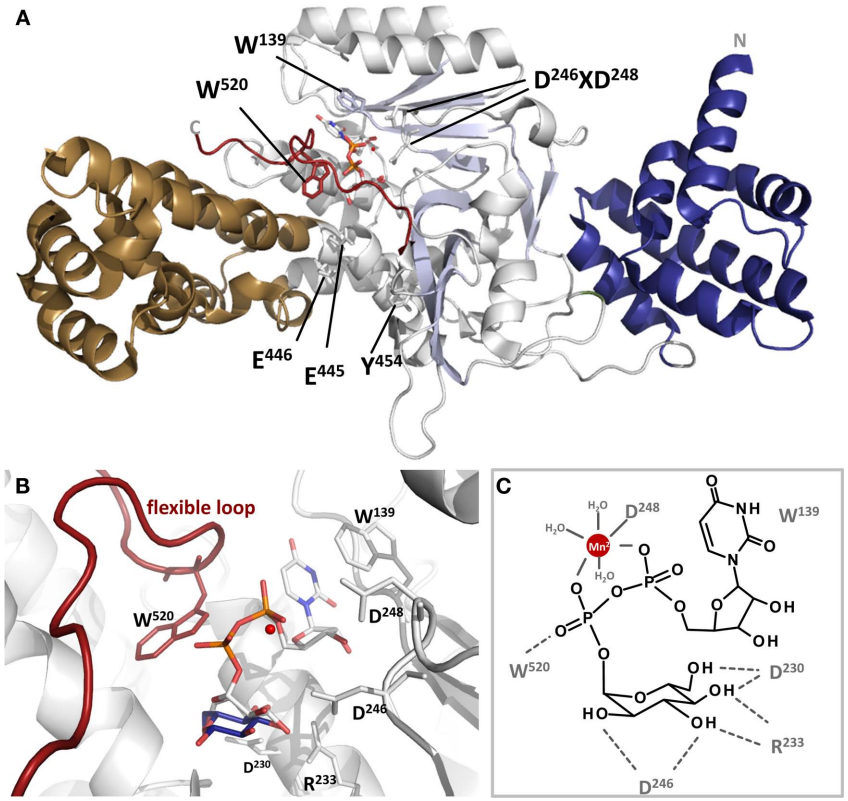

FIGURE 3 | Cartoon presentation of Lgt1 crystal structure in complex with UDP–glucose and Mg^{2+} (pdb code 3JSZ). (A) The N-terminal domain is depicted in blue, the central domain in gray and the protrusion domain in brown. The central beta sheet is shaded in light blue. UDP–glucose is shown in sticks and Mg^{2+} as a red sphere. The flexible loop region is highlighted in dark red. Aspartic acid residues of the DXD-motif, Trp-520 of the flexible loop, Trp-139 stacking the base are shown in sticks. **(B)** Magnified view on the catalytic site of Lgt1 as in **(A)** with intact UDP–glucose (white) and glucose shifted about 1.6 Å after cleavage (dark blue; deduced from pdb 2WZF). Important amino acids are shown as sticks. Trp-139 is stacking the uracil ring of the donor, Asp-230, Arg-233, and Asp-246 are orientating the distal part of glucose as a triade. Asp-248 of the DXD-motif is coordinating the divalent ion (red) in conjunction with three additional water molecules. Trp-520 of the flexible loop is marked in red. **(C)** Schematic representation of the catalytic site as in **(B)** with important amino acids marked and highlighted.

flexible loop region (Ziegler et al., 2008). Furthermore, the ion is necessary for the stabilization of the transition state during catalysis by compensating the negative charge of the β-phosphate of the nucleotide and facilitating the departure of the leaving group (Charnock and Davies, 1999; Qasba et al., 2005; Ramakrishnan et al., 2006; Hurtado-Guerrero et al., 2010).

GLYCOSYLTRANSFER MECHANISM

In general it is assumed that the reaction catalyzed by GTs follows a sequential ordered mechanism. Here, the metal ion and sugar nucleotide bind first followed by the acceptor (Qasba et al., 2005). After glycosyltransfer the product is ejected followed by the nucleotide and the metal ion. For Lgts there are some hints that the metal ion remains bound to the enzyme very tightly and is not ejected (unpublished data). The release of the products is accompanied by changes in the flexible loop region during which UDP is ejected. In Lgt1 there is one C-terminally located mobile loop (amino acid 513–525). This loop most likely rearranges upon binding to the donor substrate (**Figure 3A**; Hurtado-Guerrero et al., 2010). This conformational change is a general feature observed in GTs with GT-A and GT-B fold (Boix et al., 2001; Flint et al., 2005; Qasba et al., 2005; Gordon et al., 2006; Kubota et al., 2006; Ramakrishnan et al., 2006; Ziegler et al., 2008). In the structure of Lgt1 the loop shows only sufficient electron density in the UDP–glucose bound form (closed conformation) due to high mobility of the loop without intact donor substrate. For the closest homologous GTs, the CGT, the conformational changes of the flexible loops were crystallographically proven (Ziegler et al., 2008). In its apo-form the clostridial enzymes resemble an open conformation where the UDP–sugar has access to the donor substrate-binding pocket. Upon UDP–sugar-binding the loop closes and renders its C-terminal random coil structure to a rigid α-helix. Thereby rearrangement of a structurally conserved tryptophan residue (tryptophan-520 in toxin B) is induced resulting in an extensive movement of about 15 Å to bind the β-phosphate of the nucleotide. Mutation of this residue in *C. difficile* toxin B reduces the enzymatic and hydrolytic activity (transfer reaction to water instead of protein acceptor) of the GTs drastically implicating its decisive role in catalysis. Lgt1 harbors tryptophan-520 at the exact same position and suggests the same mechanistic function.

Extended mutational analysis of Lgt1 reveals that tryptophan-520 is not directly involved in catalysis but has rather the function to bind the acceptor substrate (Lu et al., 2010).

STEREOCHEMISTRY

Apart from the structural fold GTs are categorized and distinguished by its stereochemistry in glycosyl transfer mechanism, whether the anomeric configuration of the glycosyl moiety is retained or inversed (Coutinho et al., 2003). Lgt1 was shown to be a retaining GT. NMR structural analysis of glucosylated peptides revealed that the sugar is transferred to the acceptor with net retention of the α-anomeric configuration (Belyi et al., 2009). The mechanism for inverting GTs is well understood and follows a single nucleophilic substitution and thereby inversion of the sugar C1 configuration (Lairson et al., 2008). For a retaining mechanism two possible reaction schemes are highly discussed, the double displacement mechanism or a single S_Ni-like mechanism (Davies et al., 1997). In the double displacement strategy two subsequent S_N2-reactions occur each with inversion of the anomeric bond. For GTs the double displacement theory seems to be rejected although there are several reports of trapped glycosyl-enzymes (Mosi et al., 1997; Uitdehaag et al., 1999; Gastinel et al., 2001; Lairson et al., 2004; Ramakrishnan et al., 2006; Soya et al., 2011). Mainly the lack of an appropriate positioned conserved nucleophilic amino acid on the β-face of the sugar argues against this theory (Lairson et al., 2008). In Lgts the conserved amino acid Asn-293 is located at the N-terminus of a structurally conserved central α-helix in the obvious access pathway of the substrate eEF1A. There it is moderately positioned to act as a nucleophil on the β-face of the sugar. Although mutations of this residue lead to severely reduced enzyme activity, Asp-293 seems not to be involved in the transfer mechanism itself. It rather functions in the guidance and/or binding of the acceptor substrate (Lu et al., 2010).

In contrast to the double displacement mechanism the S_Ni-like mechanism proceeds through a short-lived oxocarbenium intermediate. This intermediate is stabilized by the enzyme and shielded on the β-face of the sugar thereby preventing a nucleophilic attack from the opposite side of the reaction center (Lairson et al., 2008). An idea for Lgt1 reacting in this manner comes from the related crystal structure of lethal toxin from *C. sordellii*. The Ca^{2+}-ligated structure presented a glucosyl half-chair conformation assuming an oxocarbenium intermediate state, the prerequisite for the S_Ni-like transfer mechanism (Ramakrishnan et al., 2006; Ziegler et al., 2008). Furthermore, studies with inhibitory glucomimetics and iminosugars, mimicking the oxocarbenium ion intermediate state conformation during catalysis, confirmed the S_Ni-like mechanism for the CGT biochemically as well as structurally (Jank et al., 2008). Lgts might react in the same manner as the related toxins but more studies are necessary.

In conclusion, the retaining reaction mechanism of the Lgts seems to follow a S_Ni-like mechanism. The glycosyltransfer reaction starts with the binding of the divalent cation to Asp-248 of the DXD-motif and the binding of the donor substrate UDP–glucose into the open cleft of the enzyme. Hereby, Trp-139 is stacking the base and Asp-230, Arg-233, and Asp-246 coordinate the distal part of the glucosyl moiety. Subsequently, the long C-terminal flexible loop rearranges to the closed conformation where Trp-520 flips into the catalytic pocket. In this state the catalytic competent conformation and the substrate-binding site is arranged. The Mn^{2+} and/or the pyrophosphoryl group abstract the proton of the incoming acceptor amino acid Ser-53 of eEF1A, leading to the positive charged oxocarbenium glucosyl-intermediate, which is then attacked by Ser-53 leading to the products UDP, Mn^{2+}, and glucosyl–eEF1A. After dissociation of gluc–eEF1A the flexible loop opens and releases UDP and the cation to start a new reaction cycle.

Lgt1–eEF1A INTERACTION

The putative acceptor binding site of Lgt1 includes two acidic residues (Glu-445 and Glu-446) located at the funnel-like entrance to the active site representing an overall negative charge. Mutation of each of these residues leads to a slightly reduced glucosyltransferase activity without reducing the affinity of UDP–glucose to the catalytic site, implicating a role in acceptor substrate-binding (Hurtado-Guerrero et al., 2010). In the substrate eEF1A the acceptor amino acid Ser-53 resides on a loop between two helices (helix A*–loop–helix A′) of the GTPase domain. Serine-53 is flanked by two conserved lysine residues protruding as a positive charge from the GTPase domain. It was assumed that this opposite electrostatic surface potential is crucial for the Lgt1–eEF1A interaction and provides the necessary affinity for the enzyme–substrate complex (Hurtado-Guerrero et al., 2010; Lu et al., 2010). Contradictory results, however, came from comprehensive biochemical analysis, bordering the minimal substrate determinants of eEF1A. Here the authors showed that these lysine residues seem not to be of fundamental importance for an efficient transfer reaction (Belyi et al., 2009). This is in line with putative substrate Hbs1, which contains only one lysine residue adjacent to Ser-53 and is remarkably modified. Using the location of the acceptor site serine-53 and the overall shape of Lgt1 and eEF1A, docking simulations have been performed, revealing an interaction model of an enzyme–substrate complex. The flexible loop of the glucosyltransferase has to adopt a slightly different conformation upon substrate-binding (Lu et al., 2010). Interface mutagenesis of the key exposed amino acid tyrosine-454 and biochemical analysis support this interaction model (Hurtado-Guerrero et al., 2010). Considering that eEF1A by itself is a rather poor substrate and structural substrate determinants can be reduced to a decapeptide, it is assumed that a particular conformation of EF1A is the preferred substrate or the non-ambiguous substrate consists of additional host factors, which are not considered in the suggested interaction model (Belyi and Aktories, 2010).

PUTATIVE GLYCOSYLTRANSFERASES IN *LEGIONELLA*

Sequence analysis of the *L. pneumophila* genome allowed identification of other putative GTs chromosomally located in T4SS effector regions (Franco et al., 2009).

A screen of 127 confirmed and putative Dot/Icm substrates for their ability to generate lethal yeast phenotypes allowed identification of a ~72-kDa protein termed subversion of eukaryotic vesicle trafficking A (SetA; Heidtman et al., 2009). Its coding sequence has ID *lpg1978* in the genome of *L. pneumophila* Philadelphia-1 strain but is not present in the genome of *L. longbeachae* (Cazalet et al., 2010; Kozak et al., 2010). Expression of this gene in *S. cerevisiae* resulted in secretory defects detected by altered trafficking/processing of yeast markers carboxypeptidase Y and alkaline phosphatase. SetA

seemed to be localized to late endosomal/lysosomal compartments, co-localizing with eukaryotic marker proteins LAMP-1 and Rab7. In *L. pneumophila*-infected cells SetA was secreted into eukaryotic cytosol in an Icm/DotA-dependent manner and demonstrated tropism for host cell membranes. One interesting structural feature of this protein is the occurrence of the GT-characteristic DXD-motif ($D^{134}SD^{136}$). Mutation of both aspartic acid residues in SetA to alanines alleviated the toxic phenotype, suggesting a link between possible GT activity of the protein and its lethal effect in yeast. Bearing in mind its subcellular distribution and altered trafficking phenotypes in yeast, transformed with the gene *setA*, these mutation experiments raise the possibility that SetA glycosylates and inactivates a factor of the endosomal protein sorting machinery.

BLAST search for proteins similar to SetA in the *L. pneumophila* Philadelphia-1 genome reveals a ~59-kDa protein, representing a product of *lpg1961* gene (**Figure 1A**). Product of this gene was also toxic for *S. cerevisiae* and caused selective defects on alkaline phosphatase processing in yeast (Heidtman et al., 2009). In accordance to several GTs this protein also possesses a DXD-motif with the canonical tyrosine, aspartic acid and arginine residues of the GT-A triad upstream the DXD-motif (**Figure 1A**). These features pointed toward a possible GT activity in this *L. pneumophila* product as well.

Recently, genome sequences of the two non-*pneumophila* species became available – *Legionella drancourtii* strain LLAP12 (Moliner et al., 2010) and *L. longbeachae* strains D-4968 and NSW150 (Cazalet et al., 2010; Kozak et al., 2010). BLAST searches within these strains disclose two proteins in *L. drancourtii* (LDG0102 and LDG0103) and two proteins in each *L. longbeachae* genomes (LLB0067/LLO1578 and LLB3681/LLO1721), showing identity of around 15% with Lgt1. Identical amino acid residues could be found predominantly in the first third of the proteins (**Figure 1B**), while homology outside this region was insignificant. Although all these four proteins possess a DXD-containing region resembling that of typical GTs, the nature of their enzymatic activity (if any), their targets and donor substrates remain to be determined. Furthermore, it is questionable whether these putative GTs are secreted *Legionella* effectors. It might be that their function lies not in virulence but rather in basic carbohydrate metabolism of the bacterium.

FUNCTIONAL CONSEQUENCES OF GLYCOSYLATION AND OPEN QUESTIONS

Major targets of Lgt-catalyzed glucosylation are crucial components of translational machinery of eukaryotic cells, e.g., eEF1A and Hbs1. Addition of Lgt1, Lgt2, or Lgt3 to *in vitro* reticulocyte or yeast translational extracts resulted in a dose-dependent inhibition of protein synthesis. Furthermore, introduction of Lgts into mammalian cells by electroporation results in eEF1A modification, protein synthesis inhibition, and death (Belyi et al., 2006, 2008). Similarly, expression of genes coding for Lgt1, Lgt2, or Lgt3 in *S. cerevisiae* resulted in yeast cell death (Heidtman et al., 2009).

Up to date, the precise mechanism of protein inhibition by Lgt-induced glucosylation of Ser53 of eEF1A is still not clear. Moreover, the role of Lgt-induced protein synthesis inhibition in the infection biology of *Legionella* is not known. One speculation is that the action of Lgt strongly decreases general metabolism and thereby antibacterial activity and, thus, makes host cells "defenseless" against proliferation of invading bacteria. On the other hand, at final stages of the intracellular life cycle, *Legionella* has to kill and escape the eukaryotic cell and eEF1A-targeting glucosyltransferases may participate in such a task as strong lethal toxins.

An alternative hypothesis about the *smart* roles of bacterial effectors of *Legionella* has been proposed recently from investigations of the T4SS effector SidI (Shen et al., 2009). The ~110-kDa protein SidI (Lpg2504) exhibits a toxic phenotype in yeast. It was demonstrated that SidI interacts with eEF1A and eEF1Bγ and inhibits protein synthesis both *in vitro* and *in vivo*. Another type of activity, associated with SidI is its participation in a stress response of eukaryotic cell.

It is known that stress response in mammalian cells is controlled by heat shock transcription factor 1 (HSF1), which is able to bind specific promoters (heat shock elements, HSE) and thus induces production of a panel of heat shock proteins, necessary to rescue eukaryotic cells, suffering under unfavorable conditions (Sarge et al., 1991). Activation of HSF1 is dependent upon formation of a multi-component complex, consisting of HSF1, eEF1A, and ~0.6 kb non-coding RNA molecule, termed heat shock RNA 1 (HSR1; Shamovsky et al., 2006). Infection of macrophage-like cells U937 with virulent *L. pneumophila*, but not with a *sidI*-negative mutant, resulted in eukaryotic stress response detected by elevated level of HFS1/eEF1A complex, increased binding of HSF1 to HSE and stimulation of *hsp70* expression. Similar phenomena were observed by transfection of target cells with SidI-coding plasmid (Shen et al., 2009). These results indicates that HSF1 is activated during *L. pneumophila* infection and SidI, shown initially to suppress protein synthesis, contributes to such an activation.

Thus, bearing in mind the multitude of its cellular functions, targeting elongation factor 1A by Lgt1/2/3 may lead to pleiotropic outcomes and the observed cytotoxicity may be a side effect of some other pro-bacterial consequence of eEF1A glucosylation (Ensminger and Isberg, 2009). The proposed modification of Hbs1 by the *Legionella* effectors further adds complexity to the list of events, which might be caused by the enzymatic activity of the glucosyltransferase Lgt.

Recent findings indicate that apart from *Clostridia* and *Legionella*, other bacteria can also possess GT activities as important virulence strategies. List of such putative glycosylating molecules includes several proteins found in *Chlamydia trachomatis* (Belland et al., 2001). One such protein, termed CT166, was shown to induce Rac-dependent actin re-organization and mammalian cell rounding, resembling action of glucosylating toxin B of *C. difficile* (Thalmann et al., 2010). Other toxins with possible glycosylation type of activity include LifA and toxin B of enteric pathogens (Stevens et al., 2004). These findings suggest that glucosylation is more often used by pathogens to prevail in a hostile environment as suggested before.

On the other hand, control of translational processes of host cells is a well-known mechanism used by various pathogenic bacteria and accomplished by different enzymatic activities, e.g., *Pseudomonas aeruginosa* exotoxin A and diphtheria toxin inhibit protein synthesis by ADP-ribosylation of eEF2, while Shiga- and Shiga-like toxins from *Shigella* and *Escherichia coli*, respectively, block host translation by *N*-glycosidase activity (Popoff, 1998). Biological purpose of inhibition of protein synthesis accomplished by the latter group of toxins is still not completely clear. But the fact that unrelated enzymatic activities (i.e., glucosylation, ADP-ribosylation, and N-glycosidation) result in termination of eukaryotic protein synthesis suggests critical importance of this targeting in host–pathogen interaction.

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Molecular characterization of exploitation of the polyubiquitination and farnesylation machineries of *Dictyostelium discoideum* by the AnkB F-box effector of *Legionella pneumophila*

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The Dot/Icm-translocated Ankyrin B (AnkB) F-box effector of *Legionella pneumophila* is essential for intra-vacuolar proliferation and functions as a platform for the docking of polyubiquitinated proteins to the *Legionella*-containing vacuole (LCV) within macrophages and ameba. Here we show that ectopically expressed AnkB in *Dictyostelium discoideum* is targeted to the plasma membrane where it recruits polyubiquitinated proteins and it *trans*-rescues the intracellular growth defect of the *ankB* null mutant, which has never been demonstrated for any effector in ameba. Using co-immunoprecipitation and bimolecular fluorescence complementation we show specific interaction of Skp1 of *D. discoideum* with the F-box domain of AnkB, which has never been demonstrated in ameba. We show that anchoring of AnkB to the cytosolic face of the LCV membrane in *D. discoideum* is mediated by the host farnesylation of the C-terminal eukaryotic CaaX motif of AnkB and is independent of the F-box and the two ANK domains, which has never been demonstrated in ameba. Importantly, the three host farnesylation enzymes farnesyl transferase, RCE-1, and isoprenyl cysteine carboxyl methyl transferase of *D. discoideum* are recruited to the LCV in a Dot/Icm-dependent manner, which has never been demonstrated in ameba. We conclude that the polyubiquitination and farnesylation enzymatic machineries of *D. discoideum* are recruited to the LCV in a Dot/Icm-dependent manner and the AnkB effector exploits the two evolutionarily conserved eukaryotic machineries to proliferate within ameba, similar to mammalian cells. We propose that *L. pneumophila* has acquired *ankB* through inter-kingdom horizontal gene transfer from primitive eukaryotes, which facilitated proliferation of *L. pneumophila* within human cells and the emergence of Legionnaires' disease.

Keywords: dot/Icm, Skp1, farnesyl, SCF1, prenylation

INTRODUCTION

Legionella pneumophila is a facultative intracellular Gram-negative bacterium that is ubiquitous in aquatic environments (Fields, 1996; Harb et al., 2000; Bitar et al., 2004; Molmeret et al., 2005). *L. pneumophila* invades and replicates within fresh water amebae and ciliated protozoa. The co-evolution and bacterial adaptation to protozoan hosts is thought to be a factor for the ability of *L. pneumophila* to proliferate within human cells and cause disease (Harb et al., 2000; Swanson and Hammer, 2000; Molmeret et al., 2005). The transmission of *L. pneumophila* to humans takes place by inhalation of *L. pneumophila*-contaminated aerosols. *L. pneumophila* reaches the alveoli, where it infects and replicates within alveolar cells leading to an atypical pneumonia known as Legionnaires' disease (Kaufmann et al., 1981). Remarkably, the life cycle of *L. pneumophila* within amebae and macrophages is similar (Fields et al., 2002). Within both host cells, the *Legionella*-containing vacuole (LCV) evades targeting to and degradation by the endosomal–lysosomal pathway and is remodeled by the endoplasmic reticulum (ER). During late stages of intracellular proliferation within macrophages and ameba, *L. pneumophila* disrupts the phagosomal membrane and escapes into the host cell cytosol where various virulence traits are triggered to enable egress of the bacteria to the extracellular environment (Molmeret et al., 2004, 2010; Al-Khodor et al., 2010).

Efficient formation of a replication vacuole and successful intracellular growth of *L. pneumophila* requires the Dot/Icm type IV secretion system (Purcell and Shuman, 1998; Vogel et al., 1998). It is estimated that >200 effectors are translocated into the host cell by the Dot/Icm secretion system, but most of the studied effectors are dispensable for intracellular proliferation (Isberg et al., 2009). The Dot/Icm-translocated AnkB effector is one of very few exceptions, since it plays a major role in intracellular proliferation within macrophages and protozoa and is essential for intrapulmonary proliferation of L. *pneumophila* in the mouse model (Al-Khodor et al., 2008, 2010; Habyarimana et al., 2008; Price et al., 2009). The majority of the structure of AnkB is composed of eukaryotic domains and motifs that include an F-box domain, two Ankyrin repeats and a C-terminal CaaX farnesylation motif (Al-Khodor et al., 2008, 2010; Habyarimana et al., 2008; Price et al., 2009).

Legionella pneumophila is one of many intracellular bacterial pathogens that exploit the host polyubiquitination machinery (Dorer et al., 2006; Price et al., 2009, 2010a,b; Al-Khodor et al., 2010). Ubiquitination is a highly conserved eukaryotic post-translational process that covalently links ubiquitin monomers to target the protein to proteasomal degradation or to modulate its function (Kerscher et al., 2006). We have shown that AnkB mimics the action of host cell F-box proteins by functioning as a platform for the docking of polyubiquitinated proteins to the LCV within evolutionarily distant hosts; macrophages and ameba (Price et al., 2009, 2010b; Al-Khodor et al., 2010). Moreover, the F-box domain of AnkB interacts with mammalian Skp1; a component of the SCF1 (Skp1, Cullin1, F-box) ubiquitin ligase complex (Zheng et al., 2002). However; it is not known whether AnkB interacts with Skp1 of ameba.

In addition to exploitation of the host cell polyubiquitination machinery by AnkB, *L. pneumophila* also exploits the host farnesylation machinery via the C-terminal CaaX motif of AnkB to anchor the F-box effector into the cytosolic face of the LCV membrane (Price et al., 2010b). Farnesylation is a post-translational modification of eukaryotic proteins, which involves farnesyl transferase (FTase)-mediated addition of a 15 carbon lipid moiety at the conserved cysteine residue of the CaaX motif (Wright and Philips, 2006). After farnesylation, the "aaX" tri-peptide is cleaved by an endoprotease (RCE1 protease; Boyartchuk et al., 1997) followed by carboxyl methylation by isoprenyl cysteine carboxyl methyl transferase (IcmT; Dai et al., 1998; Bergo et al., 2000). This post-translational modification process increases protein hydrophobicity to enable anchoring of a hydrophilic protein to the lipid bi-layer of membranes. It is not known whether farnesylation of AnkB occurs locally at the LCV within *Dictyostelium discoideum* through selective recruitment of the farnesylation enzymatic machinery or that it occurs at other cellular sites and is trafficked back to the LCV. It is also not known whether, in addition to the CaaX farnesylation motif, any of the three eukaryotic domains of AnkB are involved in specific targeting of AnkB to the LCV membrane within *D. discoideum*.

We show that anchoring of AnkB to the cytosolic face of the LCV membrane in *D. discoideum* is mediated by the ameba farnesylation machinery, and is independent of the three eukaryotic domains of AnkB (F-box and the two ANK domains). Importantly, the three farnesylation enzymes FTase, RCE-1, and IcmT of *D. discoideum* are recruited to the LCV in a Dot/Icm-dependent manner. We conclude that the farnesylation and polyubiquitination enzymatic machineries of *D. discoideum* are recruited to the LCV in a Dot/Icm-dependent manner and the AnkB effector exploits the two evolutionarily conserved eukaryotic machineries to proliferate within ameba and human cells.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CELL CULTURES

Legionella pneumophila serogroup I parental strain AA100/130b (ATCC BAA-74) and the isogenic mutants; *dotA*, *ankB*, in addition to complemented *ankB* mutants were described previously (Al-Khodor et al., 2008; Price et al., 2010a). They were grown for 72 h on buffered charcoal–yeast extract (BCYE) plates at 37°C with 5% of CO_2. The plates used for the cultivation of *dotA* and *ank* mutant strains were supplemented with kanamycin at a concentration of 50 μg/ml, and when required, chloramphenicol at concentration of 5 μg/ml. *Escherichia coli* strain DH5α was used for cloning purposes.

AMEBA CULTURE

Axenic *A. polyphaga* was cultured as adherent cells in PYG medium as previously described. The *D. discoideum* wild type strain AX2 was grown axenically at 24°C in HL5 medium supplemented with 0.6% penicillin–streptomycin and G418 20 μg/ml as needed at 24°C as we described previously (Clarke et al., 1980; Price et al., 2010b).

INTRACELLULAR GROWTH KINETICS

The infection of *D. discoideum* and *A. polyphaga* were performed as described previously (Solomon et al., 2000; Price et al., 2009, 2010b). Briefly, the exponentially growing *A. polyphaga* or *D. discoideum* were infected for 1 h with bacterial strains at a multiplicity of infection (MOI) of 10 and incubated at 24°C (*D. discoideum*) or 37°C (*A. polyphaga*). After 60 min of the infection, 50 μg/ml gentamicin was added to the medium for 1 h to kill extracellular bacteria. At the time point indicated, the infected cells were washed two times with PBS (*A. polyphaga*) or SorC buffer (*D. discoideum*), and were lysed with 0.04% Triton X-100. A dilution series of the cell lysates was plated on CYE medium for 3 days. The number of bacteria was expressed as the number of CFU/ml. At least three independent experiments, in triplicate, were performed.

CONFOCAL LASER SCANNING MICROSCOPY

Analyses of infected cells by confocal microscopy were performed as described previously for both hosts (Habyarimana et al., 2008; Price et al., 2009, 2010b). Briefly, at the time point indicated, the infected cells were washed three times with cold SorC buffer (*D. discoideum*) or PBS (*A. polyphaga*) and fixed with 4% paraformaldehyde in PBS for 30 min. The fixed cells were washed and were permeabilized (cold methanol 30 s) and blocked for 60 min. The 3XFLAG-tagged proteins were labeled with polyclonal rabbit anti-AnkB (1/200 dilution) antiserum, followed by Alexa-Fluor 488-conjugated donkey secondary anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA, USA). Bacteria were labeled with monoclonal anti-*L. pneumophila* antibodies and Alexa-Fluor 647-conjugated donkey anti-mouse antibody. For ectopic expression of FLAG-*ankB* in *D. discoideum*; FLAG-*ankB* was labeled with polyclonal rabbit anti-AnkB (1/200 dilution) antiserum. To label the polyubiquitinated proteins, anti-polyubiquitin FK1 mouse monoclonal antibodies were used (BIOMOL International/Affiniti, Exeter, UK), followed by appropriate Alexa-Fluor conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). Polyclonal rabbit anti-(FT-α or RCE-1) or polyclonal goat anti-IcmT antisera were used at 1:50 dilutions (Santa Cruz). The cells were examined by Olympus Fv1000 laser scanning confocal microscope as we described previously. On average, 8–15, 0.2 μm serial Z sections of each image were captured and stored for further analyses, using Adobe Photoshop CS3.

ISOLATION OF LCVs

Phagosomes were isolated from post-nuclear supernatants (PNS) of infected *D. discoideum* as we described previously (Berger and Isberg, 1993; Price et al., 2009, 2010b). Post-exponentially grown

L. pneumophila were introduced onto monolayers at MOI of 10 by 10 min centrifugation at 300×*g*, the infection was allowed to proceed for 1 h. After removing the extracellular bacteria by washing the cells three times with 10 ml of cold SorC, infected cells were scraped from the dish using 10 ml of cold SorC. The cells were pelleted by centrifugation (5 min, 1000 rpm, 4°C), and re-suspended in 2 ml of homogenization buffer (20 mM Hepes/KOH pH = 7.2, 250 mM sucrose, 5 mM EGTA) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma) and lysed in a Dounce homogenizer. The homogenate was transferred to microfuge tubes to separate LCVs from unbroken cells and nuclei (3 min, 1500 rpm, 4°C). The PNS containing the LCVs was spun for 5 min at 4°C onto poly L-lysine coated coverslips, and immobilized by 4% paraformaldehyde for 60 min. The LCVs were labeled with polyclonal rabbit anti-AnkB (1/200 dilution) antiserum and *L. pneumophila* was labeled with DAPI stain followed by Alexa-Fluor tagged anti-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, CA, USA).

CHEMICAL INHIBITION OF FARNESYLATION

The inhibitor FTI-277 was re-suspended in DMSO + 0.4 mM DTT and used immediately for experimental assays, as we described previously. *D. discoideum* were treated with 5.0 μM FTI-277 1 h before the infection. *L. pneumophila* was used to infect *D. discoideum* at MOI of 10 for 1 h followed by treatment with 50 μg/ml gentamicin for 1 h to kill extracellular bacteria. FTI-277 was maintained in the growth media throughout the experiment. After 2 h, semi-purified LCVs were purified and were fixed. Samples were labeled with polyclonal goat anti-*Legionella* and rabbit anti-AnkB antisera. Alexa-Fluor 488-tagged antibodies against rabbit IgG and Alexa-Fluor 555-conjugated donkey anti-goat IgG antibodies were used as secondary antibodies (Invitrogen, Carlsbad, CA, USA).

IN VIVO CO-IMMUNOPRECIPITATION

Dictyostelium discoideum were infected with *L. pneumophila* strains for 2 h using MOI 50. Semi-purification of LCVs was performed as mentioned above. The supernatants that contain the semi-purified LCVs were incubated overnight at 4°C with polyclonal rabbit anti-AnkB antibodies. One hundred microliter of immobilized protein G (Pierce, Rockford, IL, USA) were added to the reaction and incubated for 4 h at 4°C. After removing the unbound proteins by washing the beads five times with cold PBS, samples were heated at 96°C for 5 min in sample buffer and subjected to 10.4–15% gradient SDS-PAGE gel electrophoresis. For AnkB-Skp1 interaction, samples were subjected to immunoblot analysis using an polyclonal rabbit anti-AnkB antibodies (1/60000 dilution; Price et al., 2010b) followed by anti-Skp1 (1/200 dilution) antibodies. To test if AnkB is being modified by farnesylation; samples were immunoblotted with anti-AnkB antibodies followed by anti-farnesylation antibodies (1/200 dilution). Immunoblots were visualized using SuperSignal West Femto substrate (Thermo Scientific).

BiFC AND TRANSFECTION OF *DICTYOSTELIUM*

To generate *D. discoideum* fusion constructs; total RNA was extracted from *D. discoideum* using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. Total RNA was treated with DNase I (Ambion, Austin, TX, USA) at 37°C for 30 min. Total RNA was used for cDNA synthesis with Superscript III Plus RNase H reverse transcriptase (RT; Invitrogen, Carlsbad, CA, USA) and random primers. Primers listed in **Table 1** were used to amplify Skp1 from the generated cDNA. The resulting PCR products were cloned in pDM314 (Veltman et al., 2009) to generate GST-Skp1 fusion or pDXA-CFP/YFP to generate Skp1-YC (Knetsch et al., 2002) using the standard procedure.

To create NY-*ankB* and NY-*ankB* ΔF-box in pDXA-CFP/YFP; the *ankB* gene was amplified from the genome of *L. pneumophila AA100/130b* strain. The pBCSK+ vectors that harbors *ankB*-ΔF-box mutant alleles was used as templates to generate *ankB*-ΔF-box fusion as described previously (Price et al., 2009). Primers were used are listed in **Table 1**. The PCR product was treated with restriction enzymes that are mentioned in the table and was sub-cloned. The ligation products were transformed into *E. coli* DH5α.

Table 1 | List of primers used to generate corresponded fusions.

Constructs	Primer sequences (5′–3′)	Restriction sites
NY-*ankB*	F 5′-AGATCTATGAAAAAGA ATTTTTTTTCTGATCTTC-3′ R 5′-ACTAGT TTA ACAAACA AGGCACTTG-3′	BsrGI, XbaI
NY-*ankB* Δ*F-box*	F 5′-AGATCTATGAAAAAGC AACAGCATATAAA-5′ R 5′-ACTAGT TTA ACAAA CAAGGCACTTG-3′	BsrGI, XbaI
skp1-YC	F 5′-CTCGAGATGTCTTTAG TTAAATTAGAATCTTC-3′ R 5′-ITCTAGATTAGTTTCCA CCTTTATCTTCACACC-3′	XhoI, XbaI
3XFLAG-*ankB*	AnkBBglIF; 5′-AGATCTat gaaaaagaatttttttctgatcttc-3′ AnkBSpeIR; 5′-ACTAGT TTA ACAAACAAGGCACTTG-3′	BglII, SpeI
3XFLAG-Δ*F-box*	F-boxBglIF; 5′-AGATCTat gaaaaagcaacagcatataaa-3′ F-boxSpeIR; 5′-ACTAGT TTA ACAAACAAGGCACTTG-3′	BglII, SpeI
3XFLAG-*ankBΔ*^{9}*L*^{10}*P/AA*	AnkBΔ9L10P/AABglIF 5′-AGATCTatgaaaaaga attttttttctgatg-3′ AnkBΔ9L10P/AASpeIR 5′-ACTAGTTTAACAAACA AGGCACTTG-3′	BglII, SpeI
3XFLAG-*ankBΔA1A2*	AnkBΔA1A2BglIF 5′-AGATCTatgaaaaa gaatttttttctgatcttc-3′ AnkBΔA1A2 SpeIR 5′-ACTAGT TTA ACAAA CAAGGCACTTG-3′	BglII, SpeI
3XFLAG-*ankBΔ*^{169}*C/A*	AnkBΔ169C/A BglIF 5′-AGATCTatgaaaaaga attttttttctgatcttc-3′ AnkBΔ169C/A speIR 5′-ACTAGTTAACAAA CAAGAGCCTTG-3′	BglII, SpeI

D. discoideum were transfected by electroporation following standard protocols (Pang et al., 1999). Cells were harvested at log phase and washed two times in cold H50 buffer and re-suspended in H50 at a concentration of 2×10^7 cells/ml. One hundred microliters of cells was then added to a cold 1-mm electroporation cuvette containing 4 μg of plasmid DNA. Cells and DNA were mixed and then incubated on ice for 5 min. Two consecutive pulses of 0.85 kV with a capacitance of 25 mF were applied to the cuvette with a 5-s recovery between pulses. After 5 min of incubation on ice, the cells from each transformation were plated onto a 100-mm culture dish containing 10 ml of HL5 and were allowed to recover for 24 h. Then, the medium was replaced by HL5 containing G418 20 μg/ml.

STATISTICAL ANALYSIS

All experiments were performed at least three times and the data shown are representatives of one experiment. To analyze for statistically significant differences between different sets of data, the two-tail Student's *t*-test was used and the *p*-value was obtained.

RESULT

ECTOPICALLY EXPRESSED AnkB IN *D. DISCOIDEUM* MEDIATES DOCKING OF POLYUBIQUITINATED PROTEINS TO THE PLASMA MEMBRANE AND *TRANS*-RESCUES THE *AnkB* NULL MUTANT

We have previously shown that AnkB functions as platforms for the docking of polyubiquitinated proteins to the LCVs within *D. discoideum* and macrophages (Price et al., 2009, 2010a; Al-Khodor et al., 2010). We examined whether an ectopically expressed FLAG-tagged AnkB in *D. discoideum* exhibited functional activity in recruitment of polyubiquitinated proteins. Co-localization of FLAG-AnkB with polyubiquitinated proteins was observed at the plasma membrane of FLAG-AnkB-transfected *D. discoideum*, where AnkB was exclusively localized (**Figure 1**). To determine the role of the F-box domain and its two conserved LP residues in the biological function of ectopically expressed AnkB, we transfected *D. discoideum* with the FLAG-AnkB-^{9}L^{10}P/AA or the FLAG-AnkB-ΔF-box constructs. The data showed that the F-box domain of AnkB was indispensable for the biological function of the effector, since ectopically expressed FLAG-AnkB-^{9}L^{10}P/AA and FLAG-AnkB-ΔF-box proteins failed to function as platforms for the docking of polyubiquitinated proteins despite their localization to the host plasma membrane (**Figure 1**). The C-terminal CaaX farnesylation motif of AnkB was indispensable for targeting AnkB to the plasma membrane, since ectopically expressed FLAG-AnkB^{169}C/A failed to be targeted to the plasma membrane, which resulted in loss of biological function in recruiting polyubiquitinated proteins to the plasma membrane (**Figure 1**). Interestingly, FLAG-AnkBΔA1,2 ectopically expressed in *D. discoideum* was localized to the plasma membrane and also distributed throughout the cytosol, but it had no biological function as it failed to recruit polyubiquitinated proteins. We conclude that ectopically expressed AnkB in *D. discoideum* is biologically functional as platforms for the docking of polyubiquitinated proteins to the plasma membrane. The farnesylation motif and the ANK domains are required for targeting AnkB to the plasma membrane of *D. discoideum*. However, the F-box domain is not involved in localization of ectopically expressed AnkB to the plasma membrane of *D. discoideum*.

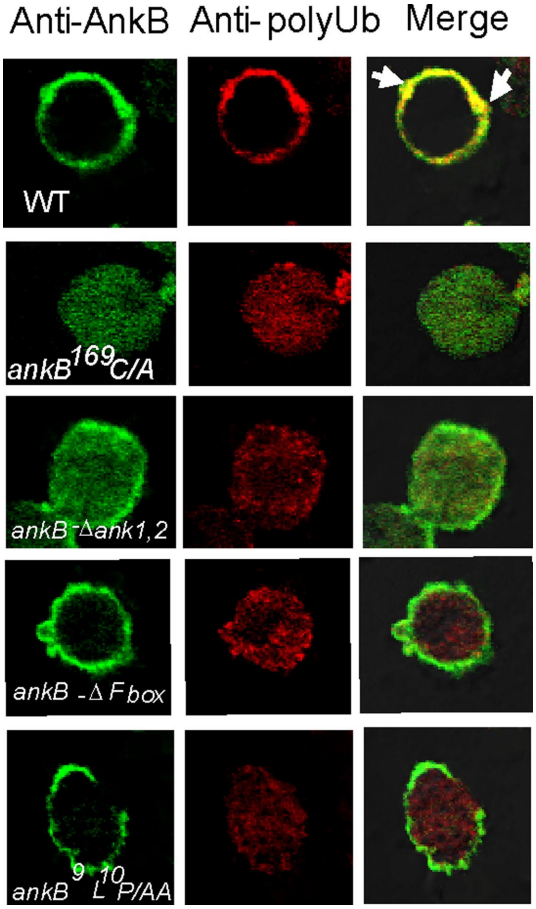

FIGURE 1 | Biological function of AnkB as platforms for the recruitment of polyubiquitinated proteins to the plasma membrane of AnkB-transfected *D. discoideum*. *D. discoideum* was transfected with the FLAG-AnkB, or FLAG-AnkB^{169}C/A, FLAG-ΔAnkB1,2, FLAG-ΔF-box, or AnkB^{9}L^{10}P/AA. Localization of FLAG-AnkB fusion proteins was examined by confocal laser scanning microscopy. Cells were labeled with anti-AnkB antibodies (green) and anti-polyubiquitin antibodies (red). The data are representatives of three independent experiments.

To examine whether the ectopically expressed AnkB would restore intracellular proliferation to the *ankB* mutant, *D. discoideum* cells transfected with FLAG-AnkB were infected with either the wild type strain AA100 or the *ankB* null mutant for 1 h, incubated for a total of 2 and 12 h and analyzed by microscopy for formation of replicative vacuoles, as we described previously (Price et al., 2009). The data revealed that there was no detectable proliferation of the *ankB* mutant in untransfected *D. discoideum*. However, the *ankB* mutant replicated similar to the wild type strain by 12 h post-infection of transfected *D. discoideum* (**Figure 2**). The *dotA* translocation-defective mutant was not *trans*-rescued by ectopically expressed FLAG-AnkB (data not shown). Interestingly, the ectopically expressed FLAG-AnkB protein was detected only at the plasma membrane but not on the LCV. We conclude that ectopically expressed AnkB in *D. discoideum* exhibits its biological function by acting as a platform for the docking of polyubiquitinated proteins at the plasma membrane and that is sufficient to *trans*-rescue the intracellular growth defect of the *ankB* mutant

within the LCV. This is the first demonstration of a *trans*-rescue of a mutant of *L. pneumophila* defective in intracellular proliferation by ectopic expression of the mutated gene in ameba.

INTERACTION OF Skp1 OF *D. DISCOIDEUM* WITH AnkB *IN VIVO*

We have recently shown that AnkB interacts with the mammalian Skp1, but whether AnkB interacts with Skp1 of ameba is not known. Therefore, a bimolecular fluorescence complementation (BiFc) approach was used to determine whether AnkB interacts with Skp1 of *D. discoideum in vivo*. In the BiFc approach, the yellow fluorescence protein (YFP) is expressed as N-terminal (YN) and C-terminal (YC) non-fluorescent fragments. Restoration of YFP fluorescence occurs when the two fragments are brought into proximity by an interaction between two proteins that have been fused to the YN and YC fragments, respectively (Hu et al., 2002). Either *ankB*-YN or *ankB*-ΔF-box–YN fusion proteins were co-expressed with YC–*skp1* in *D. discoideum*. As a negative control; untransfected cells were used to rule out any auto-fluorescence. The results showed that a fluorescent protein was detected in cells transformed with AnkB–YN and YC–Skp1. Importantly, the F-box domain of AnkB was essential for Skp1-AnkB interaction, since the AnkB-ΔF-box–YN fusion did not interact with YC–Skp1, which confirmed the specificity of the interaction (**Figure 3A**).

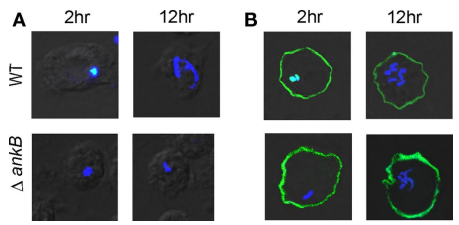

FIGURE 2 | *Trans*-rescue of the *ankB* null mutant for intra-ameba growth defect in AnkB-transfected *D. discoideum*. Representative confocal microscopy images of *D. discoideum* to determine the formation of replicative LCVs. **(A)** Untransfected and **(B)** Transfected *D. discoideum* with FLAG-AnkB were infected with either the WT strain or the Δ*ankB* mutant for 1 h and examined at 2 and 12 h post-infection. Cells were labeled with anti-Lpn antibodies (blue) and anti-AnkB antibodies (green). Rescue was determined by the observations of replicative vacuoles for the *ankB* mutant. The data are representatives of three independent experiments.

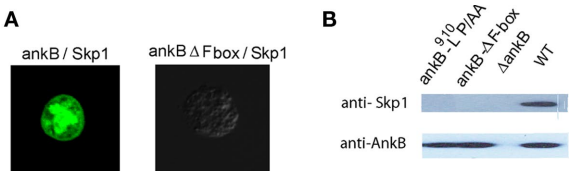

FIGURE 3 | *In vivo* interaction of AnkB with Skp1 of *D. discoideum*. **(A)** Representative confocal images of co-transfected *D. discoideum* with constructs expressing fusions of AnkB-YN and Skp1-YC or AnkB-ΔF-box-YN and Skp1-YC. The data are representatives of three independent experiments. **(B)** *D. discoideum* were infected with *L. pneumophila* strains for 2 h. Skp1 was immunoprecipitated from semi-purified LCVs using anti-AnkB antibodies and then analyzed by immunoblotting with anti-AnkB antibodies followed by anti-Skp1 antibodies. The experiments were performed twice and representative examples are shown.

To confirm the BiFC results, the LCVs were semi-purified at 2 h post-infection of *D. discoideum* and were processed for Co-IP using anti-AnkB antibodies and analyzed by western blots probed with anti-Skp1 and anti-AnkB antibodies. The results showed that endogenous Skp1 of *D. discoideum* interacted with AnkB. In contrast, the AnkB-ΔF-box or AnkB-^{9}L^{10}P/AA variants failed to interact with Skp1 (**Figure 3B**). Taken together, we conclude that the F-box domain of AnkB interacts specifically with Skp1 of *D. discoideum in vivo*.

POST-TRANSLATIONAL MODIFICATION OF AnkB BY THE FARNESYLATION MACHINERY OF *D. DISCOIDEUM* AND ITS ROLE IN ANCHORING AnkB TO THE LCV MEMBRANE

We have previously shown that substitution of the cysteine residue in the CaaX motif with alanine (AnkB^{169}C/A) abolishes anchoring of AnkB to the LCV membrane (Price et al., 2010b). It is not known whether the LCV-anchored AnkB was farnesylated by the host farnesylation machinery. To test if AnkB anchored to the LCV membrane was modified by the farnesylation machinery of *D. discoideum*, we infected *D. discoideum* with the wild type strain, the a*nkB* null mutant, the *ankB^{169}C/A* mutant or the translocation-defective *dotA* mutant as a negative control. Co-immunoprecipitation of semi-purified LCVs was performed using anti-AnkB antibodies, followed by western blots probed with anti-AnkB followed by anti-farnesyl antibodies. The data showed that AnkB but not the AnkB^{169}C/A variant was detected by anti-farnesyl antibodies (**Figure 4**). As expected, AnkB expressed by the translocation-defective *dotA* mutant was not farnesylated.

To confirm farnesylation of AnkB on the LCV within *D. discoideum*, immunoprecipitation was performed on LCVs harvested from *D. discoideum* that was pre-treated with the FTase inhibitor FTI-277 (Lerner et al., 1995). The data showed that inhibition of FTase blocked recognition of AnkB by the anti-farnesyl antibodies, similar to the AnkB^{169}C/A variant in non-treated cells (**Figure 4**). We conclude that AnkB anchored to the LCV membrane is farnesylated by *D. discoideum*.

Inhibition of the farnesylation machinery has been shown to block intracellular proliferation (Price et al., 2010b). We examined whether inhibition of the FTase of *D. discoideum* by FTI-277 would prevent anchoring AnkB to the cytosolic face of LCV membrane. Therefore, *D. discoideum* was infected with the wild type strain AA100, the *ankB* null mutant or the *ankB^{169}C/A* mutant. The LCVs were isolated from untreated or FTI-277-treated *D. discoideum* to

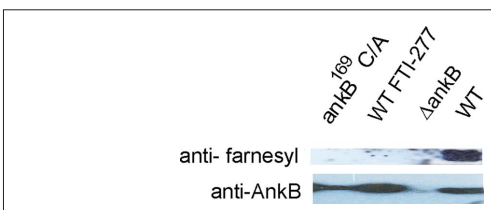

FIGURE 4 | Ankyrin B is modified by the host cell farnesylation machinery. *D. discoideum* were infected with the *L. pneumophila* strains. The infection was performed for 1 h and the cells were examined at 2 h post-infection. The AnkB proteins were immunoprecipitated from semi-purified LCVs using anti-AnkB antibodies and then analyzed by immunoblotting with anti-AnkB and by anti-farnesyl antibodies. The data are representatives of independent experiments.

determine whether AnkB was localized to the cytosolic face of the LCV membrane. The inhibitor had no effect on viability of the cells (data not shown). AnkB was labeled with anti-AnkB antibodies prior to or after permeabilization of membranes. In untreated cells, permeabilized and non-permeabilized LCVs containing the *ankB* mutant failed to bind the anti-AnkB antibodies. Prior to permeabilization of membranes, the LCVs harboring the WT strain bound the anti-AnkB antibodies (**Figure 5**). In untreated cells, the LCVs harboring the *ankB*^{169}*C/A* mutant did not bind the anti-AnkB antibodies prior to permeabilization but did bind after permeabilization. Importantly, in LCVs that harbored the WT strain isolated from FTI-277-treated *D. discoideum*, AnkB was not anchored to the cytosolic face of the LCV membrane, similar to the *ankB*^{169}*C/A* mutant in untreated cells. We conclude that farnesylation of AnkB by *D. discoideum* is essential for anchoring AnkB to the cytosolic face of the LCV membrane.

THE F-BOX AND ANK DOMAINS ARE DISPENSABLE FOR ANCHORING AnkB TO THE LCV MEMBRANE BUT ARE ESSENTIAL FOR BIOLOGICAL FUNCTION WITHIN *D. DISCOIDEUM*

Our data above showed that farnesylation was indispensable for targeting AnkB to the plasma membrane of *D. discoideum* during ectopic expression but that the ANK domains contributed to this localization. Therefore, we examined whether the two ANK domains also contributed to localization of AnkB to the LCV membrane during infection of *D. discoideum* by *L. pneumophila*. Semi-purified LCVs from infected *D. discoideum* harboring the wild type strain of *L. pneumophila* or its isogenic mutants, were labeled with anti-AnkB antibodies prior to or after permeabilization of membranes, as described previously (Price et al., 2010b). After permeabilization, *L. pneumophila* was labeled with DAPI. To ensure that isolation of the LCVs did not disrupt their integrity, anti-SidC antibodies were used to label the LCVs, since the SidC effector is localized to the cytosolic face of the LCV membrane (Ragaz et al., 2008). Prior to permeabilization of membranes, anti-AnkB antibodies recognized AnkB on the LCVs that harbor the WT strain, as expected, indicating localization of AnkB to the cytosolic face of the LCV membrane (**Figure 6**; Price et al., 2010b). Both permeabilized and non-permeabilized LCVs containing the *ankB* mutant failed to bind the anti-AnkB antibodies (student's *t*-test, $p < 0.005$; **Figure 6**; Price et al., 2010b). The AnkB-ΔF-box or AnkB-^{9}L^{10}P/AA variant forms of AnkB were also detected on the LCV prior to permeabilization of membranes. Interestingly, the AnkBΔA1, AnkBΔA2, AnkBΔA1A2 variants forms of AnkB were also localized to the cytosolic face of the LCV membrane. This is in contrast to the ectopic expression where the two ANK domains contributed to targeting of AnkB to the plasma membrane (**Figure 1**). We conclude that the two ANK domains and the F-box domain do not contribute to targeting of AnkB to the LCV membrane within *D. discoideum*.

Although the two eukaryotic-like ANK domains of AnkB were not involved in targeting the effector to the LCV membrane, we examined whether the two domains contributed to the biological function of AnkB in intracellular proliferation of *L. pneumophila* within ameba. The intracellular growth kinetics analysis was performed in *D. discoideum* and *A. polyphaga*. Mutants with in-frame deletions of either or both of the two ANK domains (*ankB*ΔA1, ankBΔA2, and *ankB*ΔA1A2), the wild type strain, or the *ankB* null mutant were used to infect *D. discoideum* and *A. polyphaga*. The

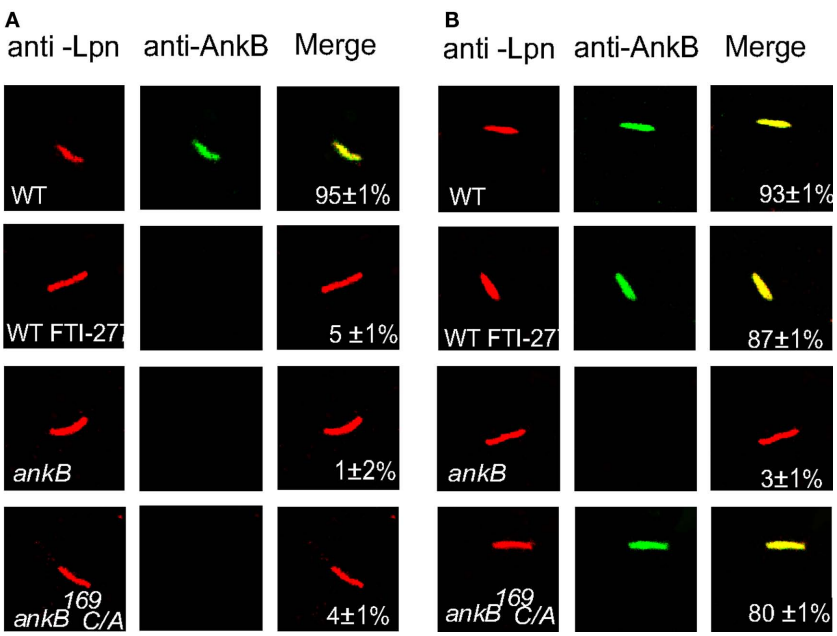

FIGURE 5 | Farnesylation by *D. discoideum* anchors AnkB to the LCV membrane. The infection was performed for 1 h and the cells were lysed at 2 h post-infection. The LCVs were isolated from untreated or FTI-277-treated *D. discoideum*. The LCVs were labeled with **(A)** anti-AnkB antibodies prior to permeabilization (green). After permeabilization, the LCVs were labeled with anti-*L. pneumophila* (Lpn, red). **(B)**. The LCVs were permeabilized then labeled with anti-AnkB and anti-*L. pneumophila* antibodies. Samples were analyzed by confocal microscopy and analyses were based on examination of 100 LCVs from different coverslips from triplicate samples. The data are representatives of three independent experiments.

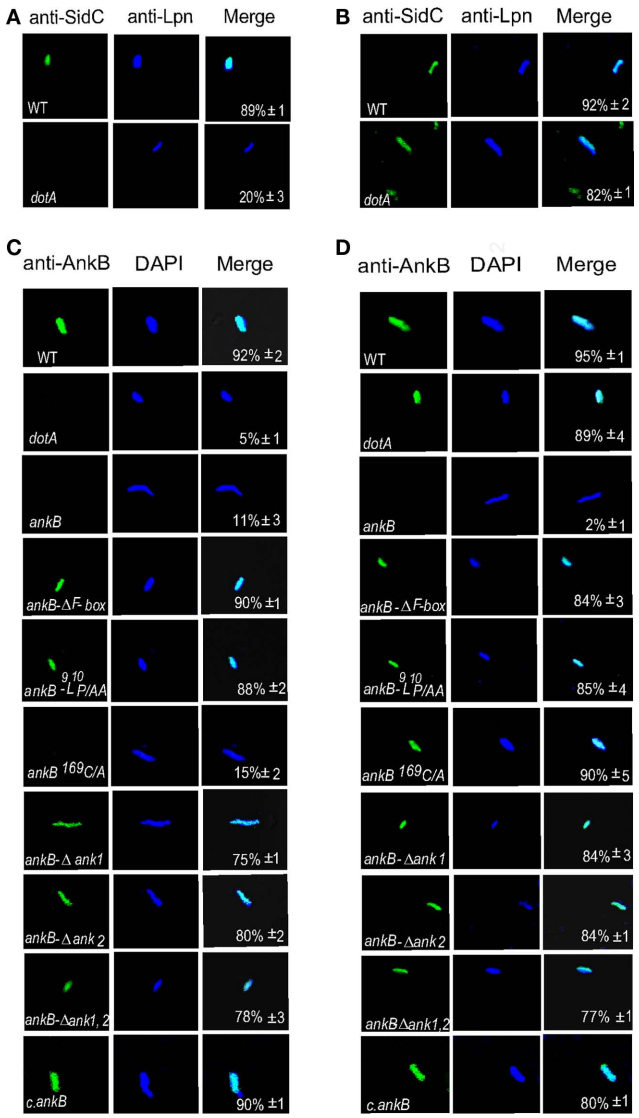

FIGURE 6 | The two ANK domains are dispensable for anchoring AnkB to the cytosolic face of the LCV membrane. The infection was performed for 1 h and the cells were lysed at 2 h post-infection to purify the LCVs. Semi-purified LCVs were analyzed by confocal microscopy. Representative confocal microscopy images that show location of AnkB at the cytosolic face of LCVs. **(A)** The LCVs were probed with anti-SidC prior to permeabilization of membranes (green). After permeabilization of membranes, the LCVs were stained with DAPI to visualize *L. pneumophila* (Lpn blue). **(B)** The LCVs were permeabilized then labeled with anti-SidC and DAPI stain. **(C,D)** Integrity of the membrane of semi-purified LCVs from *D. discoideum* was verified by **(C)** labeling with anti-AnkB antibodies (green) prior to permeabilization of the LCVs. After permeabilization, the LCVs were labeled with anti-Lpn antibodies (blue) within the LCVs. **(D)** The LCVs were permeabilized followed by labeling with anti-AnkB and anti-Lpn antibodies (blue). Quantification is shown in the merged panels, where the numbers represent the percentage +SD of LCVs that showed localization of AnkB to the cytosolic face of the LCV membrane. Quantitation was based on analyses of 100 LCVs from different coverslips. The data are representatives of three independent experiments.

complemented *ankB* mutant and the translocation-defective *dotA* mutant were used as positive and negative controls, respectively. The cells were infected with MOI of 10 for 1 h. Gentamicin treatment was followed for another hour to kill extracellular bacteria. The data showed that the *ankB* mutant exhibited a severe intracellular growth defect within *D. discoideum* and *A. polyphaga* and the defect was complemented by the native *ankB*, as expected (**Figure 7**). The kinetics of the intracellular growth of the ANK domains deletion mutants showed a partial defect in intracellular growth at 24 and 48 h but significant (Student's *t*-test, $p < 0.05$). As expected, the negative control *dotA* mutant strain did not replicate within *D. discoideum* or *A. polyphaga*. Therefore, the ANK domains are not required for targeting AnkB to the cytosolic face of the LCV membrane but they are indispensable for full biological function of AnkB in promoting intracellular proliferation within ameba.

DotA/ICM-DEPENDENT RECRUITMENT OF FTase RCE-1, AND Icmt TO THE LCV WITHIN *D. DISCOIDEUM*

The FTase is cytosolic while the other two processing enzymes RCE-1 and Icmt are located in the ER, and the three enzymes are highly conserved through evolution at the structural and functional levels. Since AnkB is only detectable on the LCV during infection of ameba, we hypothesized that ameba-mediated post-translational modification of the effector and its subsequent anchoring to the LCV membrane occurred locally at the ER-derived LCV membrane. To determine whether the three enzymes were recruited to the LCV in ameba, confocal microscopic analyses were performed after 2 h of infection of *D. discoideum* with the wild type strain AA100, the Δ*ankB* null mutant, the *dotA* translocation-defective mutant, or the *ankB*^{169}*C/A* mutant. Our data showed that 76% of the WT strain-containing LCVs co-localized with the host FTase, and ~60% co-localized with RCE-1 and Icmt (**Figure 8**). Importantly, the LCVs that harbored the translocation-defective *dotA* mutant failed to recruit the three farnesylation enzymes. Interestingly, LCVs that harbor the ΔankB mutant and the *ankB*^{169}*C/A* mutant also co-localized with the three host enzymes; FTα, RCE-1, and Icmt. We conclude that the three farnesylation enzymes FTase, RCE-1, and Icmt of *D. discoideum* are recruited to the LCVs in a Dot/Icm-dependant manner, but AnkB is dispensable for this recruitment.

DISCUSSION

It has been generally believed that *L. pneumophila* has evolved through frequent interaction with various protozoa, which has facilitated its infection of mammalian cells. We have previously shown that AnkB is necessary for decorating the LCV with polyubiquitinated proteins and is essential for intracellular proliferation within protozoan hosts, mammalian cells and for intrapulmonary proliferation in the mouse model of Legionnaires' disease (Al-Khodor et al., 2008; Habyarimana et al., 2008; Price et al., 2009). The AnkB effector is the first remarkable example of how *L. pneumophila* exploits conserved eukaryotic processes, which are the ubiquitination and farnesylation machineries to proliferate within the two evolutionarily distant hosts, mammalian and protozoan cells (Price et al., 2009, 2010a,b; Al-Khodor et al., 2010; Price and Abu Kwaik, 2010).

Our data show that the *ankB* mutant that is defective in intracellular proliferation is *trans*-rescued for its defect within *D. discoideum* ectopically expressing AnkB that is biologically functional as platforms for the docking of polyubiquitinated proteins to the plasma membrane of *D. discoideum*. This is the first demonstration

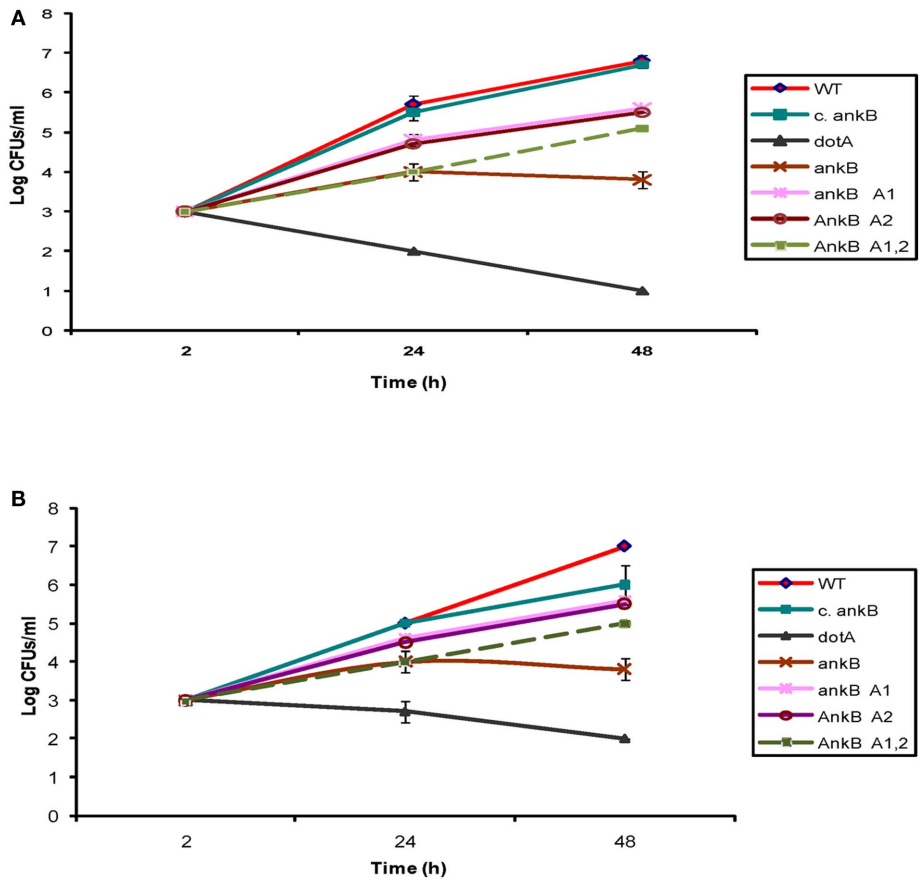

FIGURE 7 | The two ANK domains of AnkB are essential for the biological function of AnkB in intracellular growth of *L. pneumophila* in *D. discoideum*. **(A)** *D. discoideum* or **(B)** *A. polyphaga* were infected with the WT strain, the *ankB* mutant, or the *ankB* mutant harboring one of the mutant alleles *ankB*ΔA1, *ankB*ΔA2, or *ankB*ΔA1A2. The *ankB* mutant complemented with native WT *ankB* (c.*ankB*) and the *dotA* were used as controls. The infection was carried out for 1 h using an MOI of 10 followed by treatment with gentamicin for 1 h. The infected monolayers were lysed at different time points and plated onto agar plates for colony enumeration. The results are representative of three independent experiments performed in duplicate.

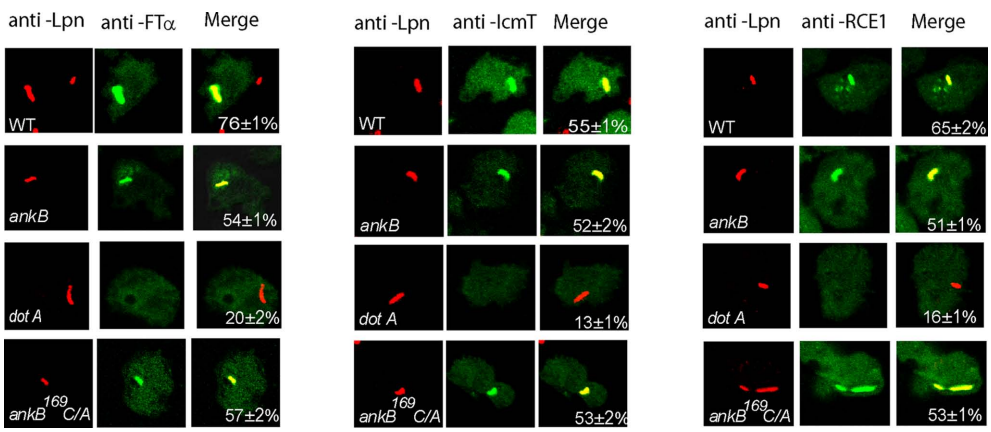

FIGURE 8 | Dot/Icm-dependent recruitment of the three farnesylation enzymes FTα, RCE-1, and Icmt to the LCV within *D. discoideum*. *D. discoideum* were infected with various strains for 1 h. At 2 h after infection, the cells were labeled with anti-Lpn antibodies (red) and anti-FTα, anti-RCE-1, or anti-IcmT antibodies (green) and analyzed by confocal microscopy, and analyses were based on examination of 100 LCVs from different coverslips from triplicate samples. The data are representatives of three independent experiments.

of *trans*-rescue of a *L. pneumophila* mutant in ameba by ectopic expression of the lost protein. Remarkably, similar phenomenon is also exhibited in human-derived cells (Price et al., 2009). It is unclear why ectopically expressed AnkB is targeted to the plasma membrane but not at the LCV. The mechanism by which this *trans*-rescue occurs is not known. We speculate that it is possible that the host factors ubiquitinated by AnkB on the LCV are also ubiquitinated during ectopic expression of AnkB, which would ensure formation of a replicative niche, but we find that to be unlikely. It is important to note that ectopic expression is an artificial system that may not represent what is exhibited during infection as the case in here. Interestingly, during ectopic expression in *D. discoideum* the two ANK domains contribute to localization of AnkB to the plasma membrane and are required for polyubiquitination. The two ANK domains of AnkB are also required for the recruitment of polyubiquitinated proteins to the plasma membrane during ectopic expression in *D. discoideum*. However, during infection, the two ANK domains are dispensable for localization of AnkB to cytosolic face of the LCV membrane. It is possible that the ANK domains interact with host cell targets that are located in the plasma membrane, which may be supported by recent work that the AnkB allele of the Paris strain of *L. pneumophila* interacts with Parvin B located in the plasma membrane (Lomma et al., 2010).

Our data show that the F-box domain of AnkB interacts specifically with the Skp1 protein the component of the SCF1 ubiquitin ligase complex of *D. discoideum*, similar to mammalian cells. Other studies have shown that orthologs of AnkB in the Philadelphia (legU13) and Paris (lpp2082) strains of *L. pneumophila* interact with mammalian Skp1 (Ensminger and Isberg, 2010; Lomma et al., 2010). Further investigations are needed to verify the interaction of AnkB with other components of the SCF1 complex and identify the substrates that are polyubiquitinated.

During infection by *L. pneumophila*, AnkB is modified by the farnesylation machinery of *D. discoideum* and this post-translation modification of the microbial effector is essential for anchoring AnkB to the LCV membrane, which is indispensable for the biological function of the effector. Remarkably, the three host enzymes (FTase, RCE-1, and IcmT) that constitute the farnesylation enzymatic machinery are recruited to the LCV within *D. discoideum* by a Dot/Icm-dependent process but AnkB is dispensable for this recruitment. Since IcmT and RCE-1 are localized to the ER and the LCV is ER-derived, we postulate that these two enzymes are part of the LCV membrane that is derived from the ER in a Dot/Icm-dependent manner. Since farnesyl transferase is cytosolic and its recruitment is Dot/Icm-dependent, it is likely that another Dot/Icm-translocated effector(s) of *L. pneumophila* is involved in recruiting this host cytosolic enzyme to the LCV membrane. It is likely that recruitment of the three farnesylation enzymes would ensure local post-translational farnesylation and processing of the C-terminus of AnkB to anchor the effector to the LCV membrane, without exporting AnkB to the cytosol where it may disrupt various cellular membranes. This compartmentalized hijacking of the polyubiquitination and farnesylation machineries by the LCV is likely to be a major factor in the success of this pathogen to proliferate intracellularly while maintaining a viable host cell that can sustain intracellular proliferation of the pathogen.

The AnkB microbial effector that is injected into the host cell is mostly composed of eukaryotic-like domains that include an F-box domain and two ANK domains, in addition to a C-terminal eukaryotic CaaX motif (Al-Khodor et al., 2010; Price et al., 2010b). The three eukaryotic domains of AnkB are essential for the biological function of AnkB in intracellular proliferation of *L. pneumophila* within *D. discoideum*, similar to mammalian cells. We propose that it is more likely that this effector has been acquired by *L. pneumophila* through inter-kingdom horizontal gene transfer from a primitive unicellular or multicellular eukaryotic host (Al-Khodor et al., 2010). This hypothesis is supported by the domain architecture of this F-box effector that resembles those of F-box proteins of unicellular eukaryotes where ANK domains constitute the protein–protein interaction domains that determine specificity of F-box proteins (Al-Khodor et al., 2010, Price and Abu Kwaik, 2010). In contrast, mammalian F-box proteins do not have ANK domains, but do have WD or LRR as protein–protein interaction domains, instead. However, convergent evolution of AnkB may not be excluded at this time. It is interesting that the ANK domains are also essential for intracellular proliferation of *L. pneumophila* within evolutionarily distant host cells, human macrophages, and ameba. It would be interesting to identify the host substrates that bind the ANK domains within the two evolutionarily distant hosts. Based on our findings, we speculate that these substrates are likely to be evolutionarily conserved.

In summary, our data show the hijacking of two evolutionarily conserved eukaryotic processes by the AnkB effector and the remarkable similarities in the molecular and biochemical events orchestrated in evolutionarily distant eukaryotic hosts. We propose that such hijacking of evolutionarily conserved eukaryotic machineries through inter-kingdom horizontal gene transfer of the F-box effector from primitive eukaryotes to *L. pneumophila* is a factor in the ability of this organism to proliferate within human macrophages and the emergence of Legionnaires' disease in humans. However, the pulmonary tissue tropism of *L. pneumophila* and its exploitation of pro- and anti-apoptotic processes of higher eukaryotes (Amer, 2010) suggest additional processes are involved in the evolution of this human pathogen.

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Host-mediated post-translational prenylation of novel Dot/Icm-translocated effectors of *Legionella pneumophila*

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The Dot/Icm type IV translocated Ankyrin B (AnkB) effector of *Legionella pneumophila* is modified by the host prenylation machinery that anchors it into the outer leaflet of the *Legionella*-containing vacuole (LCV), which is essential for biological function of the effector *in vitro* and *in vivo*. Prenylation involves the covalent linkage of an isoprenoid lipid moiety to a C-terminal CaaX motif in eukaryotic proteins enabling their anchoring into membranes. We show here that the LCV harboring an *ankB* null mutant is decorated with prenylated proteins in a Dot/Icm-dependent manner, indicating that other LCV membrane-anchored proteins are prenylated. *In silico* analyses of four sequenced *L. pneumophila* genomes revealed the presence of eleven other genes that encode proteins with a C-terminal eukaryotic CaaX prenylation motif. Of these eleven designated Prenylated effectors of *Legionella* (Pel), seven are also found in *L. pneumophila* AA100. We show that six *L. pneumophila* AA100 Pel proteins exhibit distinct cellular localization when ectopically expressed in mammalian cells and this is dependent on action of the host prenylation machinery and the conserved cysteine residue of the CaaX motif. Although inhibition of the host prenylation machinery completely blocks intra-vacuolar proliferation of *L. pneumophila*, it only had a modest effect on intracellular trafficking of the LCV. Five of the Pel proteins are injected into human macrophages by the Dot/Icm type IV translocation system of *L. pneumophila*. Taken together, the Pel proteins are novel Dot/Icm-translocated effectors of *L. pneumophila* that are post-translationally modified by the host prenylation machinery, which enables their anchoring into cellular membranes, and the prenylated effectors contribute to evasion of lysosomal fusion by the LCV.

Keywords: Legionnaires', *Agrobacterium*, CaaX, Dot/Icm, prenylation, ankyrin, AnkB, Pel, F-box,RCE-1, IcmT, PFT, PGGT

INTRODUCTION

Exploitation of eukaryotic cellular processes is essential for proliferation of intracellular microbial pathogens. The Legionnaires' disease causing bacterium, *Legionella pneumophila*, replicates within alveolar macrophages causing pneumonia (Isberg et al., 2009). The organism is transmitted to humans from the aquatic environment where *L. pneumophila* replicates within ameba and ciliates (Molmeret et al., 2005; Franco et al., 2009). Co-evolution and adaptation of *L. pneumophila* to the intracellular lifestyle within ameba in the aquatic environment is believed to have played a major role in its ability to exploit evolutionarily conserved eukaryotic processes that enables its proliferation within human alveolar macrophages (Molmeret et al., 2005; Franco et al., 2009). Within both evolutionarily distant host cells, *L. pneumophila* evades endocytic fusion and intercepts ER-to-Golgi vesicle traffic to remodel its phagosome into an ER-derived vacuole (Kagan and Roy, 2002; Molmeret et al., 2005; Shin and Roy, 2008; Isberg et al., 2009).

The *L. pneumophila* Dot/Icm type IV secretion system (Segal et al., 1998; Vogel et al., 1998) injects into the host cell a cadre of ~200 effectors to modulate a myriad of cellular processes to re-program the host cell into a proliferation niche (de Felipe et al., 2008; Shin and Roy, 2008; Isberg et al., 2009). The Ankyrin B (AnkB) effector is injected into the host cell by the Dot/Icm system upon bacterial attachment to the plasma membrane, and exploits an evolutionarily conserved eukaryotic machinery within mammalian and protozoan cells (Al-Khodor et al., 2008, 2010a,b; Habyarimana et al., 2008; Price et al., 2009, 2010a,b; Lomma et al., 2010). The F-box domain of AnkB interacts with the host Skp1 component of the SCF1 ubiquitin ligase complex and functions as a platform for the docking of polyubiquitinated proteins to the *Legionella*-containing vacuolar (LCV) membrane within human cells, *Acanthamoeba*, and *Dictyostelium discoideum* (Dorer et al., 2006; Habyarimana et al., 2008; Price et al., 2009, 2010a; Al-Khodor et al., 2010a,b; Lomma et al., 2010).

In addition to hijacking the host ubiquitination machinery, prenylation of AnkB by the host cell anchors it to the membrane of the LCV and that the three host enzymes involved in prenylation are recruited to the LCV in a Dot/Icm-dependent manner, and are essential for the biological function of AnkB (Price et al., 2010b). Prenylation (farnesylation or geranylgeranylation) is a highly conserved post-translation lipid modification of eukaryotic proteins that confers hydrophobicity on the modified protein, and its targeting to membranes (Wright and Philips, 2006). Prenylation is mediated by protein geranylgeranyl transferase I (PGGT), protein farnesyl transferase (PFT), or by Rab geranylgeranyl transferase (RGGT) (Wright and Philips, 2006). Prenylated proteins

often undergo further post-translational modifications at the ER membrane by the activity of the RCE-1 and ICMT enzymes (Wright and Philips, 2006), which cleave the terminal "-aaX" tripeptide and methylate the terminal prenylated cysteine residue, respectively. This post-translational modification plays a key role in functional activity of numerous eukaryotic proteins, including Rab proteins, Ras, G proteins, and protein kinases (Casey et al., 1989; Hancock et al., 1989; Mumby et al., 1990; Yamane et al., 1990; Wang et al., 1992). Prenylation of the AnkB effector is essential for its biological function in proliferation of *L. pneumophila* within the two evolutionarily distant hosts, mammalian and protozoan cells, and for intrapulmonary bacterial proliferation in the mouse model (Al-Khodor et al., 2008; Price et al., 2009).

A myriad of effectors are injected into the host cell by elaborate type III-VII translocation systems of intra-vacuolar pathogens. Although many injected bacterial effectors are anchored into the pathogen-containing vacuolar membrane or other endo-membranes, the mechanism of this membrane-anchoring is not well understood. Many intracellular bacterial pathogens capable of injecting effectors into host cells encode proteins with predicted prenylation C-terminal CaaX motif (Price et al., 2010b). Here we show that the *Legionella*-containing vacuole (LCV) is decorated with prenylated proteins other than AnkB. This led us to examine the genomes of *L. pneumophila* strains for proteins harboring the eukaryotic CaaX motif. In this study we identified 11 new C-terminal CaaX motif-containing proteins in *L. pneumophila*, which we designated Prenylated effectors of *Legionella* (PelA-K). Seven of these Pel proteins were found in *L. pneumophila* strain AA100/130b genome and six of these exhibited distinct cellular localization in mammalian cells that was dependent on a functional CaaX motif and the host protein prenylation machinery. Five Pel proteins were translocated into host cells by the Dot/Icm type IV translocation system of *L. pneumophila* and represent novel *L. pneumophila* effectors that contribute to evasion of lysosomal fusion by the LCV. These data show exploitation of the host post-translational modification through prenylation by a novel set of Dot/Icm-translocated effectors of *L. pneumophila* that are targeted into various host membranes. It is likely that exploitation of host prenylation to anchor injected microbial effectors into various host membranes is a common theme utilized by microbes that are capable of injecting effectors into host cells.

RESULTS

IDENTIFICATION OF *L. PNEUMOPHILA* C-TERMINAL CAAX MOTIF-CONTAINING PROTEINS

Our previous study showed that host cell prenylation of AnkB anchors it to the membrane of the LCV, and that the three host enzymes (PFTase, IcmT, and RCE-1) involved in prenylation and processing of the prenylated C-terminus are recruited to the LCV in a Dot/Icm-dependent manner and are essential for the biological function of AnkB (Price et al., 2010b). We were interested to determine if other *L. pneumophila* proteins were modified by the host prenylation machinery to be anchored into host membranes. To test if the LCV is decorated with other prenylated proteins apart from AnkB, we analyzed semi-purified LCVs by confocal microscopy for the presence of prenylated proteins. The U937 human macrophage cell line was infected with the WT *L. pneumophila* strain AA100, the *ankB* mutant or the *ankB* mutant complemented with the native *ankB* allele (*ankB*/c.*ankB*) or a substitution variant allele (*ankB*/c.*ankB*^{169}C/A) defective in prenylation. The translocation-defective isogenic *dotA* mutant was used as a control. After 2 h of infection, semi-purified LCVs were isolated and allowed to adhere to glass coverslips for confocal microscopy. To determine whether the farnesylated proteins were localized to the cytosolic face of the LCV membrane, which is impermeable to antibodies, the LCVs were incubated with an anti-farnesyl antibody prior to permeabilization of membranes. Following this, the LCVs were permeabilized and incubated with a monoclonal anti-*Legionella* antibody. Bound primary antibodies were detected using Alexa-Fluor conjugated secondary antibodies. The data showed that 88% of LCVs harboring the WT strain were decorated with prenylated proteins, while only 18% of LCVs harboring the type IV translocation-defective *dotA* mutant showed the presence of prenylated proteins (**Figure 1**). This showed that decoration of the LCV with prenylated proteins was dependent on a functional type IV secretion apparatus. Interestingly, 58% of LCVs harboring the *ankB* mutant were still decorated with prenylated proteins at the LCV outer surface (**Figure 1**). In addition, 63% of the LCVs harboring the *ankB* mutant complemented with *ankB*^{169}*C/A* were decorated with prenylated proteins, similar to the *ankB* mutant, which was significantly different for the WT strain (Student *t*-test, $p < 0.01$) (**Figure 1**). The LCVs harboring the *ankB* mutant complemented with a WT copy of the *ankB* gene exhibited a frequency of decoration by prenylated proteins similar to the WT bacteria (85% positive) (**Figure 1**). Taken together, LCV membrane-anchored proteins other than AnkB at were prenylated and their presence requires a functional Dot/Icm type IV secretion. Some of the prenylated proteins that decorated the LCV were likely Dot/Icm effectors, since their presence was dependent on a function translocation system. It is also possible that some of the prenylated proteins were host proteins that were recruited by Dot/Icm effectors.

To investigate whether some of the prenylated proteins decorating the LCV were of bacterial origin we analyzed the genomes of four *L. pneumophila* strains (Philadelphia, Paris, Lens, and Corby) for genes encoding proteins that have a C-terminal CaaX motif similar to that found in AnkB (Price et al., 2010b). We identified a total of eleven new CaaX motif-containing proteins in strains Philadelphia, Paris, Lens, and Corby (**Table 1**). We designated these proteins as Prenylated effectors of *Legionella* (PelA-K). Of these 11, 5 [PelA, B, E (LegG1), H, and I (PepO)] are shared between all the sequenced strains. PelD is found in Lens, Paris, and Corby, while PelF is found in Philadelphia, Paris, and Corby. PelC (LegC1) is found only in Philadelphia, while PelJ and K are only found in Lens. Interestingly, PelB in strain Lens has a mutation that resulted in substitution of the crucial cysteine residue in the CaaX motif to alanine. In addition, PelD in strains Lens and Corby has a mutation resulting in substitution of the crucial cysteine residue of the CaaX motif to a tyrosine.

DISTINCT LOCALIZATION OF ECTOPICALLY EXPRESSED PEL PROTEINS IN MAMMALIAN CELLS

By using PCR we identified that *L. pneumophila* AA100 also harbored PelA, B, D, E, F, H, and I., the Pel proteins that are shared amongst the sequenced strains (**Table 1**). Our previous work showed that

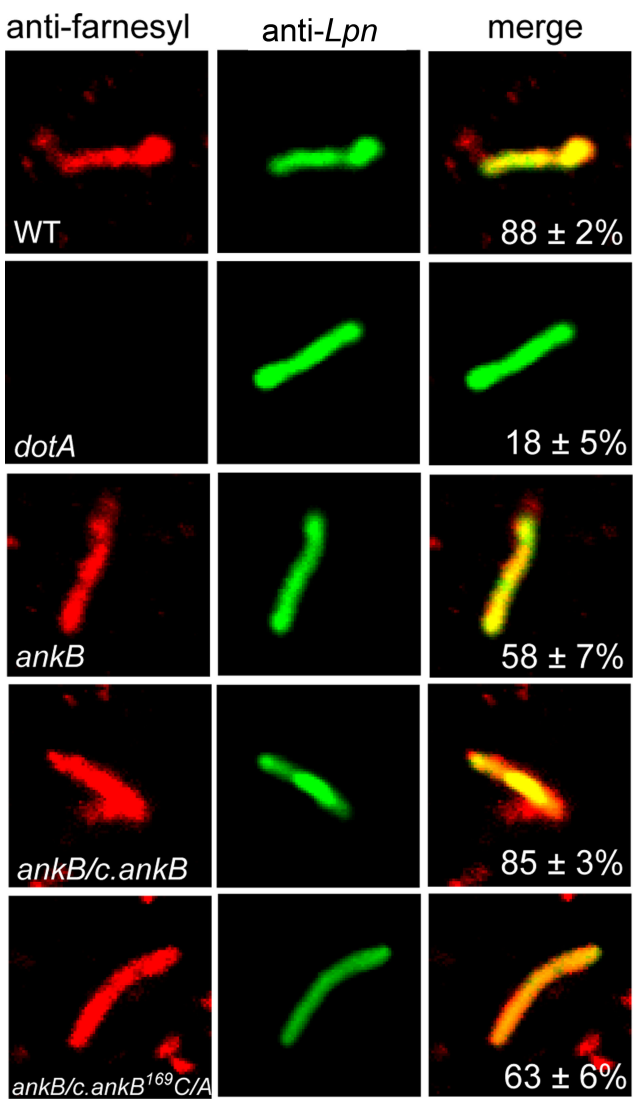

FIGURE 1 | The LCV membrane is decorated with prenylated proteins in a Dot/Icm-dependent manner. Infected U937 cells were lysed after 2 h infection by the WT, *dotA*, *ankB*, *ankB*/c.*ankB* or *ankB*/c.*ankB*^{169}C/A bacteria. The LCVs were isolated and were probed with anti-farnesyl antiserum prior to permeabilization (red) and examine by confocal microscopy to determine whether the prenylated proteins were localized to the cytosolic side of the LCV membrane. After permeabilization, the LCVs were probed with mouse anti-*L. pneumophila* (Lpn) monoclonal antibodies (green) to visualize the bacteria within the LCV. Quantitation is shown in the merged panels, where the numbers represents percentage plus standard deviation of LCVs that bound anti-farnesyl antiserum prior to permeabilization. Analyses were based on examination of 100 LCVs from triplicate samples. All experiments were performed in triplicate and representative examples are shown. All the results in this figure are representative of three independent experiments.

ectopically expressed 3X-FLAG AnkB in HEK293 cells exhibits a striking localization at the cell periphery (Price et al., 2009) and this localization is dependent on prenylation of the C-terminal CaaX motif of AnkB (Price et al., 2010b). To determine if the Pel proteins also exhibit distinct membrane localization in mammalian cells we generated 3X-Flag fusions to the N-terminus of these proteins, and transfected HEK293 cells. Following 24 h of transfection, HEK293 cells were fixed and permeabilized and incubated with anti-Flag antibody and examined by confocal microscopy (**Figure 2A**). The 3X-Flag AnkB control exhibited distinct localization to the cell plasma membrane as expected (**Figure 2A**). Interestingly all the Pel proteins were not diffusely distributed throughout the cytosol but showed distinct localization in the cell (**Figure 2A**). 3X-Flag PelA concentrated around the periphery of enlarged vesicles and as distinct punctate spots within the cell (**Figure 2A**). 3X-Flag PelB localized as intense punctate spots in HEK293 cells, indicating vesicular localization (**Figure 2A**). 3X-Flag PelD, E and F localized similarly in HEK293 cells, as punctate spots and weakly around the cell periphery (**Figure 2A**). 3X-Flag PelH localized strongly around the cell periphery and as punctate spots in the cell, reminiscent of that observed for 3X-Flag AnkB (**Figure 2A**). This shows that ectopically expressed Pel proteins are targeted to distinct locations within mammalian cells. We could not detect ectopic expression of 3X-Flag PelI (PepO), which may be due to potentially detrimental effects of over-expressing a metalloprotease in a cell.

SPECIFIC LOCALIZATION OF THE PELS IN MAMMALIAN CELLS THROUGH HOST-MEDIATED PRENYLATION OF THE C-TERMINAL CAAX MOTIF

We utilized two independent and complementary approaches to determine whether the distinct localization of the Pel proteins in mammalian cells was due to host-mediated prenylation, resulting in association of these proteins with cellular membranes. Our first approach was to inhibit the host prenylation machinery to examine whether that would alter localization of the Pel proteins in the host cell. To address this, HEK293 cells were pre-treated with the prenylation inhibitor FTI-277 (Lerner et al., 1995) and then transfected with the 3X-FLAG Pel constructs, and analyzed by confocal microscopy (**Figure 2B**). As expected, the 3X-Flag AnkB control was no longer localized to the plasma membrane in cells treated with FTI-277 and was found throughout the cytosol (**Figures 2A,B**). Interestingly, all the Pel proteins exhibited a shift in cellular localization following chemical inhibition of PFTase (**Figure 2B**). The 3X-Flag PelA in prenylation-inhibited cells was no longer found around enlarged vacuoles but became more evenly distributed throughout the cytosol (**Figures 2A,B**). The distribution of 3X-Flag PelB, which is found as distinct punctate spots in untreated cells, completely shifted to a more homogenous cytosolic distribution in prenylation-inhibited cells (**Figures 2A,B**). The 3X-Flag PelD, E and F which were localized, in untreated cells, to small punctate spots throughout the cytoplasm and on the plasma membrane exhibited an even distribution in the cytosol in prenylation-inhibited cells (**Figures 2A,B**). Localization of 3X-Flag PelH, which exhibits similar plasma membrane localization to 3X-Flag AnkB, was completely altered to a cytoplasmic distribution in prenylation-inhibited cells (**Figures 2A,B**). These data show that the specific localization of the Pel proteins when expressed in HEK293 cells is dependent on the activity of the host prenylation machinery.

Our second approach to determine whether localization of the Pel proteins to distinct cellular locations was directly due to prenylation modification was a genetic approach from the microbe side. The conserved cysteine residue in the C-terminal CaaX motif of each Pel protein was substituted by alanine. We have previously shown that mutation of the conserved cysteine residue of the

CaaX motif of AnkB abolish prenylation and anchoring of AnkB to the host plasma membrane (Price et al., 2010b). Examination by confocal microscopy showed that ectopically expressed 3X-FLAG AnkB^{169}C/A control was distributed evenly throughout the cytosol in HEK293 cells, as expected (**Figures 3A,B**). Interestingly, mutation of the conserved cysteine residue in the CaaX motif of all the Pel

Table 1 | Genes in the four sequenced *L. pneumophila* genomes that harbor a C-terminal CaaX motif. Genes were identified using the Legiolist website (http://genolist.pasteur.fr/LegioList/) and the "search pattern" tool.

Gene name	Philadelphia	Lens	Paris	Corby	CaaX motif	Gene designation	Domains
PelA	lpg0254	lpl0307	lpp0324	lpc0331	CVLM	Hypothetical	–
PelB	lpg0770	lpl0811*	lpp0835	lpc2522	CLIK	Hypothetical	–
PelC	lpg1312	–	–	–	CTII	LegC1	Coiled-coils
PelD	–	lpl1858**	lpp1863	lpc1344**	CSLL	Hypothetical	–
PelE	lpg1976	lpl1953	lpp1959	lpc1462	CNLL	LegG1	ATS1
AnkB	lpg2144	lpl2072	lpp2082***	lpc1593	CVLC	AnkB	F-box, ankyrins
PelF	lpg2375	–	lpp2440	lpc2117	CSIL	Hypothetical	–
PelG	lpg2525	–	–	–	CSIL	Hypothetical	–
PelH	lpg2541	lpl2462	lpp2607	lpc1927	CTIM	Hypothetical	–
PelI	lpg2607	lpl2530	lpp2660	lpc0534	CIIW	PepO	Peptidase M13
PelJ	–	lpl2477	–	–	CTIM	Hypothetical	HAD-SF-IIIC
PelK	–	lpl2806	–	–	CVIS	Hypothetical	–

**Lens CaaX motif is mutated to ALAK, **Lens and Corby CaaX motif is missing the conserved cysteine residue (YSLL), ***Paris 2082 has a frame shift mutation resulting in an 18 amino acid truncation at the C-terminus, abolishing the CaaX motif. AnkB is only known translocated CaaX motif-containing protein.*

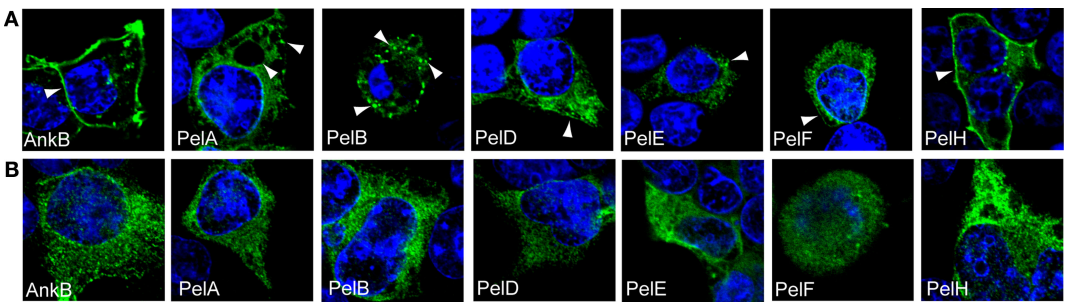

FIGURE 2 | Cellular distribution of ectopically expressed 3X-FLAG Pel proteins is dependent on host prenylation machinery. Representative confocal microscopy images of untreated **(A)** or FTI-277-treated **(B)** HEK293 cells ectopically expressing 3X-FLAG tagged AnkB, PelA, PelB, PelD, PelE, PelF or PelH fusion proteins. Green indicates labeling with anti-FLAG M2 antibody and the nucleus is stained blue (DAPI). The arrowheads indicate strong localization of the various 3X-Flag fusions at distinct cellular locations. All experiments were performed three times and representative examples are shown.

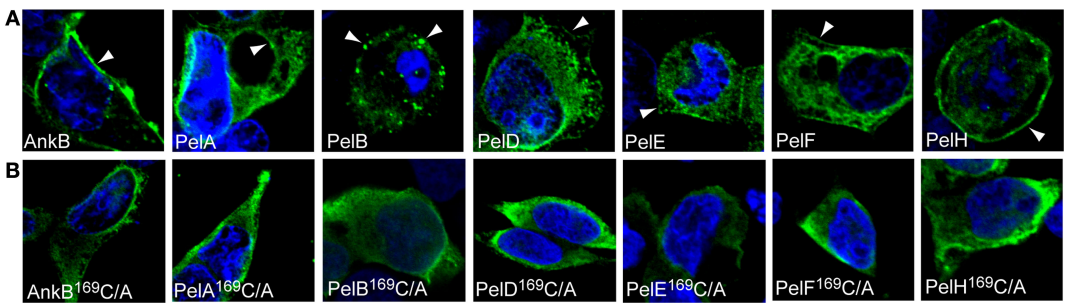

FIGURE 3 | The CaaX motifs of the Pels are indispensable for their distinct cellular distribution. (A) Representative confocal microscopy images of HEK293 cells ectopically expressing 3X-FLAG tagged AnkB, PelA, PelB, PelD, PelE, PelF or PelH fusion proteins. Green indicates labeling with anti-FLAG M2 antibody and the nucleus is stained blue (DAPI). The arrowheads indicate strong localization of the various 3X-Flag fusions at distinct cellular locations. **(B)** Representative confocal microscopy images of HEK293 cells ectopically expressing 3X-FLAG tagged AnkB^{169}C/A, PelA^{169}C/A, PelB^{169}C/A, PelD^{169}C/A, PelE^{169}C/A, PelF^{169}C/A or PelH^{169}C/A fusion proteins. All experiments were performed three times and representative examples are shown.

proteins resulted in a re-distribution of these proteins throughout the cytosol, similar to the results obtained upon chemical inhibition of prenylation (**Figures 3A,B**). This clearly shows that the cysteine residue is crucial in the localization of the Pel proteins to distinct cellular locations, and these are likely to be various host membranes. Taken together, these data indicate that the Pel proteins are modified by the host prenylation machinery that enables their targeting to specific cellular location, which is most likely various host membranes.

TRANSLOCATION OF PEL PROTEINS INTO MACROPHAGES

To be prenylated by the host machinery, the CaaX motif-containing Pel proteins must be translocated out of the LCV. To examine whether the Pel proteins were translocated into host cells by the Dot/Icm type IV translocation system of *L. pneumophila*, we utilized the calmodulin-dependent adenylate cyclase reporter fusion assay using ELISA (Sory and Cornelis, 1994; Al-Khodor et al., 2008). The U937 cells were infected for 1 h with the WT strain harboring the vector alone, *ralF*-CyaA and *ankB*-CyaA as positive controls, or *Pel*-CyaA fusions. The translocation-defective *dotA* mutant harboring the various CyaA fusions was used to determine whether translocation was Dot/Icm-dependent. The data showed that PelA, B, D, E, F, and I were translocated into the host cell cytosol similar to AnkB and the RalF effector controls (**Figure 4**). Translocation of PelA and PelB was reproducibly lower compared to the controls and other Pel proteins. However, translocation of both PelA and PelB was higher compared to the empty vector negative control, was Dot/Icm-dependent and this was statistically significant (Student *t*-test, $p < 0.01$). Furthermore, translocation of all the Pel proteins was not observed in the translocation-defective *dotA* mutant, indicating that translocation of the Pel proteins was Dot/Icm-dependent. PelH was not translocated using this assay, which may be due to interference by the Cya fusion. Interestingly, when ectopically expressed in cells, this protein exhibited similar localization to AnkB. Further studies are required to rule out the possibility PelH is not translocated. These data show that *L. pneumophila* strain AA100/130b harbors a group of novel translocated effectors, in addition to AnkB, that are modified by the host prenylation machinery that anchors them into host membranes.

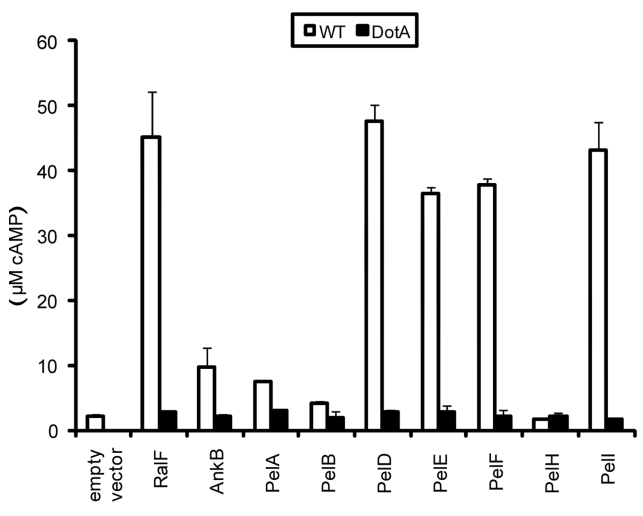

FIGURE 4 | Dot/Icm-dependent injection of the Pel proteins of *L. pneumophila* into human macrophages. Translocation of the Pels into U937 cells was determined at 1 h post-infection. WT or *dotA* mutant bacteria harbored either empty vector (pCya) or Cya hybrids of RalF, AnkB or PelA, PelB, PelD, PelE, PelF, PelH or PelI. All experiments were performed three times and representative examples are shown. The data are the mean of triplicate samples and the error bars are the standard deviations. Translocation of all Pel proteins was statistically significant compared to bacteria harboring empty vector (Students *t*-test, *p*-value < 0.01).

EFFECT OF HOST PRENYLATION OF TRAFFICKING OF THE LCV

Upon phagocytosis *L. pneumophila* evades endocytic fusion and intercepts ER-to-Golgi vesicle traffic to remodel its phagosome into an ER-derived vacuole, which is essential for intra-vacuolar proliferation (Kagan and Roy, 2002; Molmeret et al., 2005; Shin and Roy, 2008; Isberg et al., 2009). Since inhibition of the host prenylation machinery blocks intracellular proliferation of *L. pneumophila*, we determined whether that was due to alteration in trafficking of the LCV by examination of colocalization of the LCV with the late endosomal/lysosomal marker Lamp2 and the lysosomal enzyme Cathepsin D. The U937 cells were pre-treated with prenylation inhibitor and then infected with WT or formalin-killed WT bacteria. Following 2 h of infection, fixed cells were incubated with anti-*Legionella* and anti-Lamp2 or anti-cathepsin D antibody and analyzed by confocal microscopy. The data showed that LCVs harboring the WT bacteria did not localize with both Lamp2 and Cathepsin D staining in untreated U937 cells (28 and 26% localization respectively), indicating WT bacteria evaded endocytic fusion (**Figures 5A,B**). In prenylation-inhibited cells, there was a moderate increase in WT bacteria co-localizing with both Lamp2 and Cathepsin D (35% and 37% positive, respectively (**Figures 5A,B**) and this was statistically significant (Student *t*-test, $p < 0.05$). Over 83% of formalin-killed WT bacteria localized with Lamp2 or cathepsin D in untreated or prenylation-inhibited cells (**Figures 5A,B**) showing that inhibition of the prenylation machinery did not affect the activity of endosomal-lysosomal pathway in the cell. Taken together, these data show that host prenylation machinery contributes to the ability of living *L. pneumophila* to evade endocytic fusion.

DISCUSSION

Although numerous bacterial effectors injected by various intravacuolar pathogens are localized to the pathogen-containing vacuolar membrane and other host membranes, the mechanisms of this localization are not well known. We have recently shown that host-mediated prenylation of the AnkB effector of *L. pneumophila* represents a new paradigm for anchoring microbial effectors to the pathogen-containing vacuolar membrane (Price et al., 2010b). The presence of putative CaaX motif proteins in >20 species of extracellular and intracellular bacterial pathogens of mammals and plants and endosymbionts that are capable of injecting effectors into host cell suggests that hijacking the host prenylation pathway may be a common theme utilized by microbial pathogens that inject effectors into host cells (Price et al., 2010b). Prior to this study, the AnkB F-box effector was the only microbial translocated effector known to be modified by the host prenylation machinery where this host-mediated post-translational modification was essential

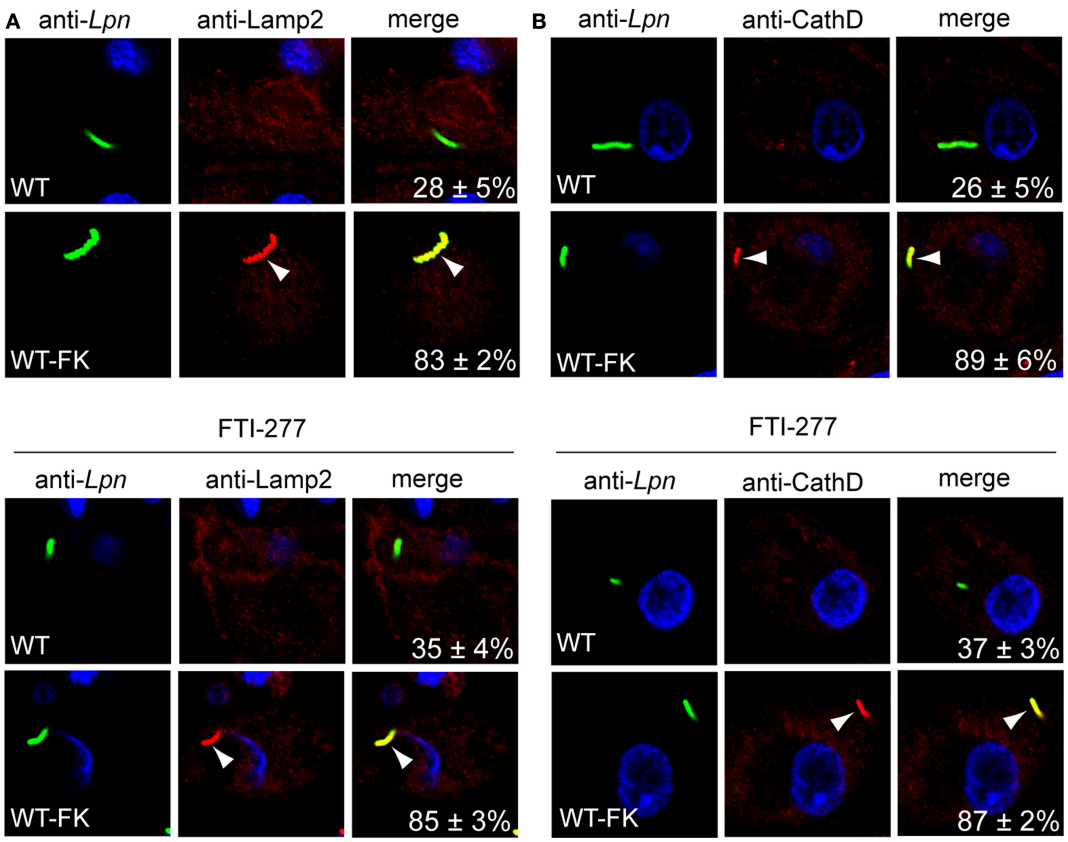

FIGURE 5 | Trafficking of the LCV in prenylation-inhibited U937 cells. (A,B) Untreated or FTI-277–treated U937 cells infected for 1 h with WT or WT formalin-killed bacteria (WT-FK). After 2 h of infection, the cells were labeled with anti-Lamp2 (red) or anti-cathepsin D (CathD; red) antibodies and analyzed by confocal microscopy. Arrowheads indicate colocalization of the LCVs with LAMP-2 or cathepsin D. The formalin-killed WT were used as a positive control for Lamp2 and cathepsin D colocalization. Quantitation is shown in the merged panels, where the numbers represent the percentage of LCVs that colocalized with the respective marker. Analyses were based on the examination of 100 infected cells from multiple coverslips. Data represent means ± standard deviation. Results are representative of three independent experiments performed in triplicate. Lpn, *L. pneumophila*. Increased localization of WT LCVs to Lamp2 and cathepsin D in FTi-277 treated cells was statistically significant (Students *t*-test, p-value < 0.05).

for intra-vacuolar proliferation of the pathogen (Al-Khodor et al., 2008, 2010a,b; Habyarimana et al., 2008; Price et al., 2009, 2010a,b). This study has significantly expanded the list of known translocated effectors modified by the host prenylation machinery. The only other confirmed bacterial effector previously shown to be prenylated is SifA of *S. typhimurium* (Boucrot et al., 2003; Reinicke et al., 2005). SifA is translocated by the type III secretion system and is localized to the *Salmonella*-induced filaments that connect *Salmonella*-containing vacuoles, but its biological function is not known (Boucrot et al., 2003; Reinicke et al., 2005). Prenylation of SifA by PGGT is dispensable for its biological function, since a prenylation-defective *sifA* substitution mutant is fully competent in intracellular proliferation and virulence in the mouse model (Reinicke et al., 2005). In contrast, prenylation of AnkB is essential for its biological function *in vitro* and for intrapulmonary proliferation in the mice model of Legionnaires' disease (Price et al., 2010b).

Our data show that *L. pneumophila* translocates several novel effectors that are modified by the host prenylation machinery, similar to AnkB (Price et al., 2010b). Our data have clearly shown that the Pel effectors exhibit distinct localization in the cells and the host-mediated prenylation of the CaaX motif was essential for this localization, which is most likely various host membranes. Currently the cellular localization and function of the Pel proteins during infection of macrophages is unknown, but studies are underway to investigate these questions. It is possible that the Pel proteins target to the LCV membrane similar to AnkB (Price et al., 2010b). When ectopically expressed in mammalian cells, 3X-Flag AnkB localizes to the inner leaflet of the plasma membrane, but native AnkB translocated by bacteria during infection is found solely on the cytosolic face of the LCV membrane (Price et al., 2010b). We have shown that PFTase, IcmT, and RCE-1 are all recruited to the LCV in a Dot/Icm-dependent manner (Segal et al., 1998; Vogel et al., 1998; Price et al., 2010a). Prenylated proteins often undergo further post-translational modifications at the ER membrane by the activity of the RCE-1 and ICMT enzymes (Wright and Philips, 2006), which cleave the terminal "-aaX" tri-peptide and methylate the terminal prenylated cysteine residue, respectively. RCE-1 and ICMT are localized to the ER-derived LCV membrane in a Dot/Icm-dependent manner (Price et al., 2010b). The presence of

PFTase, RCE-1 and IcmT at the LCV membrane may enable local prenylation of Pel proteins immediately following translocation. It is likely that some of the Pels integrate into the outer leaflet of the LCV membrane, similar to what we have observed with AnkB (Price et al., 2010b) and may account for the presence of prenylated proteins other than AnkB at the LCV surface. It is also possible that some of the prenylated proteins decorating the LCV are host proteins that are recruited to the LCV by Dot/Icm-translocated effectors, and prenylation of these host proteins may occur locally at the LCV membrane.

It is important to note that the exact nature of the prenylation modification (farnesylation or geranylgeranylation) of the Pel proteins is currently unknown. The inhibitor used in this study, FTI-277 is a peptidomimetic inhibitor of PFTase but also exhibits cross-inhibition of PGGT (Lerner et al., 1995). Therefore, we cannot determine whether the Pels are geranylgeranylated or farnesylated. Interestingly, mammalian K-Ras and N-Ras are preferentially farnesylated, however when PFTase is inhibited, these two proteins can still be geranylgeranylated by PGGT and be functional (James et al., 1995; Whyte et al., 1997; Geryk-Hall et al., 2010). It will be interesting to determine if this phenomenon also occurs in Pel prenylation. Since inhibition of prenylation blocks intra-vacuolar replication of *L. pneumophila* and related *Legionella* species (Price et al., 2010b), understanding the exact lipid modifications of prenylated effectors will be critical in understanding how host-mediated prenylation of bacterial effectors contributes to bacterial replication, which may facilitate the development of novel anti-*Legionella* therapeutics.

Avoidance of the endocytic pathway is crucial for the success of *L. pneumophila* to replicate in macrophages and in ameba (Kagan and Roy, 2002; Molmeret et al., 2005; Shin and Roy, 2008; Isberg et al., 2009). We have shown that blocking host prenylation activity results in reduced ability of *L. pneumophila* to avoid trafficking to the lysosome. Membrane bound prenylated AnkB is unlikely to play a role in intracellular trafficking of the LCV, since the *ankB* null bacteria avoid trafficking to the lysosome similar to WT bacteria (Al-Khodor et al., 2008). However the roles of the Pel proteins are unknown. Perhaps when integrated into membranes, these prenylated proteins may directly assist in evading the endocytic pathway, thus contribute to the ability of *L. pneumophila* to establish a safe replicative niche. It is important to note that contribution of the Pels to evasion of the lysosomal pathway by the LCV is very modest. Therefore, other more important non-prenylated effectors play the major role in evasion of the endosomal-lysosomal pathway by the LCV. We also cannot rule out the possibility that prenylated host proteins, hijacked by *L. pneumophila*, also contribute to evasion of the endocytic pathway by the LCV. The function of the Pels and potential prenylated host proteins in remodeling the LCV into a proliferative niche represents exciting new avenues of research.

Manipulation of host activities is crucial for the success of intracellular pathogens. This is often achieved by translocation of effectors that harbor eukaryotic protein domains that mimic the function of host proteins (Franco et al., 2009; Galan, 2009; Hauser, 2009; Ibarra and Steele-Mortimer, 2009; Isberg et al., 2009). We show that this intimate host/pathogen relationship goes even further with the ability of microbes to translocate effectors that take advantage of host post-translational modification such as prenylation, to enable the correct cellular localization of effectors. Given the abundance of CaaX motif-containing proteins in *L. pneumophila* and putative CaaX-containing proteins in other extracellular and intracellular pathogens of mammals and plants such as *Mycobacterium*, *Salmonella*, *Anaplasma*, *Bartonella*, *Brucella*, *Agrobacterium*, and *Toxoplasma* (Price et al., 2010b), it is likely that the ability to exploit the host prenylation machinery may be a common theme utilized by microbial pathogens and endosymbionts capable of injecting effectors in host cells to anchor the injected effectors into specific host membranes.

MATERIALS AND METHODS

BACTERIAL STRAINS, CELL CULTURES, AND INFECTIONS

Legionella pneumophila strain AA100/130b (ATCC BAA-74) and the isogenic mutants *dotA*, *ankB*, and complemented *ankB* mutants were grown as described previously (Al-Khodor et al., 2008). Maintenance of U937 and HEK293 cells were performed as previously described (Price et al., 2009). Infection studies for purification of the LCVs and effector translocation were performed as we described previously (Al-Khodor et al., 2008; Price et al., 2009). Briefly, macrophages were infected at a multiplicity of infection (MOI) of 10 (LCVs) or 50 (Cya assay). Measurement of cAMP in cell lysates for adenylate cyclase fusion assays was performed using the Direct Cyclic AMP Enzyme Immunoassay kit (Assay Designs), as we described previously (Al-Khodor et al., 2008). The Student *t*-test was used to determine if differences in cAMP levels were significant in strains harboring pCYA-Pel fusions compared to the strain harboring the empty vector and whether translocation was Dot/Icm-dependent. Purification of post-nuclear supernatant containing LCVs was performed as previously described (Price et al., 2010b). For analysis of LCV trafficking, U937 cells on glass coverslips were infected with WT or formalin-killed bacteria for 1 h followed by treatment with 50 μg/ml gentamicin for 1 h to kill extracellular bacteria. Infected cells were then fixed and permeabilized with ice cold methanol for 5 min.

TRANSFECTIONS AND INHIBITORS

Cloning of the 3X-FLAG tagged *Pel* alleles was performed as described previously (Price et al., 2009), using specific primers (**Table A1** in Appendix). Mutations in the CaaX motif were performed as described previously (Price et al., 2010b), using specific primers (**Table A1** in Appendix). HEK293 cells were transfected using Fugene HD reagent (Roche, Mannheim), as we described previously (Price et al., 2009). The inhibitor FTI-277 (Calbiochem, Gibbstown, NJ) was re-suspended in DMSO +0.4 mM DTT and used immediately, and maintained in the growth media throughout the experiment.

CONFOCAL LASER SCANNING MICROSCOPY

Processing of infected cells for confocal microscopy was performed as we described previously (Price et al., 2009). Polyclonal rabbit anti-*L. pneumophila* antiserum and monoclonal mouse anti-*L. pneumophila* antibodies were detected by Alexa-Fluor 488-conjugated donkey anti-rabbit or -mouse IgG (Invitrogen, Carlsbad, CA, USA). Prenylation was detected with a rabbit anti-farnesyl antibody (1/50 dilution) (Abcam, Cambridge, MA, USA) followed by Alexa-Fluor 555-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) (Price et al., 2010b). Mouse anti-Lamp2

and mouse anti-cathepsin D antiserum was used as previously described (Al-Khodor et al., 2008). Alexa-fluor tagged antibodies against mouse IgG were used as secondary antibodies (Invitrogen, Carlsbad, CA, USA). 3X-flag fusion proteins were detected using anti-Flag M2 antibody (Sigma, St Louis, MO, USA) and Alexa-fluor conjugated antibodies. The cells were examined with an Olympus FV1000 laser scanning confocal microscope as we described previously (Price et al., 2009). On average, 8–15 0.2 μm serial Z sections of each image were captured and stored for further analyses, using Adobe Photoshop CS3. The Student *t*-test was used to determine if differences in localization of LCV with Lamp2 and CathD were statistically significant.

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APPENDIX

Table S1 | Primers used in this study.

Primer name	Nucleotide sequence
pelA cya F	GGATCCTTATGAGTGAATATTTGG
pelA cya R	CTGCAGCTACATGAGCACACAAACAG
pelB cya F	GGATCCTTATGTCAAATACTGTTTTAG
pelB cya R	CTGCAGTTATTTAATCAGGCAATC
pelD cya F	GGATCCTTGTGTTCAAAAAAAAGC
pelD cya R	CTGCAGTCACAATAAAGAACAATTA
pelE cya F	GGATCCTTTTGCATCTTGAATTGC
pelE cya R	CTGCAGTCATAACAAATTGCATGG
pelF cya F	GGATCCTTATGCGTAGCAGAACAGAA
pelF cya R	CTGCAGTTAGAGAATGCTGCATTGC
pelH cya F	GGATCCTTGTGCTAATGGAATTCG
pelH cya R	CTGCAGTTACATTATTGTACAACGG
pelI cya F	GGATCCTTATGAAATTTAAGATTGC
pelI cya R	CTGCAGTTACCATATGATGCAACGA
3x pelA F	GCGGCCGCATGAGTGAATATTTGG
3x pelA R	TCTAGACTACATGAGCACACAAACAG
3X pelB F	GCGGCCGCATGTCAAATACTGTTTTAG
3X pelB R	TCTAGATTATTTAATCAGGCAATC
3X pelD F	GCGGCCGCATGTTCAAAAAAAAGC
3X pelD R	TCTAGATCACAATAAAGAACAATTA
3X pelE F	GCGGCCGCATGCATCTTGAATTGC
3X pelE R	TCTAGATCATAACAAATTGCATGG
3X pelF F	GCGGCCGCATGCGTAGCAGAACAGAA
3X pelF R	TCTAGATTAGAGAATGCTGCATTGC
3X pelH F	GCGGCCGCATGCTAATGGAATTCG
3X pelH R	TCTAGATTACATTATTGTACAACGG
3X pelI F	GCGGCCGCATGAAATTTAAGATTGC
3X pelI R	TCTAGATTACCATATGATGCAACGA
3X pelA CA F	/5Phos/GCTGTGCTCATGTAGTCTAGAGGATCCCGGG
3X pelA CA R	/5Phos/AACAGATTCTTTCTCTGGTG
3X pelB CA F	/5Phos/GCCCTGATTAAATAATCTAGAGGATCCCGGG
3X pelB CA R	/5Phos/ATCAATATAATTATTAATTCG
3X pelD CA F	/5Phos/TGTTCTTTATTGTGATCTAGAGGATCCCGGG
3X pelD CA R	/5Phos/ATTATTTGAGCTGATAATACACC
3X pelE CA F	/5Phos/TGCAATTTGTTATGATCTAGAGGATCCCGGG
3X pelE CA R	/5Phos/TGGCGAGAATTTACTAATTTTC
3X pelF CA F	/5Phos/TGCAGCATTCTCTAATCTAGAGGATCCCGGG
3X pelF CA R	/5Phos/TTGCTCTGTGAACTTTGGTTTG
3X pelH CA F	/5Phos/TGTACAATAATGTAATCTAGAGGATCCCGGG
3X pelH CA R	/5Phos/ACGGTGTTTTTTAGTATCTTTG
3X pelI CA F	/5Phos/TGCATCATATGGTAATCTAGAGGATCCCGGG
3X pelI CA R	/5Phos/ACGATTCTTACTTATCATAGG

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Control of host cell phosphorylation by *Legionella pneumophila*

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Phosphorylation is one of the most frequent modifications in intracellular signaling and is implicated in many processes ranging from transcriptional control to signal transduction in innate immunity. Many pathogens modulate host cell phosphorylation pathways to promote growth and establish an infectious disease. The intracellular pathogen *Legionella pneumophila* targets and exploits the host phosphorylation system throughout the infection cycle as part of its strategy to establish an environment beneficial for replication. Key to this manipulation is the *L. pneumophila* Icm/Dot type IV secretion system, which translocates bacterial proteins into the host cytosol that can act directly on phosphorylation cascades. This review will focus on the different stages of *L. pneumophila* infection, in which host kinases and phosphatases contribute to infection of the host cell and promote intracellular survival of the pathogen. This includes the involvement of phosphatidylinositol 3-kinases during phagocytosis as well as the role of phosphoinositide metabolism during the establishment of the replication vacuole. Furthermore, *L. pneumophila* infection modulates the NF-κB and mitogen-activated protein kinase pathways, two signaling pathways that are central to the host innate immune response and involved in regulation of host cell survival. Therefore, *L. pneumophila* infection manipulates host cell signal transduction by phosphorylation at multiple levels.

Keywords: *Legionella*, phosphatidylinositol 3-kinase, NF-κB, mitogen-activated protein kinase

INTRODUCTION

In the eukaryotic genome, protein kinases comprise one of the largest families of proteins (Manning et al., 2002a) and together with their counteracting protein phosphatases, they regulate a common post-translational modification observed in intracellular signaling. Activation of protein kinases and phosphatases typically occurs in response to extracellular stimuli as well as to intracellular stresses, and the resulting changes in the phosphorylation state of proteins lead to specific cellular responses (Cohen, 2000; Moorhead et al., 2009; Pidoux and Tasken, 2009). Eukaryotic kinases themselves often require activation by phosphorylation (Nolen et al., 2004) and are distinguished by their target residue specificities, which for the purposes of this review are either serine/threonine or tyrosine residues (Olsen et al., 2006; Moorhead et al., 2009; Pidoux and Tasken, 2009). Changes in the phosphorylation state have a broad impact on the cell, altering many processes such as the subcellular localization of proteins, the activity, or substrate specificity of enzymes, as well as specific protein–protein interactions. Furthermore, signals can be amplified within the cell by a cascade of substrate phosphorylation events, while fine-tuning and temporal control can be modulated by opposing phosphatases. This interplay allows spatial and temporal separation of intracellular signaling (Cohen, 2000; Moorhead et al., 2009; Pidoux and Tasken, 2009; Scott and Pawson, 2009).

Cellular processes that rely on phosphorylation have been extensively described in prokaryotes and eukaryotes and include transcription, translation, transport and energy flux, cell cycle, phagocytosis, and the innate immune response to pathogens (Manning et al., 2002a,b; Ryan and Shapiro, 2003; Lindmo and Stenmark, 2006; Back et al., 2009). In order to ensure a proper balance between the unphosphorylated and the phosphorylated state of proteins, tight regulation of protein kinases, and phosphatases is of crucial importance for the cell and imbalance often results in aberrant signaling leading to disease (Manning et al., 2002b).

Interference with the host phosphorylation machinery is a common strategy used by pathogens to promote growth and survival in host tissues. For instance, the gastric pathogen *Helicobacter pylori* directs host cell cytoskeletal rearrangements by delivery of the CagA protein into host cells (Covacci et al., 1993; Segal et al., 1996, 1999; Backert et al., 2000; Stein et al., 2002). Following translocation into the cell, CagA is phosphorylated by Src host tyrosine kinases and subsequently induces changes in cell morphology due to cytoskeleton rearrangement (Segal et al., 1996; Stein et al., 2002). Src kinases along with the host focal adhesion kinase are activated as a result of the interaction of the bacterial CagL protein with the integrin $\alpha_5\beta_1$ receptor (Kwok et al., 2007). Therefore, *H. pylori* proteins can both activate and serve as substrates for host kinases.

Many intracellular pathogens exploit the host phosphorylation machinery by interfering with phosphoinositide (PI) metabolism and thereby target a major signaling pathway controlling membrane trafficking, actin rearrangement, and cell survival (Toker and Cantley, 1997; De Matteis and Godi, 2004; Krauss and Haucke, 2007; Duronio, 2008; Weber et al., 2009a). Phosphatidylinositol 3-kinases (PI3Ks) play a major role in signal transduction during phagocytosis and therefore in the uptake of many pathogens (Ireton et al., 1996; Toker and Cantley, 1997; Lindmo and Stenmark, 2006; Weber et al., 2009a). *Listeria monocytogenes* is one

such pathogen that is internalized in a PI3K-dependent fashion, as 3-phosphorylated phosphatidylinositol phosphates are required for cytoskeletal rearrangements involved in this process (Ireton et al., 1996; Mostowy and Cossart, 2009). *Mycobacterium tuberculosis* controls PI metabolism in a different fashion, forcing entry into a replication compartment that has a low phosphatidylinositol-3-phosphate [PI(3)P] content. This association appears to be linked with evasion of the lysosomal network, as PI(3)P is a key component of early endosomes that mature into endolysosomes (Lindmo and Stenmark, 2006; Philips, 2008). One of the strategies utilized by *Mycobacteria* to keep the PI(3)P content low in the membrane surrounding the replication compartment involves the secretion of PI, protein, and lipid phosphatases (Vergne et al., 2005; Beresford et al., 2007). *M. tuberculosis* proteins, therefore directly target phosphorylation events associated with host PI metabolism.

Similar to *M. tuberculosis*, the intracellular pathogen *Legionella pneumophila* resides and replicates within a specialized vacuole in the host cytosol (Horwitz, 1983a,b; Horwitz and Maxfield, 1984). The proper formation of this replication vacuole relies on the Icm/Dot type IV secretion system (Marra et al., 1992; Berger et al., 1994; Segal et al., 1998; Vogel et al., 1998). Up to 200 bacterial proteins are translocated into the cytosol, targeting a variety of host pathways contributing to efficient intracellular growth of *L. pneumophila* (Burstein et al., 2009; Huang et al., 2010). Characterized translocated proteins are known to target ER → Golgi membrane trafficking (Murata et al., 2006; Ingmundson et al., 2007; Machner and Isberg, 2007), modulate host cell survival (Laguna et al., 2006; Banga et al., 2007), or inhibit the eukaryotic translation elongation complex (de Felipe et al., 2005; Belyi et al., 2006, 2008).

In this review we discuss selected host phosphorylation pathways that are targeted by *L. pneumophila* during interactions with host cells. The strategies used by the microorganism include translocation of kinases that directly manipulate host cell phosphorylation, but also include indirect effects that result in alteration of host cell signaling in response to formation of the *L. pneumophila* replication vacuole.

L. PNEUMOPHILA PROTEIN KINASES AND PHOSPHATASES

While *M. tuberculosis* has both kinases and phosphatases that directly impact host signal transduction controlled by phosphorylation (Walburger et al., 2004; Vergne et al., 2005; Beresford et al., 2007), of the close to 200 known and putative Icm/Dot translocated substrates (Burstein et al., 2009; Huang et al., 2010) none show sequence similarity to known phosphatases or tyrosine kinases. Four translocated proteins, LegK1, LegK2, LegK3, and LegK4 contain domains that show homology to eukaryotic Ser/Thr kinases (de Felipe et al., 2005, 2008; Bruggemann et al., 2006a; Shin et al., 2008; Hervet et al., 2011). Of the LegK homologs, LegK1 and LegK2 are the best characterized. As is the case with the majority of translocated substrates, LegK1 is dispensible for intracellular growth in bone marrow-derived macrophages isolated from A/J mice and in the environmental host *Acanthamoeba castellanii* (de Felipe et al., 2005; Losick et al., 2010). LegK1 exhibits kinase activity *in vitro* and it has been proposed that it interferes with the host innate immune system by directly activating the NF-κB pathway, because ectopic expression of the protein in mammalian cells results in activation of an NF-κB-dependent promoter (Ge et al., 2009; Losick et al., 2010). The kinase activity is necessary for this activation, as a point mutation in the ATP binding domain or a catalytic residue abolishes NF-κB activity (Ge et al., 2009; Losick et al., 2010). *In vitro*, NF-κB activation by LegK1 occurs through direct phosphorylation of a component in the signaling cascade, the inhibitor IκB, resulting in degradation of the inhibitor and release of NF-κB into the host cell nucleus (Ge et al., 2009). Whether or not LegK1-mediated phosphorylation of IκB plays a role in NF-κB activation during macrophage challenge by *L. pneumophila* is unclear, as a *legK1* deletion mutant is able to efficiently activate an NF-κB regulated promoter (Losick et al., 2010). The role of LegK1 during growth within the natural host ameba is similarly unclear, as there are no known NF-κB orthologs in any sequenced amebal species.

As is true with LegK1 (Ge et al., 2009), LegK2 exhibits protein kinase activity *in vitro*, however its specific host target is not known (Hervet et al., 2011). In the amebal host *A. castellanii*, LegK2 activity plays some role in the recruitment of the ER marker calnexin and is required during early time points of intracellular replication, as a *legK2* deletion mutant displays a delayed onset of growth (Hervet et al., 2011). Less is known about the functions of LegK3 and LegK4. LegK3 has been studied in the context of NF-κB activation and the mitogen-activated protein kinase (MAPK) pathway, but failed to show an impact on either (Shin et al., 2008; Ge et al., 2009). Regarding their expression during bacterial growth in broth as well as during infection of *A. castellanii*, the LegK homologs share a similar pattern. In post-exponential phase the expression of LegK1-4 is slightly reduced in the *L. pneumophila* Lens isolate (Hervet et al., 2011) and during host cell infection, expression levels of all four genes do not significantly change (Bruggemann et al., 2006b).

Compared to other pathogens, such as pathogenic *Yersinia* species, which have well characterized kinases and phosphatases that have impact on the disease process (Viboud and Bliska, 2005; Ribet and Cossart, 2010), less is known about *L. pneumophila* proteins that directly change the phosphorylation state of host targets during infection. As will be illustrated below, *L. pneumophila* appears to modulate host cell phosphorylation pathways indirectly, via processes associated with the uptake and replication of *L. pneumophila*.

HOST PHOSPHATASES AND KINASES TARGETED DURING *L. PNEUMOPHILA* INFECTION

To obtain a comprehensive understanding of which host cell pathways are necessary for intracellular growth of *L. pneumophila*, the global transcriptional host cell response has been investigated by several groups, using mouse bone marrow-derived macrophages, human macrophage-like cell lines and amebae (Farbrother et al., 2006; Losick and Isberg, 2006; Abu-Zant et al., 2007; Shin et al., 2008; Li et al., 2009; Fontana et al., 2011). These data were complemented by chemical genetics to identify host factors that are essential for the early steps of infection and for Icm/Dot-dependent protein translocation into a macrophage cell line (Charpentier et al., 2009). The latter approach provided substantial knowledge of the host factors necessary for phagocytosis of *L. pneumophila* and Icm/Dot substrate translocation including PI3Ks (see below). In the natural host amebae, besides inducing a stress response, major transcriptional changes occurred at various time points of *L. pneumophila* challenge, including increased transcription of aminoacyl-tRNA synthetases and decreased expression of ribosomal protein genes

(Farbrother et al., 2006; Li et al., 2009). In human macrophage-like cells, encounter with *L. pneumophila* also resulted in increased transcription of stress response genes, however, the most striking transcriptional response in mammalian cells was demonstrated to be the upregulated expression of genes encoding components of the innate immune system (Losick and Isberg, 2006; Shin et al., 2008). In human macrophage-like cells these included genes regulated by NF-κB, genes encoding anti-apoptotic proteins as well as dual specificity phosphatases (DUSPs) known to be negative regulators of the MAPK pathway (Losick and Isberg, 2006). The transcriptional profile of murine bone marrow-derived macrophages also showed enhanced transcription of *dusp* genes (Shin et al., 2008). These transcriptional responses were specific to virulent *L. pneumophila,* since they were dependent on the presence of a functional Icm/Dot system.

The transcriptional analyses as well as the application of chemical genetics indicate that the host cell response to *L. pneumophila* involves differential regulation of a variety of signaling cascades that are controlled by phosphorylation and dephosphorylation. The following sections will focus on different stages of the infection cycle and the impact of host kinases and phosphatases on these pathways (**Figure 1**).

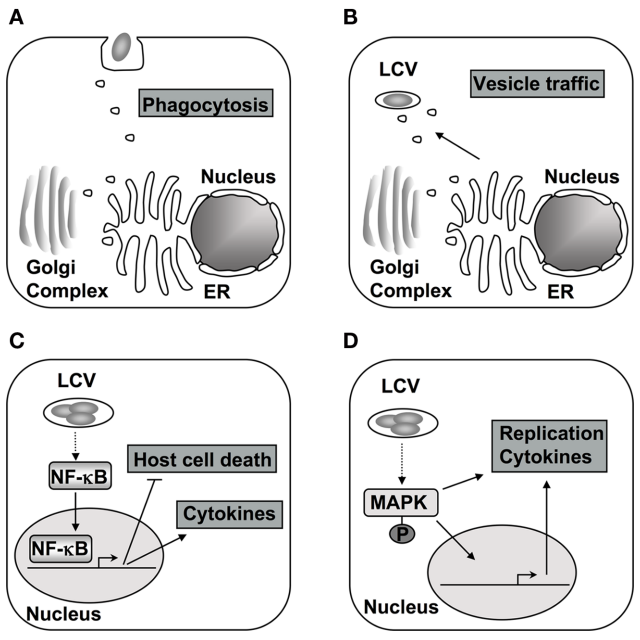

FIGURE 1 | The host phosphorylation system is targeted during different stages of *L. pneumophila* encounter with host cells. (A) The initial contact and subsequent uptake into the host cell is thought to be dependent on PI3Ks and respective downstream signaling in macrophages (Tachado et al., 2008; Charpentier et al., 2009). **(B)** Alterations in PI levels at the vacuole contribute to differential protein recruitment and could interfere with endocytic trafficking to vacuole (Weber et al., 2006, 2009b; Ragaz et al., 2008; Brombacher et al., 2009). **(C)** After contact with cells of higher eukaryotes, the NF-κB pathway is activated, which alters cytokine production, host cell survival, and intracellular replication of *L. pneumophila* (Losick and Isberg, 2006; Abu-Zant et al., 2007; Shin et al., 2008; Bartfeld et al., 2009; Fontana et al., 2011). **(D)** The MAPK signaling pathway is also modulated during infection and proper regulation is necessary for *L. pneumophila* replication in amebae and cytokine production in macrophages (Welsh et al., 2004; Losick and Isberg, 2006; Shin et al., 2008; Li et al., 2009).

HOST FACTORS INVOLVED IN PHAGOCYTOSIS AND ESTABLISHING A REPLICATION COMPARTMENT

Each cycle of infection starts with uptake of *L. pneumophila* into the host cell by phagocytosis. Uptake into human alveolar macrophages under non-opsonized conditions has been documented to occur by coiling phagocytosis (Horwitz, 1984; Charpentier et al., 2009). The mechanism of phagocytosis of *L. pneumophila*, however, has been a point of dispute for some time, with the role of PI3Ks being a particular focus of controversy. As mentioned above, pathogens may be taken up in a PI3K-dependent manner (Weber et al., 2009a). Activation of PI3K leads to downstream signaling events that involve synthesis of PI(3,4,5)P_3, which is likely to be followed by recruitment of guanidine nucleotide exchange factors that activate Rho family GTPases involved in regulation of actin rearrangements (Lindmo and Stenmark, 2006).

The role of PI3Ks during phagocytosis of *L. pneumophila* appears to differ between host systems, bacterial strains, and the experimental setup used (see below). Initially, it was postulated that uptake of virulent *L. pneumophila* JR32 (Philadelphia-1) into U937 human macrophage-like cells does not depend on PI3Ks (Khelef et al., 2001). Phagocytosis of *L. pneumophila* having an intact Icm/Dot system was not blocked by the PI3K inhibitor wortmannin, and this failure to inhibit uptake was independent of the opsonization conditions used. When actin polymerization was visualized at *L. pneumophila* entry sites, there appeared to be no affect of inhibitors of PI3K function. However, a mutant having an inactivated Icm/Dot system was taken up in a PI3K-dependent manner indicating that the presence of a functional protein translocation system targets *L. pneumophila* into a unique uptake pathway (Khelef et al., 2001).

In the ameba *Dictyostelium discoideum*, inhibition of PI3Ks by wortmannin and LY294002 reduced the uptake of *L. pneumophila* (Weber et al., 2006; Peracino et al., 2010). In spite of this reduction in uptake, deletion of class I PI3Ks appeared to have a positive influence on intracellular replication (Weber et al., 2006; Peracino et al., 2010). Deletion of genes encoding PI3Ks, as well as chemical inhibition of their activities, changes the morphology of the replication vacuole and it was postulated that this structure might stimulate intracellular replication (Weber et al., 2006). The absence of PI3Ks could result in an altered composition of PIs at the *Legionella*-containing vacuole (LCV) causing changes in host protein recruitment. Also, improved intracellular replication could be due to enhanced bypass of the endocytic pathway, which requires PI3K activity, and may compete for sequestration of the microorganism into a compartment that is restrictive for intracellular growth (Lindmo and Stenmark, 2006; Weber et al., 2006).

Besides PI3Ks, another enzyme involved in PI metabolism, the inositol polyphosphate-5-phosphatase Dd5p4, influences *L. pneumophila* infection in *D. discoideum.* Host cells lacking Dd5p4 showed a defect in uptake, but once internalized, the bacteria showed improved intracellular replication, which is similar to the phenotype observed for loss of PI3K (Weber et al., 2006, 2009b). Dd5p4 is recruited to the LCV in a Icm/Dot-dependent manner and is catalytically active, which putatively leads to conversion from PI(4,5)P_2 to PI(4)P (Weber et al., 2009b). PI(4)P is considered a lipid marker for the LCV, that serves as an anchor for Icm/Dot substrates and is detectable at the LCV dependent on the presence of an intact Icm/Dot system (Weber et al., 2006; Ragaz et al., 2008;

Brombacher et al., 2009). As in amebae, PI(4)P is also found at the LCV in infected RAW264.7 murine macrophage cells indicating that intracellular replication within ameba and mammalian cells has similar lipid requirements (Weber et al., 2006). Furthermore, the human homolog of Dd5p4, OCRL1, which plays a role in trafficking from endosomes to the *trans*-Golgi network (Lowe, 2005), could also be detected at the LCV and likely functions similarly to Dd5p4 during intracellular replication (Weber et al., 2009b). Therefore, targeting of PI metabolism seems to occur at different stages of the infectious cycle following uptake and contributes to proper establishment of the LCV (**Figure 2**).

The notion that PI3Ks do not play a major role in uptake of *L. pneumophila* was challenged by a study using the J744A.1 murine macrophage cell line (Tachado et al., 2008). In the J744A.1 murine macrophage cell line, phagocytosis of *L. pneumophila* AA100 (Wadsworth) was reduced more severely by the PI3K inhibitors wortmannin and LY294002 than had been reported with other cell lines (Khelef et al., 2001; Weber et al., 2006; Peracino et al., 2010). In addition, cells expressing a dominant-negative mutant of PI3K were also depressed for uptake of the wild type *L. pneumophila* strain. Consistent with a role for PI3K during uptake, a downstream signal of PI3K activation, protein kinase B, was activated after challenge with *L. pneumophila*. Induction of the PI3K pathway could only be observed after contact with *L. pneumophila* expressing an intact Icm/Dot system (Tachado et al., 2008). One explanation for the conflicting results is that the J744A.1 cell line supports lower levels of *L. pneumophila* growth than other cell lines. Perhaps in cells in which there is luxurious intracellular growth of the bacterium, uptake is independent of PI3Ks. However, we have observed that wortmannin inhibits uptake of *L. pneumophila* into permissive mouse macrophages and it has been reported that uptake of *L. pneumophila* is reduced after treatment of *D. discoideum* with PI3K inhibitors (Weber et al., 2006; Peracino et al., 2010). There may be subtle differences in cytoskeletal regulatory circuits that determine whether PI3K is involved in uptake in different cell types.

A chemical genetics screen to find host cell functions required for Icm/Dot-dependent substrate translocation also supports the model that PI3Ks are involved in phagocytosis, at least in the J744A.1 cell line (Charpentier et al., 2009). In this study, phagocytosis was found to be a crucial prerequisite for Icm/Dot-promoted protein translocation (Charpentier et al., 2009). Inhibitors of either actin polymerization or PI3K reduced both Icm/Dot-dependent protein translocation and bacterial uptake. Taken together these data indicate that signaling through PI3K plays an important role during phagocytosis in these models.

Other novel targets identified by the chemical genetics screen pointed to the importance of tyrosine phosphatases for phagocytosis of *L. pneumophila* (Charpentier et al., 2009). The functionally redundant receptor protein tyrosine phosphate phosphatases CD45 and CD148 together with other, as yet unidentified, tyrosine phosphatases were shown to modulate uptake. Uptake of *L. pneumophila* into bone marrow-derived macrophages isolated from mice lacking CD45 and CD148 was drastically impaired compared to wild type macrophages, without affecting bacterial adhesion to cells. A more severe reduction in uptake relative to the mutant macrophages was observed in the presence of the CD45 inhibitor RWJ-60475, indicating that additional phosphatases may be involved. These results hint at a novel role of tyrosine phosphate phosphatases in phagocytosis of *L. pneumophila*. This is particularly interesting in light of the fact that there have been few convincing demonstrations that tyrosine phosphatases stimulate phagocytosis, other than the report that the tyrosine phosphatase Shp-1 stimulates *Neisseria* uptake (Hauck et al., 1999). Generally, tyrosine phosphatase activity has been connected to interference with uptake, as exemplified by the *Yersinia* YopH tyrosine phosphatase (Adkins et al., 2007).

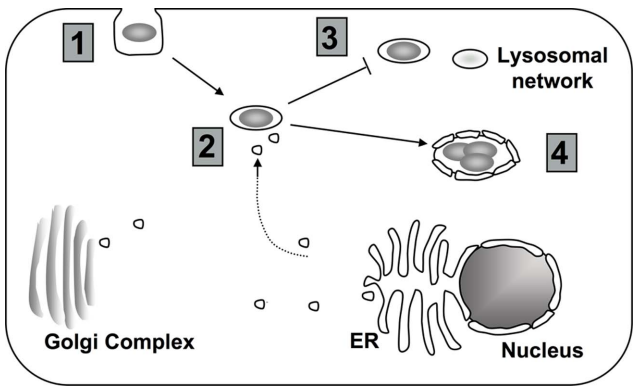

FIGURE 2 | Targeting of host cell PI metabolism by *L. pneumophila*. (1) Depending on the model system, uptake into the host cell may require PI3K signaling (Khelef et al., 2001; Weber et al., 2006; Tachado et al., 2008; Charpentier et al., 2009; Peracino et al., 2010). (2) To properly establish the LCV, *L. pneumophila* interferes with host vesicle trafficking. Changes in the composition of PIs at the LCV might contribute to altered trafficking (Weber et al., 2006, 2009b; Isberg et al., 2009). (3) Changes in the PI levels at the vacuole may help *L. pneumophila* to avoid the endocytic pathway (Lindmo and Stenmark, 2006; Weber et al., 2006, 2009a). (4) Throughout intracellular growth, PIs at the LCV likely provide scaffolding for both *L. pneumophila* translocated proteins and host proteins (Weber et al., 2006, 2009b; Ragaz et al., 2008; Brombacher et al., 2009).

ACTIVATION OF THE NF-κB PATHWAY

After phagocytosis, *L. pneumophila* resides within a membrane-bound compartment in the host cytosol. Consequently, survival of the host cell is necessary for successful replication. One way to prevent cell death involves direct interference of pro-death pathways by Icm/Dot translocated substrates (Laguna et al., 2006; Banga et al., 2007). A second mechanism of preventing host cell death during infection is to exploit proteins that are under the control of the mammalian transcription factor NF-κB, which acts as a positive regulator of genes encoding anti-apoptotic proteins (Karin and Lin, 2002). NF-κB homo- and heterodimers are master regulators of the mammalian innate immune response that control the expression of almost 400 genes (Karin and Lin, 2002; Ahn and Aggarwal, 2005; Hayden and Ghosh, 2008). NF-κB activation can result from sensing of pathogen associated molecular patterns (PAMPs; for example flagellin or peptidoglycan) by pattern recognition receptors (PRRs) that include the membrane-bound toll-like receptors (TLRs) and intracellular nod-like receptors (NLRs; Fritz et al., 2006; Kawai and Akira, 2010). Activation of these receptors triggers a signaling cascade that results in nuclear translocation of NF-κB subunits (Hayden and Ghosh, 2008). Signaling

downstream from NLRs involves Rip2 kinase whereas TLR signaling is mediated via the adaptor proteins MyD88 and Trif (Shaw et al., 2008; Kawai and Akira, 2010). Both pathways lead to activation of IκB kinases (IKKs) by phosphorylation. Once activated, IKK phosphorylates IκB family members, resulting in degradation of these inhibitory proteins that are bound to the canonical NF-κB subunits in the cell cytoplasm (Hayden and Ghosh, 2008). Degradation of IκB frees the NF-κB subunits to be translocated into the nucleus (Hayden and Ghosh, 2008). The NF-κB pathway is manipulated by different pathogens such as *H. pylori* and *Rickettsia rickettsii* as well as by *L. pneumophila* (Clifton et al., 1998; Brandt et al., 2005). Challenge of host cells with *L. pneumophila* results in increased Icm/Dot-dependent transcription of NF-κB subunits as well as NF-κB regulated genes including pro-inflammatory cytokines and antagonists of apoptosis (Losick and Isberg, 2006; Abu-Zant et al., 2007; Shin et al., 2008; Bartfeld et al., 2009; Fontana et al., 2011).

There are probably multiple mechanisms that lead to NF-κB activation during *L. pneumophila* infection. Besides the engagement of PRRs with PAMPs, direct targeting of the pathway by Icm/Dot translocated substrates, such as LegK1 has been proposed, as pointed out above (Ge et al., 2009). NF-κB activation by *L. pneumophila* is probably more complex than can be explained by the action of a single effector, and likely occurs via the synergistic interaction of PRR signaling in combination with Icm/Dot-dependent components (Losick and Isberg, 2006; Shin et al., 2008; Bartfeld et al., 2009). At face value, PRR signaling and Icm/Dot activation of NF-κB appear to have different temporal courses of action, with PRR signaling occurring with more rapid kinetics than observed for Icm/Dot activation of the pathway (Bartfeld et al., 2009). It is unclear, however, if Icm/Dot activation of NF-κB ever occurs totally independently of PRR signaling. In macrophages from mice lacking TLR signaling via MyD88 and Nod signaling via Rip2, there appears to be little Icm/Dot-dependent signaling (Shin et al., 2008), although the presence of either PRR pathway is sufficient to support Icm/Dot-dependent NF-κB activation. This argues that although neither the type of bacterial ligand nor its site of encounter within the host cell are important for signaling, there is a requirement for the host cell to sense a PAMP for there to exist a strong Icm/Dot-dependent response.

In the human alveolar epithelial cell line A549, it has been shown that NF-κB activation follows a biphasic pattern. Short-term activation, measured by NF-κB nuclear translocation, depends on TLR5 and MyD88 (Bartfeld et al., 2009). This is followed by a TLR-independent long-term activation for which a functional Icm/Dot system is required. In concert with the data on synergy, these cells still retain Nod signaling, which could facilitate the Icm/Dot-dependent response. During long-term activation, IκB is degraded and anti-apoptotic genes are expressed (Bartfeld et al., 2009). Induction of anti-apoptotic genes appears to be the common theme in different host cell types (Losick and Isberg, 2006; Abu-Zant et al., 2007). The importance of host cell survival to maintain efficient intracellular replication was demonstrated in A/J bone marrow-derived macrophages. Inhibition of NF-κB caused increased host cell death in response to *L. pneumophila* challenge. However, the presence of NF-κB enhanced cell survival and was necessary for efficient replication (Losick and Isberg, 2006). Furthermore, one of the NF-κB regulated antagonists of apoptosis had a direct positive influence on host cell survival. In bone marrow macrophages derived from mice lacking plasminogen activator inhibitor-2 (PAI-2), there was increased cell death in response to *L. pneumophila* challenge (Losick and Isberg, 2006).

In bone marrow-derived macrophages from A/J mice, *L. pneumophila* activates NF-κB via at least two pathways depending on the multiplicity of infection. At low dose infections, there is little nuclear translocation of NF-κB in the absence of Icm/Dot, indicating that PRR recognition is not sufficient to give a robust signal (Losick and Isberg, 2006). For these low dose infections, NF-κB nuclear translocation is still observed in the absence of MyD88 and Trif , but relies on Icm/Dot (Losick and Isberg, 2006; Losick et al., 2010), and presumably Nod signaling, based on the work from the Roy lab (Shin et al., 2008). At an elevated multiplicity of infection, however, MyD88-dependent NF-κB activation can be observed in the absence of Icm/Dot (Losick and Isberg, 2006).

The data on Nod signaling adds complexity to formulating models on NF-κB signaling, but the basic message is consistent with the idea that as long as at least one PRR signaling pathway is intact, an Icm/Dot-dependent signal can be detected. It is clear that in bone marrow-derived macrophages capable of TLR signaling, Icm/Dot-dependent activation of NF-κB occurs in the absence of Nod1 or Rip2, even under conditions of low multiplicity challenge with *L. pneumophila* (Losick and Isberg, 2006; Losick et al., 2010). However, in HEK293T cells, which do not express TLRs that efficiently engage *L. pneumophila*, knockdown of Nod1 reduces NF-κB activation (Losick et al., 2010). These results complement results indicating that Icm/Dot-dependent NF-κB activation in the absence of MyD88 is only seen when Rip2 is present (Shin et al., 2008). Therefore, crosstalk between Icm/Dot translocated substrates and PAMP signaling must exist, but the source of the PAMP or the site in the cell that PAMP signaling is initiated do not appear to be critical (Losick et al., 2010). TLR engagement acts together with Icm/Dot in cells lacking Rip2 signaling, while similarly, Nod signaling collaborates with Icm/Dot in cells that lack the TLR pathway. This indicates that in the case of collaboration with Icm/Dot, the NLR, and TLR pathways could be redundant.

The significant impact of Icm/Dot on the activation of NF-κB led to two investigations to identify translocated substrates that could directly induce activation of this protein. NF-κB can be stimulated as a response to many different cellular insults, with ER stress and disruption of the actin cytoskeleton being two important examples (Nemeth et al., 2004; Ahn and Aggarwal, 2005; Schroder, 2008). Using similar approaches, two laboratories have identified Icm/Dot translocated substrates that are able to induce an NF-κB reporter when ectopically expressed in HEK293T cells. As described above, LegK1 was one of these substrates (Ge et al., 2009). In addition, there were a number of activators that showed modest activation of the reporter (Ge et al., 2009; Losick et al., 2010) as well as another strong inducer, the translocated substrate LnaB (Losick et al., 2010). When bacteria are grown into post-exponential phase, LnaB was shown to be required to fully activate the NF-κB reporter after challenge of HEK293T cells with *L. pneumophila* (Losick et al., 2010). The C-terminal coiled coil domain of LnaB was required for NF-κB induction. This suggests that this

domain of LnaB interacts with a protein in the signaling cascade upstream of NF-κB or it may contribute to a cellular activity that increases NF-κB signaling. However, the role of LnaB in a cellular process leading directly or indirectly to NF-κB activation is not known (Losick et al., 2010). Although there is no evidence that LegK1 induces NF-κB after *L. pneumophila* challenge (Losick et al., 2010), as described above, *in vitro* experiments demonstrate a direct interaction with the signaling cascade upstream of NF-κB (Ge et al., 2009), and its pathway of activation could be very different from that observed with LnaB.

Besides LnaB and LegK1, five Icm/Dot translocated inhibitors of host translation exhibited NF-κB inducing activity accompanied by a distinctive transcriptional response including *IL23a* and *Csf2* induction (Fontana et al., 2011). It is possible that many Icm/Dot translocated substrates may lead to activation of the NF-κB pathway via a stress response rather than directly modulating the activity of proteins that regulate the nuclear translocation of this protein. The *L. pneumophila* proteins Lgt1, Lgt2, Lgt3, SidI, and SidL are known to cause an inhibition of host translation. This interferes with the synthesis of the unstable IκB inhibitory protein, which releases cytoplasmic NF-κB, allowing subsequent translocation into the nucleus (Hayden and Ghosh, 2008; Fontana et al., 2011). This inhibition of host protein synthesis caused by Icm/Dot substrates appears to be a key to causing sustained activation of NF-κB that can synergize with either TLR or Rip2-dependent signaling, as a *L. pneumophila* mutant lacking several translocated substrates that act as protein synthesis inhibitors is defective for NF-κB activation (Fontana et al., 2011; **Figure 3**).

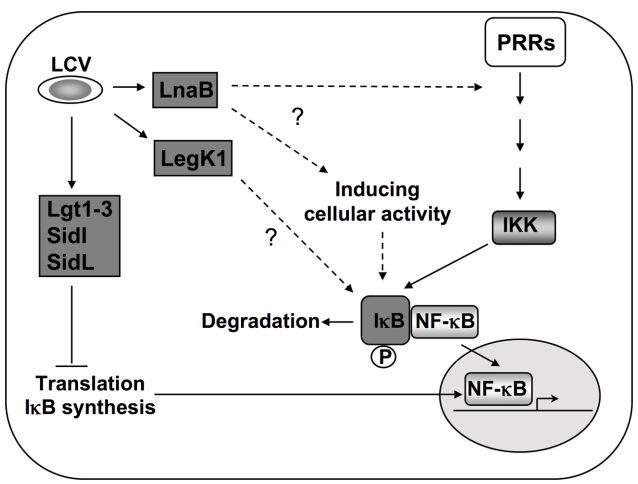

FIGURE 3 | Activation of NF-κB during *L. pneumophila* infection. In addition to PRR mediated activation, Icm/Dot translocated substrates induce NF-κB by multiple mechanisms. During induction of the NF-κB pathway, the inhibitor IκB is phosphorylated by IKK and degraded, leading to nuclear translocation of the transcription factor (Hayden and Ghosh, 2008). Five Dot/Icm translocated substrates are known to target this pathway by inhibiting host translation (Fontana et al., 2011). This inhibition of translation interferes with the synthesis of the unstable inhibitor IκB and frees NF-κB subunits to translocate into the nucleus (Hayden and Ghosh, 2008; Fontana et al., 2011). The Ser/Thr kinase LegK1 has the ability to act directly on the NF-κB pathway by phosphorylating IκB (Ge et al., 2009), resulting in its degradation. The translocated protein LnaB activates NF-κB by an as yet unknown mechanism (Losick et al., 2010).

MAP KINASES AND DUAL SPECIFICITY PHOSPHATASES

In addition to the NF-κB pathway, which is only found in multicellular eukaryotes, the MAP kinase pathway is a second component of the innate immune system that is targeted during *L. pneumophila* infection (Welsh et al., 2004; Losick and Isberg, 2006; Shin et al., 2008; Li et al., 2009). The natural amebal hosts employ the MAP kinase pathway, so there is good reason to believe that selective pressures for effective interaction with MAP kinases must have taken place to facilitate intracellular replication of the bacterium. MAPKs regulate diverse cellular processes such as gene expression, cytoskeletal integrity, cell death, mitosis, and the induction of inflammatory mediators (Johnson and Lapadat, 2002; Jeffrey et al., 2007; Pullikuth and Catling, 2007; Huang et al., 2009). A cascade of sequentially active kinases, MAPKKKs and MAPKKs, activate MAPKs by threonine and tyrosine phosphorylation (Johnson and Lapadat, 2002; Huang et al., 2009). Activated MAPKs in turn phosphorylate specific substrates such as transcription factors, other kinases, or cytoskeletal proteins (Johnson and Lapadat, 2002). The four best characterized families of MAPKs found in higher eukaryotes, ERK, JNK, p38, and ERK5 respond to various stimuli, and are activated by specific MAPKKKs, resulting in both signal and target-specific responses (Johnson and Lapadat, 2002; Huang et al., 2009). MAPK signaling can be induced by activation of TLRs or NLRs as well as other stress response signals (Johnson and Lapadat, 2002; Huang et al., 2009). Linkage of NLRs to MAPKs occurs via Rip2 and Card9 while TLR signaling to MAPKs is MyD88-dependent (Hsu et al., 2007; Liu et al., 2007; Ting et al., 2010).

In order to ensure a proper balance of activation, MAPKs are regulated on a variety of levels including elaborate feedback loops and spatial separation of signaling (Jeffrey et al., 2007). After activation by the MAP kinase relay, inactivation occurs by dephosphorylation of Thr and Tyr residues of MAPKs by DUSPs (Lang et al., 2006; Jeffrey et al., 2007). DUSP family members are under tight control both at the transcriptional level and by post-translational modifications (Patterson et al., 2009) and in order to ensure specific targeting of MAPKs, they differ in their expression pattern and cellular localization (Lang et al., 2006; Jeffrey et al., 2007). Despite these differences, a common feature of many DUSPs is their transcriptional activation downstream from MAPK signaling, providing important feedback control of MAPK activation. In addition to transcriptional control, DUSP protein stability is influenced by MAPKs (Jeffrey et al., 2007).

Unlike the NF-κB pathway, MAPKs are also found in lower eukaryotes such as yeast and ameba (Molina et al., 2010). *D. discoideum* contains two enzymes similar to the mammalian ERK family (Gaskins et al., 1994; Segall et al., 1995). In *D. discoideum*, ERK-1 is phosphorylated shortly after *L. pneumophila* challenge with either a wild type or a Icm/Dot-deficient mutant (Li et al., 2009). This activation is transient with peak activation 1-h post infection. Inactivation of ERK-1 most likely relies on the tyrosine kinase/dual specificity phosphatase DupA, as there is constitutive activation of the MAPK in strains lacking DupA. The correct temporal regulation of ERK-1 activation has a significant impact on intracellular growth and host gene expression. In a mutant lacking DupA, intracellular replication of *L. pneumophil*a is impaired, and this accompanied by hyperphosphorylation of ERK-1 relative to wild type amebae (Li et al., 2009). The resulting transcriptional response in cells having

hyperactivated ERK-1 included over 500 misregulated genes that were also impacted in wild type amebae after challenge with *L. pneumophila*. Interestingly, these genes include those encoding proteins hypothesized to play a role in the amebal response to pathogens (Li et al., 2009).

As in amebae, MAPK activation was also observed as a response to infection in murine bone marrow-derived macrophages (Shin et al., 2008). Here, ERK is activated independently of Icm/Dot and in the absence of the TLR signaling. Activation of p38 and SAPK/JNK follows a different pattern from mammalian ERK and is composed of an initial MyD88-dependent, Icm/Dot-independent component, as well as a delayed prolonged MyD88-independent component that relies on Icm/Dot. The kinetic data are reminiscent of MAPK activation in amebae, in that p38 and SAPK/JNK activation peaks at 1-h post infection and continues for 4 h. The high and sustained MAPK activity in response to *L. pneumophila* is necessary for increased cytokine production and requires MyD88-dependent, Icm/Dot-independent and MyD88-independent, Icm/Dot-dependent signaling. As Icm/Dot is required to fully activate MAPK signaling it was proposed that translocated substrates may play a role in this process. This idea was supported by data showing that the pore forming activity of the type IV secretion system alone is not sufficient to activate p38 and SAPK/JNK (Shin et al., 2008). Signaling through p38 and SAPK/JNK may involve Icm/Dot substrates that directly target the MAPK pathway or substrates that exhibit inducing activity by acting on a different host process and thereby indirectly activate MAPKs (**Figure 4**). However, the most likely Icm/Dot substrates containing Ser/Thr kinase domains, LegK1, Legk2, and LegK3, could be excluded as direct p38 activators (Shin et al., 2008). As described for NF-κB induction (Fontana et al., 2011), Icm/Dot translocated substrates that inhibit host protein synthesis could also explain the Icm/Dot-dependent mechanism of MAPK activation.

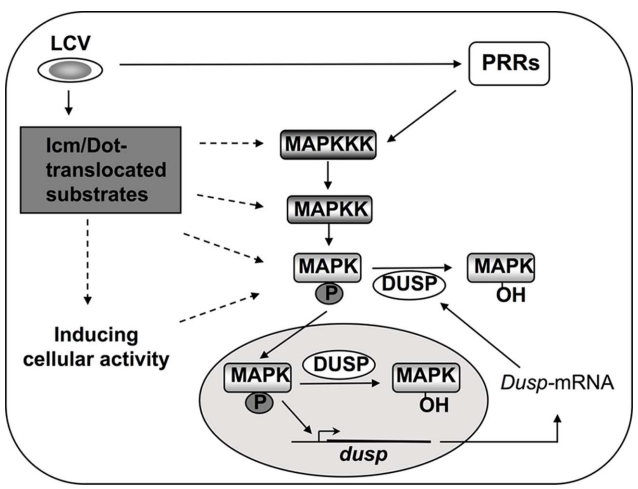

FIGURE 4 | Mechanisms of MAPK activation during *L. pneumophila* infection. MAPKs are activated by sequentially induced kinases and in turn phosphorylate cellular and nuclear proteins such as transcription factors (Johnson and Lapadat, 2002; Huang et al., 2009). Inactivation of MAPKs results from dephosphorylation by dual specificity phosphatases (DUSPs; Lang et al., 2006; Jeffrey et al., 2007). DUSPs are regulated on many levels to ensure proper signaling through MAPKs, and MAPKs themselves can control DUSPs at the level of transcription and protein stability (Jeffrey et al., 2007). During *L. pneumophila* challenge of host cells, induction of the MAPK signaling pathway occurs through PRR signaling as well as by Icm/Dot-dependent activity in macrophages, and via unidentified sensors in amebae (Shin et al., 2008; Li et al., 2009). Whether Icm/Dot translocated substrates target kinases upstream of MAPK, or, whether interference with protein synthesis is sufficient to alter MAPK activity, is unknown (Shin et al., 2008).

As observed with DupA in amebae, DUSPs also play a role during infection in mammalian cells. An Icm/Dot-dependent increase in transcription of *dusp* genes was observed in the human macrophage-like U937 cell line (Losick and Isberg, 2006). In mouse bone marrow-derived macrophages, it was shown that Icm/Dot-dependent induction of *dusp1* transcription did not require MyD88 or Rip2 kinase (Shin et al., 2008). Since *dusp* transcription is upregulated by MAPKs, this increase might be due to enhanced MAPK activity during infection. However, an increase of DUSP protein levels that would be expected to accompany increased transcription of the gene could not be observed, perhaps because of the presence of the *L. pneumophila* translocated substrates that interfere with protein synthesis in the host cell.

Mitogen-activated protein kinase activation appears to be a common response to *L. pneumophila* infection in lower and higher eukaryotes. Even though a direct participation of *L. pneumophila* proteins in MAPK activation seems likely, so far no Icm/Dot substrate was shown to act directly on the MAPK pathway. Concerning the function of DUSPs in regulating MAPKs during infection, the requirement of DUSPs to ensure proper balance of MAPK signaling is obvious from results in *D. discoideum*, as misregulation of ERK-1 interferes with intracellular growth of the bacterium (Li et al., 2009). The role of DUSPs in mammalian cells is not clear, however, especially since the induction of gene expression does not appear reflected in increased protein levels of DUSPs. In fact, the Icm/Dot-dependent induction of *dusp* transcription may be the result of MAPK activation caused by interference of host protein synthesis by *L. pneumophila*, so both transcriptional induction, and the lack of a translational response, are promoted by the same translocated substrates. *Dusp* expression is no longer elevated as a response to *L. pneumophila* infection in the absence of the five Icm/Dot translocated inhibitors of host translation, Lgt1, Lgt2, Lgt3, SidI, and SidL.

CONCLUDING REMARKS

Legionella pneumophila is known to interfere with many host cell processes such as the ubiquitination machinery (Kubori et al., 2008, 2010; Ivanov and Roy, 2009; Ensminger and Isberg, 2010; Price et al., 2010), host translation (de Felipe et al., 2005; Belyi et al., 2006, 2008), or vesicle trafficking (Murata et al., 2006; Ingmundson et al., 2007; Machner and Isberg, 2007). Here we have presented a selected overview of targeted host cell pathways that mediate signal transduction through changes in the phosphorylation state of proteins and lipids. As phosphorylation and dephosphorylation are among the most common modifications in cell signaling, *L. pneumophila* exploits host phosphorylation at all stages of infection. Targeting of the host phosphorylation machinery may involve direct modification of host factors by *L. pneumophila* proteins that act as kinases or phosphatases as well as sensing of cellular processes during infection that indirectly change the phosphorylation

state of host proteins. In the case of PI metabolism, the importance of controlling flux and recruitment of this important lipid during intracellular replication in amebae was clearly demonstrated (Weber et al., 2006, 2009b; Ragaz et al., 2008; Brombacher et al., 2009; Peracino et al., 2010), whereas the role of PI3Ks during uptake of *L. pneumophila* is not yet resolved and differs depending on the model system (Khelef et al., 2001; Weber et al., 2006; Tachado et al., 2008; Charpentier et al., 2009; Peracino et al., 2010). The requirement of Icm/Dot translocation for NF-κB and MAPK activation implies that translocated substrates contribute to these regulatory changes in host cells. Besides the inducing activity of inhibitors of host translation, no other mechanism for this activation has been proposed based on *in vivo* studies, so in the future it will be very interesting to determine what other factors induce these pathways apart from PRR signaling. With the emerging knowledge on the function of Icm/Dot translocated substrates, details regarding how *L. pneumophila* manipulates major signaling pathways involving phosphorylation should be forthcoming shortly.

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Targeting one of its own: expanding roles of substrates of the *Legionella pneumophila* Dot/Icm type IV secretion system

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Facing the many host defense mechanisms at different stages of the infection, a pathogen needs to employ corresponding arsenals either to hijack host cellular processes for its own use or to thwart the attacks from the host. For numerous bacterial pathogens, one of the effective weapons is effector protein. Type IV protein secretion systems are associated with the virulence of many important pathogens such as *Helicobacter pylori*, *Bartenella pertussis*, *Brucella* spp., *Coxiella burnetii*, and *Legionella pneumophila* (Backert and Meyer, 2006). Although the structures of these transporters appear similar, their protein substrates differ drastically not only in functions but also in abundance. For example, despite extensive efforts, CagA is the only effector identified for the Cag system of *H. pylori* (Hatakeyama, 2008). On the other hand, there are at least 274 experimentally confirmed protein substrates of the *L. pneumophila* Dot/Icm system (Zhu et al., 2011). This count is close to 10% of the predicted genes in the *L. pneumophila* genome (Cazalet et al., 2004; Chien et al., 2004; Schroeder et al., 2010), making the Dot/Icm system arguably the most prolific protein transporter in term of the number of translocated substrates. Similar to effectors of other types of secretion systems, Dot/Icm substrates function to target various host cellular processes, such as vesicle trafficking, cell death, ubiquitination, lipid metabolism, and innate immunity, thus allowing the biogenesis of an intracellular niche permissive for bacterial replication (Isberg et al., 2009; Hubber and Roy, 2010).

To cope with the dynamic response from the host, bacterial pathogens can employ several strategies to achieve temporal regulation of the activity of their virulence factors. The most commonly used mechanism is to regulate expression of virulence factors at the transcriptional level in response to environmental cues present during different phases of infection. For example, many *Legionella* effector genes are induced when bacteria enter the post-exponential phase and are ready to infect (Bruggemann et al., 2006). The second is to control the translocation efficiency of effectors at post-translational level, which is exemplified by small RNA-mediated regulation of effector transfer in *Salmonella typhimurium* (Padalon-Brauch et al., 2008). The third is to code for effectors capable of causing opposite cell biological effects to neutralize or reverse the effects caused by other effectors. In *S. typhimurium*, bacterial entry is induced by SopE, a guanine nucleotide exchange factor (GEF) for the Rho family of small GTPases, an activity that is antagonized by the GTPase activation protein (GAP) SopE. In this scenario, temporal control is achieved by the inherent differences in the sensitivity of these two proteins to host proteasome degradation (Kubori and Galan, 2003). Similarly, *L. pneumophila* reverses the SidM/DrrA-mediated activation of the small GTPase Rab1 by the GAP protein LepB (Ingmundson et al., 2007). However, the mechanism underlying the temporal control remains elusive. Third, the pathogens can inhibit pathways triggered by themselves. For example, as a result of apoptosis induced by *L. pneumophila* challenge, infected cells contains active caspases 3 and 7, but the activity of these enzymes presumably can be inhibited by IAPs induced by the bacterium itself (Abu-Zant et al., 2005, 2007; Losick and Isberg, 2006; Nogueira et al., 2009). Unfortunately, the bacterial proteins involved in such manipulation remain unknown as does the mechanism underlying the temporal regulation.

Almost all characterized virulence factors exert their effects by targeting one or more host molecules. The discovery by Kubori et al. (2010) extended the roles of bacterial effectors into a completely new domain: direct regulation of the function of other effectors, in this particular case, by targeting a different effector protein for proteasome degradation. LubX is a U-box-type E3 ubiquitin ligase that was previously found to target the host kinase Clk1 (Kubori et al., 2008). In this new study, the authors reported that LubX polyubiquitinates the effector SidH and targets it for degradation (Kubori et al., 2010). Moreover, their data showed that in a fruit fly infection model, deletion of *lubX* caused a hyper-lethality phenotype and reduced bacterial replication. Thus, this discovery established a new mechanism used by a bacterial pathogen to temporally control the activity of its virulence factors. Their results also substantiated some earlier observations on *L. pneumophila* infection. First, they showed that LubX is expressed and translocated by the Dot/Icm system only until after the initial phase of infection has been established, peaking at 10 h post infection (Kubori et al., 2008, 2010), which is in line with the observation that the Dot/Icm system is active for at least 8 h after bacterial internalization (Liu et al., 2008). Second, targeting of SidH did not occur in the initial phase of infection, implying that SidH is important in the early phase of infection and becomes unnecessary or even detrimental to further development of the bacterial phagosome as infection proceeds. Such prediction is consistent with the potential role of SidH in inhibition of host cell death (Laguna et al., 2006), because continued arrest of apoptosis presumably is counterproductive when the infection comes to a close.

This study also provided an explanation to one of the perplexing questions in the study of *L. pneumophila* pathogenesis: Why does this bacterium code for so many effectors? At least two models can be used to explain this phenomenon. The first is that there is a tremendous functional redundancy among effectors targeting a particular host cellular process. This model is supported by the fact that mutations eliminating one single effector gene rarely caused defects in intracellular bacterial growth (Ensminger and Isberg, 2009). Second, these effectors

may be necessary for successful colonization of the taxonomically diverse protozoan hosts encountered by *L. pneumophila* in the environment. The study by Nagai and colleagues added a novel dimension of targets regulated by *L. pneumophila* virulence factors.

Given the complexity of the interactions between *L. pneumophila* and its hosts, it will not be surprising to identify more metaeffectors with modes of action different from that of LubX in future study. It is possible that inhibition of effector activity by a second effector can be achieved by post-translational modifications. An effector can directly reverse the modification made by another effector on its target molecule. For example, such regulation conceivably can be achieved by a pair of effectors with kinase and phosphatase activity, respectively. Alternatively, one effector can directly modify another effector at post-translational level to activate or inactivate its function. As novel post-translational modifications such as AMPylation (Roy and Mukherjee, 2009; Muller et al., 2010) and farnesylation (Ivanov et al., 2010; Price et al., 2010) continued to be identified in *Legionella* effectors, it will be interesting to know whether these modifications are subjected to effector-mediated regulation. Although the existence of these regulatory mechanisms in the control of *L. pneumophila* effector activity is uncertain, it is certain that more excitement will be generated in our continuing study of these several hundreds interesting proteins.

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Small regulatory RNA and *Legionella pneumophila*

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Legionella pneumophila is a gram-negative bacterial species that is ubiquitous in almost any aqueous environment. It is the agent of Legionnaires' disease, an acute and often under-reported form of pneumonia. In mammals, *L. pneumophila* replicates inside macrophages within a modified vacuole. Many protein regulators have been identified that control virulence-related properties, including RpoS, LetA/LetS, and PmrA/PmrB. In the past few years, the importance of regulation of virulence factors by small regulatory RNA (sRNAs) has been increasingly appreciated. This is also the case in *L. pneumophila* where three sRNAs (RsmY, RsmZ, and 6S RNA) were recently shown to be important determinants of virulence regulation and 79 actively transcribed sRNAs were identified. In this review we describe current knowledge about sRNAs and their regulatory properties and how this relates to the known regulatory systems of *L. pneumophila*. We also provide a model for sRNA-mediated control of gene expression that serves as a framework for understanding the regulation of virulence-related properties of *L. pneumophila*.

Keywords: CsrA, RsmY, RsmZ, 6S RNA, cyclic di-GMP, CRISPR

INTRODUCTION

Legionella pneumophila is the causative agent of Legionnaires' disease, an acute form of pneumonia (Fraser et al., 1977). It is a common, but often underestimated, cause of community-acquired and nosocomial pneumonia. The case-fatality rate of Legionellosis ranges between 10 and 40% and may approach 50% in nosocomial outbreaks, particularly among individuals with compromised health status (Benin et al., 2002). In Germany, where pneumonia causes are systematically investigated, *Legionella* is a leading cause of community-acquired pneumonia (von Baum et al., 2008).

Legionella pneumophila is commonly found in almost all natural and engineered water systems where it replicates in a variety of phagocytic protozoa, including *Hartmannella vermiformis*. Transmission mechanisms are still unclear, but a clear association was found between local watershed hydrology and Legionellosis risk in Toronto (Ng et al., 2008), which indicates that environmental factors are key players in transmission to humans. In people, infection is thought to occur by inhalation of contaminated water droplets.

Once in the lungs, *L. pneumophila* infects and replicates inside alveolar macrophages. To successfully infect and grow inside host cells, *L. pneumophila* circumvents normal endocytic trafficking pathways and inhibits phagosome acidification and fusion with lysosomes to establish a permissive replication niche called the *Legionella* containing vacuole (LCV) (Franco et al., 2009). The LCV is characterized by recruitment of early secretory vesicles, mitochondria, and membrane vesicles derived from the Golgi and endoplasmic reticulum (Roy and Tilney, 2002; Molofsky and Swanson, 2004; Shin and Roy, 2008). Central to the formation of the LCV and intracellular growth is the Icm/Dot type IVB secretion system, which translocates approximately 200 diverse effector proteins to the cytosol and LCV membrane (Segal and Shuman, 1998; Segal et al., 1998; Vogel et al., 1998; Cazalet et al., 2004; Chien et al., 2004; de Felipe et al., 2005; Burstein et al., 2009; Hubber and Roy, 2010; Faucher et al., 2011; Huang et al., 2011; Zhu et al., 2011).

Because *L. pneumophila* has evolved in a variety of niches, including aquatic environments, biofilms as well as within diverse hosts, different stress response pathways and virulence pathways must be correctly regulated. Although little is known about gene regulation in natural or engineered aquatic environments, several two-component systems are known to be involved in the regulation of stress response pathways and virulence factors required during host cells infection. These include PmrA/PmrB (Zusman et al., 2007), CpxR/CpxA (Altman and Segal, 2008) and LetA/LetS (Hammer et al., 2002). In addition, the sigma factor RpoS (σ^S) has been shown to regulate a number of known virulence factors including many Icm/Dot effectors (Hovel-Miner et al., 2009) and is required for intracellular multiplication in ameba and primary macrophages but not in macrophage-like cell lines, probably because of their reduced antimicrobial capacity (Hales and Shuman, 1999; Abu-Zant et al., 2006).

There is an increased awareness of the role of small regulatory RNAs (sRNAs) in the regulation of virulence factors and other processes in bacterial pathogens (Papenfort and Vogel, 2010). sRNAs are short (40–500 nt) RNA molecules that typically do not encode proteins and mainly perform regulatory functions. They can originate from either primary transcripts, meaning the sRNA is transcribed from its own promoter and its transcription stops at a Rho-independent terminator, or from the processing of larger transcripts. The vast majority of sRNAs are post-transcriptional regulators that can either inhibit or enhance mRNA translation of the target mRNAs (Waters and Storz, 2009). Other sRNAs regulate gene expression by binding to and interfering with regulatory proteins and have global effects on gene expression. Riboswitches and untranslated regions (UTR) are not sRNA *per se*, being an intrinsic part of the mRNA, but they are often found by the methodologies used to identify small RNA molecules.

Putative sRNA molecules expressed by *L. pneumophila* were identified by both a bioinformatic approach as well as by deep RNA-sequencing from growth in broth and inside *A. castellanii* (Faucher et al., 2010; Weissenmayer et al., 2011). In addition, a number of sRNAs have been implicated in the regulation of virulence factors of *L. pneumophila*, including the CsrB homologs RsmY and RsmZ (Rasis and Segal, 2009a; Sahr et al., 2009) and the RNA polymerase (RNAP) regulator 6S RNA (Faucher et al., 2010). This review aims to describe the current knowledge about sRNAs in general and provide a global perspective of the involvement of sRNA regulation systems in the behavior of *L. pneumophila*.

BASE-PAIRING sRNAs

The most common type of regulatory sRNA are base-pairing sRNAs. They are short, highly structured RNA molecules that are complementary to some degree to their target mRNAs and are therefore often called antisense sRNAs (Brantl, 2007). Base-pairing sRNAs can have a positive or a negative effect on expression of the target gene. Binding of the sRNA at or near the ribosomal binding site (RBS) prevents recognition by the ribosome and subsequent translation (**Figures 1B,C**). Alternatively, binding of the sRNA could change the secondary structure of the mRNA and free the RBS to permit translation initiation (**Figure 1C**). sRNA-binding to the mRNA can also induce its degradation by recruiting RNases (Waters and Storz, 2009). Base-pairing sRNAs can be encoded in cis or in trans.

CIS-ENCODED BASE-PAIRING sRNAs

Cis-encoded sRNAs are antisense RNA molecules encoded on the complementary strand of their target RNA gene (**Figure 1B**). Therefore, they share extensive sequence complementarity with the target mRNA but do not necessarily form long RNA duplexes (Brantl, 2007). Thirty-three sRNAs were recently identified in *L. pneumophila* that were at least partially complementary to genes encoding protein, some being known virulence factors (Weissenmayer et al., 2011; **Table 1**). Lpr0020 is encoded antisense to *lpg0644*, which encodes a homolog of RtxA involved in intracellular survival and modification of trafficking (Cirillo et al., 2001, 2002). Another sRNA, Lpr0050, is found antisense to the Icm/Dot effector SdeA (*lpg2157*; Bardill et al., 2005). Two sRNAs, Lpr0003 and Lpr0004, are antisense to the gene encoding the Icm/Dot effector LegA10, and are expressed during intracellular growth in *A. castellanii*.

Lpr0018 is encoded antisense to *comEC* (also known as *comA*, *lpg0626*) and would form a duplex with the 5′ end of the coding sequence and partially with a putative 5′UTR. ComEC is predicted to

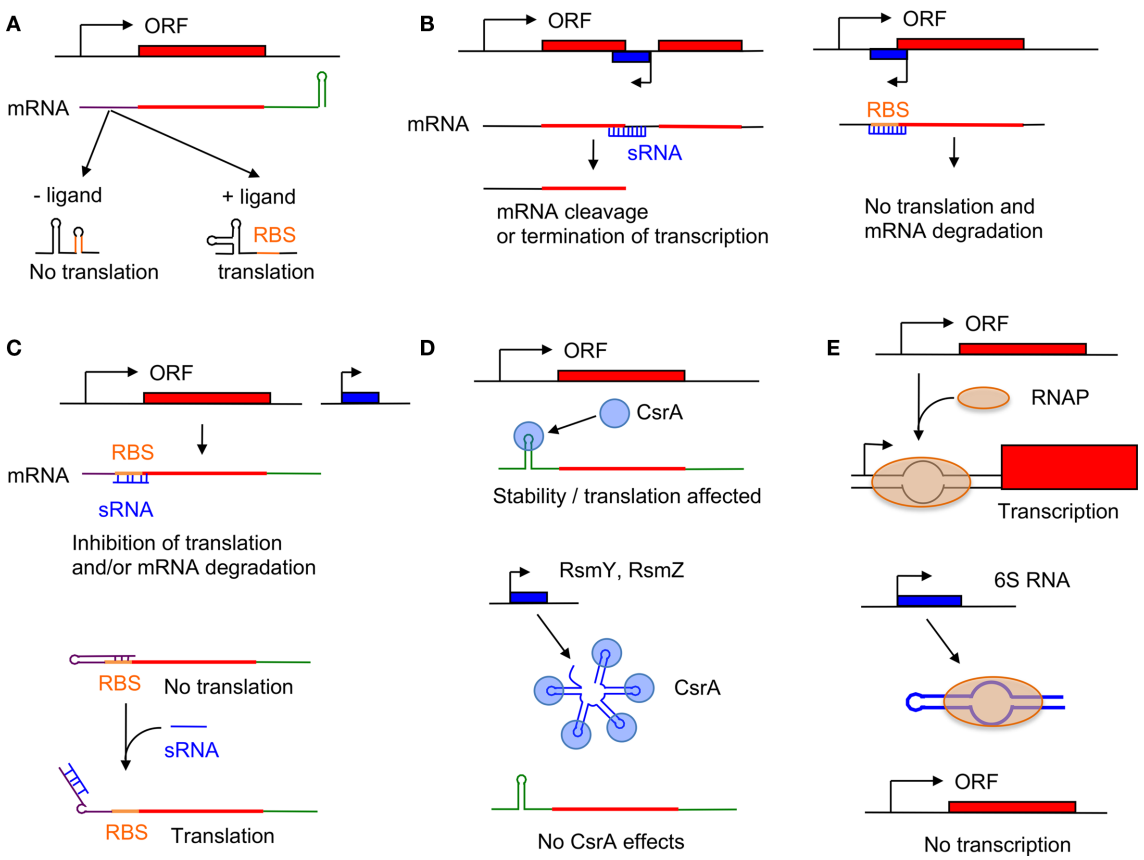

FIGURE 1 | Mode of action of sRNAs. (A) Riboswitch; **(B)** cis-encoded base-pairing sRNA; **(C)** trans-encoded base-pairing sRNA; **(D)** CsrA system; **(E)** 6S RNA. See text for details.

Table 1 | Small RNA molecules identified in *L. pneumophila*.

Name	5′ end[1]	3′ end[1]	Size (nt)	Regulator	Target[5]	Note	Reference
PROTEIN-BINDING sRNAs							
RsmY	7168	7059[2]	110[2]	LetA, RpoS	CsrA	Δ*rsmYZ* is defective for intracellular multiplication	Rasis and Segal (2009a), Sahr et al. (2009),
RsmZ	1892720	1892592	132	LetA, RpoS	CsrA		Hovel-Miner et al. (2009)
6S RNA	951819	951673, 951638	147, 182		RNAP	Required for optimal intracellular multiplication	Faucher et al. (2010)
6S2 RNA	81013	80800	213				Weissenmayer et al. (2011)
6S2 RNA antisense	80859	81037	178				Weissenmayer et al. (2011)
CIS-ENCODED BASE-PAIRING sRNAs							
lpr0002	33166	33516	350		lpg0027		Weissenmayer et al. (2011)
lpr0003	45316	45539	223		lpg0038		Weissenmayer et al. (2011)
lpr0004	45714	45904	190		lpg0038		Weissenmayer et al. (2011)
lpr0006	74969	74729	240		lpg0066		Weissenmayer et al. (2011)
lpr0008	262101	262297	196		lpg0228		Weissenmayer et al. (2011)
lpr0009	291705	291852	147		lpg0245		Weissenmayer et al. (2011)
lpr0012	369510	369457	53		lpg0320		Weissenmayer et al. (2011)
lpr0015	425773	425602	171		lpg0384		Weissenmayer et al. (2011)
lpr0016	532291	532155	136		lpg0494		Weissenmayer et al. (2011)
lpr0017	539616	539866	250		lpg0499, lpg0500		Weissenmayer et al. (2011)
lpr0018	662203	662439	236		lpg0626		Weissenmayer et al. (2011)
lpr0019	662451	663193	742		lpg0627, lpg0628		Weissenmayer et al. (2011)
lpr0020	686010	685864	146		lpg0644		Weissenmayer et al. (2011)
lpr0021	744637	744929	292		lpg0691		Weissenmayer et al. (2011)
lpr0025	825408	825265	143		lpg0754		Weissenmayer et al. (2011)
lpr0026	837669	837871	202		lpg0766		Weissenmayer et al. (2011)
lpr0028	871501	871409	92		lpg0796		Weissenmayer et al. (2011)
lpr0029	1046275	1046144	131		lpg0959		Weissenmayer et al. (2011)
lpr0031	1135290	1135190	100		lpg1035		Weissenmayer et al. (2011)
lpr0033	1330562	1330454	108		lpg1202		Weissenmayer et al. (2011)
lpr0036	1385926	1386008	82		lpg1259		Weissenmayer et al. (2011)
lpr0037	1425122	1425478	356		lpg1297		Weissenmayer et al. (2011)
lpr0043	2040383	2040499	116		lpg1821		Weissenmayer et al. (2011)
lpr0044	2122994	2122901	93		lpg1903		Weissenmayer et al. (2011)
lpr0049	2389440	2389117	323		lpg2142		Weissenmayer et al. (2011)
lpr0050	2418574	2418506	68		lpg2157		Weissenmayer et al. (2011)
lpr0053	2564155	2564056	99		lpg2261		Weissenmayer et al. (2011)
lpr0054	2574935	2574715	220		lpg2271		Weissenmayer et al. (2011)
lpr0058	2867524	2867415	109		lpg2535, lpg2536		Weissenmayer et al. (2011)
lpr0062	2948692	2948783	91		lpg2612		Weissenmayer et al. (2011)
lpr0065	3095189	3095277	88		lpg2744		Weissenmayer et al. (2011)
lpr0071	3351555	3351860	305		lpg2961		Weissenmayer et al. (2011)
lpr0072	3374990	3375092	102		lpg2981		Weissenmayer et al. (2011)
TRANS-ENCODED PUTATIVE BASE-PAIRING sRNAs							
LprA	2013775	2013510	265	RpoS, OxyR		Correspond to Lpr0041	Faucher et al. (2010), Weissenmayer et al. (2011)
LprB	2022555	2022672	117	OxyR			Faucher et al. (2010)
LprC	978559	978676	117				Faucher et al. (2010)
LprD	3321618	3321516	103				Faucher et al. (2010)
LprE	33394003	33393503	<50[3]				Faucher et al. (2010)
lpr0001	18080	18214	134			Functional[6]	Weissenmayer et al. (2011)
lpr0005	51416	51182	234				Weissenmayer et al. (2011)
lpr0007	262199	262033	166			Functional[6]	Weissenmayer et al. (2011)
lpr0010	341341	341434	93			Very unstable	Weissenmayer et al. (2011)

(Continued)

Table 1 | Continued

Name	5′ end[1]	3′ end[1]	Size (nt)	Regulator	Target	Note	Reference
lpr0011	360467	360391	76				Weissenmayer et al. (2011)
lpr0013	411825	412167	342				Weissenmayer et al. (2011)
lpr0014	413345	413495	150				Weissenmayer et al. (2011)
lpr0022	753291	753083	208				Weissenmayer et al. (2011)
lpr0023	753084	753379	295			Functional[6]	Weissenmayer et al. (2011)
lpr0024	816705	816590	115			Functional[6]	Weissenmayer et al. (2011)
lpr0027	861601	861363	238				Weissenmayer et al. (2011)
lpr0030	1102961	1103162	201				Weissenmayer et al. (2011)
lpr0032	1215340	1215182	158			Functional[6]	Weissenmayer et al. (2011)
lpr0034	1333886	1334233	347			Functional[6]	Weissenmayer et al. (2011)
lpr0035	1355695	1355444	251				Weissenmayer et al. (2011)
lpr0038	1444737	1444509	228				Weissenmayer et al. (2011)
lpr0039	1869948	1869698	250				Weissenmayer et al. (2011)
lpr0040	2003953	2003691	262				Weissenmayer et al. (2011)
lpr0042	2013722	2013773	51				Weissenmayer et al. (2011)
lpr0045	2233311	2233172	139			Functional[6]	Weissenmayer et al. (2011)
lpr0046	2317451	2317603	152				Weissenmayer et al. (2011)
lpr0047	2358694	2358599	95				Weissenmayer et al. (2011)
lpr0048	2360881	2360970	89			Functional[6]	Weissenmayer et al. (2011)
lpr0051	2432864	2432952	88				Weissenmayer et al. (2011)
lpr0052	2549075	2548822	253				Weissenmayer et al. (2011)
lpr0055	2769045	2768903	142				Weissenmayer et al. (2011)
lpr0056	2768934	2769061	127				Weissenmayer et al. (2011)
lpr0057	2862083	2862349	266				Weissenmayer et al. (2011)
lpr0059	2877260	2877374	114				Weissenmayer et al. (2011)
lpr0060	2921311	2921667	356			Functional[6]	Weissenmayer et al. (2011)
lpr0061	2921870	2921081	789				Weissenmayer et al. (2011)
lpr0063	2981537	2981411	126				Weissenmayer et al. (2011)
lpr0064	3068413	3068327	86				Weissenmayer et al. (2011)
lpr0066	3099914	3099986	72				Weissenmayer et al. (2011)
lpr0067	3284621	3284705	84				Weissenmayer et al. (2011)
lpr0068	3294905	3295056	151				Weissenmayer et al. (2011)
lpr0069	3303482	3303401	81				Weissenmayer et al. (2011)
lpr0070	3338909	3338797	112				Weissenmayer et al. (2011)
3′UTRs							
lpg0165 –3′UTR	195661[3]	195572[3]	89				Faucher et al. (2010)
infA-3′UTR	1976419[3]	1976584[3]	165				Faucher et al. (2010)
gltX-3′UTR	2132087[3]	2131915[3]	172				Faucher et al. (2010)
rpsU-3′UTR[4]	2663501[3]	2663567[3]	66				Faucher et al. (2010)
rpsU-3′UTR[4]	2663501[3]	2663681[3]	180				Faucher et al. (2010)
lpg2505 –3′UTR	2824648[3]	2824828[3]	180				Faucher et al. (2010)
OTHER							
tmRNA	172820	173374	554				Weissenmayer et al. (2011)
Rnase P	1944961	1944585	375				Weissenmayer et al. (2011)

[1]The position of the sRNA is given relative to L. pneumophila Philadelphia-1 genome.

[2]The 3′ end and the size given for RsmY are based on published results in L. pneumophila Paris. In L. pneumophila Philadelphia-1, the 3′ end of RsmY is at position 7090 for a size of 79 nt, as determined by 3′ RACE.

[3]Estimated based on genomic sequence and size on Northern Blot. For 3′UTR, the size given is the distance between the predicted Rho-independent terminator and the stop codon of the upstream gene. The size of all 3′UTR was higher than 500 nt as observed on northern blot.

[4]Two overlapping putative sRNAs were predicted in this region with distinc predicted terminators.

[5]For cis-encoded sRNAs, the target correspond to the gene on the complementary strand of the sRNA.

[6]Trans-encoded sRNAs identified by Weissenmayer et al. (2011) were predicted as functional if the predicted structure was found to be stable.

be part of the machinery involved in DNA uptake in *L. pneumophila*. Competence for natural transformation is induced by treatment that triggers stalling of the replication fork, such as UV irradiation and exposure to bicyclomycin (Charpentier et al., 2011). Some evidence previously suggested that sRNA could be involved in regulation of competence in *L. pneumophila*. First, deletion of the *rnr* gene, encoding RNase R, was found to induce competence and resulted in the accumulation of small RNA molecules originating from highly structured 16S rRNA and tmRNA (see below; Charpentier et al., 2008). Whether or not these two phenotypes are related requires clarification. Second, the *Escherichia coli* homolog of the *L. pneumophila* competence repressor ProQ (Sexton and Vogel, 2004) was found to work as a RNA chaperone to allow translation of *proP* mRNA, involved in the uptake of osmoprotectants (Chaulk et al., 2011). Taken together, these facts could lead one to hypothesize a regulatory model in which ProQ is essential to inhibit degradation, by RNase R, of the sRNA Lpr0018, which would mediate degradation of *comEC* mRNA, similar to the mechanism depicted in **Figure 1B**. Therefore, in the absence of ProQ or RNase R, *comEC* would be stabilized and efficiently translated. Alternatively, the sRNA Lpr0018 could stabilize *comEC* mRNA, allowing its transcription, while ProQ could act as a negative regulator of Lpr0018, potentially by targeting it for degradation. However, to our knowledge, such a mechanism has yet to be described for cis-encoded sRNA. Another sRNA, Lpr0019, is 742 nt long and is complementary to the 5′ end of *lpg0627* and to the 3′ end of *lpg0628*. Both genes are part of a predicted polycistronic RNA composed of *lpg0632–lpg0627* encoding subunits of the type IV pili, which was associated with competence (Stone and Kwaik, 1999). Lpr0019 could possibly be involved in induction of competence in a manner similar to what we suggested for Lpr0018. Of course, those hypotheses will need to be tested experimentally. Nonetheless, the finding that two sRNAs are encoded antisense to key players of DNA uptake by *L. pneumophila* strongly suggest that its induction is regulated at the post-transcriptional level. Recently, induction of competence in *Vibrio cholerae* was found to be dependent on the expression of a trans-encoded sRNA (TfoR), which allows translation of the positive regulator TfoX (Yamamoto et al., 2011).

Lpr0036 is encoded antisense to *lvrA* (*lpg1259*), the first gene of the *lvr/lvh* locus encoding a Type IVA secretion system, involved in conjugation (Segal et al., 1999). However, the role of LvrA is currently unknown and it is difficult at this point to speculate a possible role for this sRNA.

TRANS-ENCODED BASE-PAIRING sRNAs

In contrast to cis-encoded sRNA, trans-encoded base-pairing sRNAs are not physically linked to their mRNA target and the formation of RNA duplexes are mediated by short imperfect RNA interactions (**Figure 1C**). The function of many of the trans-encoded base-pairing sRNAs depends on the RNA-binding protein Hfq, which is thought to enhance the likelihood of a productive interaction between the sRNA and its target (Waters and Storz, 2009). This is in contrast to cis-encoded base-pairing sRNA that do not generally require the participation of a RNA chaperone (e.g., Hfq) to bind their target mRNA (Brantl, 2007).

In bacterial pathogens, deletion of the *hfq* gene often leads to a reduction in virulence, as was observed for *E. coli, Salmonella, Shigella, Yersinia,* and *Listeria* (Reviewed in Chao and Vogel, 2010). For example, in *E. coli,* Hfq was shown to regulate the locus of enterocytes effacement (LEE) encoding a type III secretion system (TTSS; Hansen and Kaper, 2009; Shakhnovich et al., 2009). In the intracellular pathogen *Salmonella enterica* serovar Typhimurium, Hfq is necessary for optimal growth in epithelial cells and macrophages (Sittka et al., 2007). *Burkholderia cenocepacia* encodes two Hfq homologs and both of them are required for optimal resistance to stress and virulence (Ramos et al., 2011). Deletion of the *hfq* gene of *Staphylococcus aureus* has no effect on metabolism but reduces virulence (Bohn et al., 2007; Liu et al., 2010). However, in *Neisseria gonorrhoeae*, deletion of *hfq* leads to only a weak reduction of virulence (Dietrich et al., 2009). Moreover, in some bacteria, Hfq is required for the function of some sRNAs but dispensable for others. For example, in *V. cholerae*, Hfq is required for the control of the quorum sensing systems by the sRNAs Qrr1–Qrr4, but dispensable for the repression of *ompA* by VrrA (Lenz et al., 2004; Song et al., 2008). It is noteworthy that *Helicobacter pylori* does not encode an Hfq homolog but still expresses hundreds of sRNAs (Sharma et al., 2010). This suggests that in some bacterial species, the function mediated by Hfq is not necessary for sRNA-mediated gene regulation or that an as yet unknown protein could carry out a similar function. Following genome-wide identification of Hfq-binding sRNAs, it was postulated that even in *E. coli*, some base-pairing sRNAs might not bind to, or use Hfq (Zhang et al., 2003). Careful review of the Hfq-related literature lead Jousselin et al. (2009) to postulate that the need for Hfq in mRNA–sRNA interaction is related to a number of factors. First, the higher the overall GC content of the bacterial genome the more likely Hfq is required and Hfq seems to be dispensable in bacteria whose genomes display a low GC value, such as *S. aureus* (32% GC). Second, Hfq is dispensable when the sRNA–mRNA interaction is mediated by long (>30) and uninterrupted pairing. Third, they observed a correlation between a requirement for Hfq and the C-terminal extension length of Hfq, which forms an mRNA interaction surface. Hfq proteins that have a short C-terminus tend to be found in bacteria in which Hfq is dispensable.

In *L. pneumophila*, deletion of the *hfq* gene affects the duration of the lag phase after inoculation in fresh broth (McNealy et al., 2005). Moreover, the *L. pneumophila hfq* mutant shows a reduced growth rate in chemically defined medium containing low concentrations of iron and a reduction in the expression of the ferric uptake regulator (*fur*). In *E. coli*, the RyhB sRNA negatively regulates expression of *fur* in a Hfq-dependant manner (Vecerek et al., 2007). In addition, the *L. pneumophila hfq* mutant shows a small reduction in intracellular growth (McNealy et al., 2005). The somewhat limited effect of deleting the *hfq* gene on *L. pneumophila* phenotypes suggests that Hfq is not critical for sRNA–mRNA interactions in this organism. The GC content of the *L. pneumophila* genome is low (38%) and alignment of its Hfq protein sequence with other homologs (**Figure 2**) reveals that the C-terminal region is short and comparable to the length of the *V. cholerae* Hfq that is not essential for all mRNA–sRNA interactions. According to the postulates of Jousselin et al. (2009), one could hypothesize that Hfq will not be required for all sRNA–mRNA interactions in *L. pneumophila*.

Nonetheless, one can speculate that in *L. pneumophila*, base-pairing sRNAs acting through Hfq may regulate iron acquisition, virulence-related functions and possibly other systems as well,

```
Eco         MAKGQSLQDPFLNALRRERVPVSIYLVNGIKLQGQIESFDQFVILLKN-TVSQMVYKHAI
Vch         MAKGQSLQDPFLNALRRERIPVSIYLVNGIKLQGQIESFDQFVILLKN-TVNQMVYKHAI
Lpn         MSKNHLLQDPFLNELRKEKVPVSVFLVNGIKLHGIIDSFDQYVVMLKN-SITQMVYKHAI
Sau         MIANENIQDKALENFKANQTEVTVFFLNGFQMKGVIEEYDKYVVSLNSQGKQHLIYKHAI
            *  .. :**  *: :: ::  *:::::**::::* *:.:*::*: *:.    :::*****

Eco         STVVPSRPVSHHSNNAGGGTSSNYHHGSSAQNTSAQQDSEETE
Vch         STVVPARPVSHHSGDRPASDRPAEKSEE---------------
Lpn         STVVPSRMVKIPAEESSGEEEGTVAD-----------------
Sau         STYTVETEGQASTESEE-------------------------
            ** .     .  : .
```

FIGURE 2 | Alignment of Hfq proteins from *E. coli* (Eco), *V. cholerae* (Vch), *L. pneumophila* (Lpn), and *S. aureus* (Sau) was performed with ClustalW2 (Chenna et al., 2003).

although Hfq function would not be essential for these. Expression profiling of a *hfq*-deficient *L. pneumophila* strain would shed light on the importance of Hfq on gene regulation and be of great help at identifying phenotypes that could be affected by it. A similar approach was used for other bacteria such as *E. coli* (Zhang et al., 2003), Typhimurium (Sittka et al., 2008), *B. cenocepacia* (Ramos et al., 2011), *Pseudomonas aeruginosa* (Sonnleitner et al., 2006), and *N. gonorrhoeae* (Dietrich et al., 2009). In addition, immunoprecipitation of Hfq with subsequent identification of bound sRNAs by enzymatic RNA-sequencing (Christiansen et al., 2006), tiling microarray (Zhang et al., 2003), or deep-sequencing (Sittka et al., 2008) would shed light on the mRNA species affected by Hfq and on the potential sRNAs whose functions are at least partially dependant on Hfq. Windbichler et al. (2008) have used an affinity chromatography procedure to identify RNA-binding proteins in *E. coli*. Briefly, they tagged a number of known sRNAs with a streptomycin-binding RNA aptamer, allowing them to bind to a streptomycin-coated column, which was then used to capture RNA-binding proteins from cellular extracts. They found that three proteins were consistently bound to a variety of sRNA sequences: Hfq, RNAP β-subunit and the small ribosomal subunit S1. Moreover, they showed that specific proteins could interact with a specific sRNA, depending on its sequence and secondary structure. Therefore, a hunt for sRNA-binding proteins is necessary to complete the sRNA-mediated regulatory landscape and to fully understand the extent of their impact on regulation of cellular functions.

In *L. pneumophila,* a number of trans-encoded base-pairing sRNA candidates have been identified but mechanistic studies are needed to evaluate their mode of action and to validate them as authentic base-pairing sRNAs (**Table 1**). Five intergenic RNAs were identified based on computer prediction by using the sRNA Predict software (Faucher et al., 2010). By searching for Rho-independent terminators in intergenic regions preceded by a sequence conserved in other *L. pneumophila* strains, 143 sRNA molecules were predicted. Using a custom-made microarray, the expression of 101 of these predicted sRNAs was monitored during growth in a variety of conditions. This two-step approach led to the identification of 12 sRNA molecules that were actively expressed, including 6S RNA, six 3′UTR, and five sRNAs that are independently transcribed (Faucher et al., 2010; **Table 1**). At this point the functions of the five identified sRNAs are unclear. Interestingly, expression of LprA during exponential growth is dependant on OxyR but dependant on RpoS during post-exponential phase (**Figure 3**). Since RpoS is an important regulator of virulence, it is tempting to speculate that LprA could be part of its regulatory cascade and plays a role in expression of virulence factors. Regardless of the growth phase, the presence of H_2O_2 induces its expression, which suggests that LprA responds to oxidative stress. This is similar to the *E. coli* sRNA OxyS, which is part of the oxidative stress response and reduces its mutagenic effects (Altuvia et al., 1997).

RNA-sequencing identified 38 sRNA molecules encoded in intergenic regions that could be considered as potential trans-encoded sRNAs (Weissenmayer et al., 2011; **Table 1**). Of these, nine were predicted to be functional based on the stability of their predicted secondary structures at 37°C. The predicted structure of one sRNA (Lpr0010) was less stable than 1000 randomly permutated sequences of the same length and base composition at 20 or 37°C, suggesting that it is under evolutionary pressure to form an unstable secondary structure. The biological relevance of this was not explored further, but one can hypothesize that the structure is only stable at low temperatures (less than 20°C) and that it could be part of a cellular response to low temperature. Interestingly five sRNA pairs were identified, for which two distinct sRNA are transcribed antisense to each other (Weissenmayer et al., 2011). In *E. coli*, the sRNAs RyeB and SraC are encoded opposite to each other and RyeB is completely complementary to the longer SraC segment (Vogel et al., 2003). The size of SraC is ≈270 nt, but when RyeB is present a shorter band (≈150 nt) is also detected. This reduction in size seems to be dependent on RNase III, suggesting that RyeB mediates degradation of SraC. For the sRNA pairs identified in *Legionella*, one sRNA can act as a negative regulator of the other, efficiently sequestering it by extended base-pairing and potentially targeting it for degradation. Moreover, mRNA can also regulates sRNAs. This mechanism, named trap-RNA, was described for the MicM sRNA that induces degradation of the YbfM porin mRNA. The *chb* polycistronic mRNA contain a sequence complementary to MicM and expression of the *chb* operon leads to MicM hybridization and degradation, resulting in stabilization of the *ybfM* mRNA (Figueroa-Bossi et al., 2009; Overgaard et al., 2009). Again, additional work is needed to understand the regulatory functions of *Legionella* trans-encoded base-pairing sRNA.

There are a number of base-pairing sRNAs encoded in other bacterial genomes that are known to affect virulence. A few examples are provided below that might be relevant in the context of

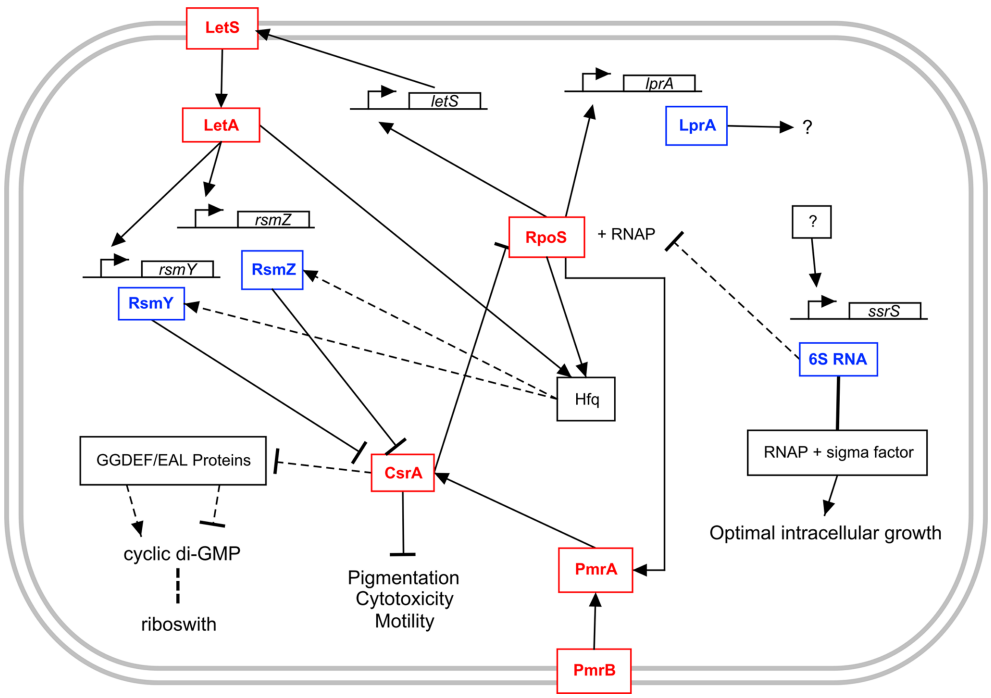

FIGURE 3 | Model of the regulatory networks involving sRNAs in *L. pneumophila*. The lines show interaction between the players: Arrow, activation; T bar, repression; dotted line, putative, or predicted interaction. See text for details.

L. pneumophila intracellular growth. One intracellular pathogen for which extensive identification and characterization of sRNA have been and are being performed is *Salmonella*. In this species, outer membrane protein (OMP) expression is regulated by a network of sRNAs. One of them, InvR, is encoded on the *Salmonella* pathogenicity island-1, acquired by horizontal gene transfer (HGT) and encoding the TTSS responsible for enterocyte invasion (Pfeiffer et al., 2007). Expression of this sRNA is dependant on HilD, a key regulator of TTSS expression. When the TTSS is expressed, InvR acts as a negative regulator of OmpD synthesis, one of the most abundant OMP in Typhimurium. Indirect evidence suggests that repression of OmpD could stabilize the membrane in the context of TTSS expression, allowing succesful translocation of bacterial effectors (Vogel, 2009). Therefore, InvR is thought to have helped establishment of the TTSS sequences after HGT by repressing expression of OMP that were incompatible with the virulence advantage provided by the TTSS (Vogel, 2009). Therefore, it is tempting to speculate that similar mechanisms exist in *L. pneumophila* to repress OMPs during expression of the Icm/Dot system, the Type IVA secretion system (*lvr/lvh*) or the Tra conjugative system. However, to date, no trans-encoded sRNAs have been identified in the vicinity of these systems, but, as described above, one cis-encoded sRNA is antisense to *lvrA* (*lpg1259*).

The sRNA VrrA of *V. cholerae* is part of the membrane stress response pathway mediated by σ^E and targets *ompA* mRNA, presumably to limit synthesis of OMPs (Song et al., 2008). Deletion of *vrrA* leads to an increase in the synthesis of outer membrane vesicles that are known to be involved in delivery of virulence factors to host cells (Mashburn-Warren and Whiteley, 2006). Moreover, VrrA seems to negatively regulate expression of the adhesion molecule Tcp and therefore affects intestinal colonization (Song et al., 2008). There is structural similarity between VrrA and LprD of *L. pneumophila* and it is tempting to speculate a role for LprD in the regulation of OMP synthesis. However, structure comparisons of trans-encoded sRNAs have been of limited help for predicting function or targets, and an experimental strategy should be taken to determine if LprD regulates OMP synthesis.

The quorum system of *V. cholerae* comprises four redundant sRNAs named Qrr1–Qrr4 and two signaling molecules, the furanosyl borate diester (AI-2) and the α-hydroxyketone Cqs (Lenz et al., 2004). At low cell density, the system positively regulates expression of Qrr1–Qrr4, which destabilize the mRNA of *hapR*, a negative regulator of virulence. Therefore, at low cell density, *hapR* is degraded allowing expression of virulence traits. *L. pneumophila* also possesses a putative quorum system, based solely on the presence of the α-hydroxyketone Lqs and the LqsR/LqsS two-components system (TCS) (Tiaden et al., 2007; Spirig et al., 2008). Beside the absence of AI-2 signaling in *L. pneumophila* the quorum system architecture of *L. pneumophila* and *V. cholerae* are quite similar (Tiaden et al., 2010). However, in *L. pneumophila* no sRNA has been implicated in this regulatory system as yet. Following RNA-sequencing, two sRNAs (Lpr0001, and Lpr0069) were found to have substantial homology both at the sequence and the secondary structure levels, which is reminiscent of the Qrr1–Qrr4 sRNAs (Weissenmayer et al., 2011). A search for homologous sequences throughout the genome revealed 20 more copies of these sRNAs, one (Lpr0049) being partially antisense to *lpg2142*, which encodes a putative ORF. The consensus structure of these sRNAs is a long stem–loop with

two central bulges comprised of ~25 nt and two small hairpins extruding from either side of the central stem 20 nt before the loop (Weissenmayer et al., 2011). Many of these sequences were found in other *Legionella* strains as well, often in the same configuration, which indicates that they are evolutionarily conserved and likely to play a beneficial role. Moreover, both the Lqs system and the homologous sRNA sequences are absent in *L. longbeachae*. These observations are only suggestive and experimental evidence is needed to link the Lqs quorum sensing system with this group of homologous sRNA sequences. It is noteworthy that deletion of all four Qrr sRNAs was needed to see a phenotype on the quorum sensing system (Lenz et al., 2004). Since only Lpr0001 and Lpr0069 seem to be expressed at good level, it might be informative to generate a double *lpr0001/lpr0069* mutant and monitor its effect on a population density-related phenotype.

Although the vast majority of base-pairing sRNAs do not encode proteins, there are at least two examples where they do. In *E. coli* the *sgrS* gene encodes a sRNA, SgrS, and a small protein, SgrT, that together regulate glucose uptake by different strategies (Wadler and Vanderpool, 2007). In *S. aureus*, the sRNA RNA III targets virulence factors and functions as a key regulator of virulence, but also encodes a 26 amino acid long hemolysin (Boisset et al., 2007). Therefore, one should keep in mind that sRNAs are not necessarily non-coding. We recently identified two small RNA molecules, LstA and LstB that are predicted to encode small proteins with transmembrane motifs (Faucher et al., 2010). Because small proteins are difficult to predict accurately from genomic sequences, the hunt for small RNA molecules also has the potential benefit of filling the gaps of genomic annotation by also identifying putative small proteins and correcting errors in genome annotation.

THE CsrA/CsrB SYSTEM

The CsrA protein was first identified in *E. coli* as a regulator of glycogen biosynthesis (Romeo et al., 1993). CsrA binds to GGA motifs in the 5′UTR of target mRNAs and affects their stability and/or their translation (Romeo, 1998). The sRNAs CsrB and CsrC contain many GGA motifs and can therefore bind multiple CsrA proteins resulting in titration/sequestration of CsrA, thus relieving CsrA effects on the expression of its target mRNAs (**Figure 1D**). Transcription of CsrB and CsrC is regulated by the BarA/UvrY TCS. Both sRNAs are degraded by a pathway involving RNase E and CsrD, a cyclic di-GMP binding protein (Suzuki et al., 2006).

Legionella pneumophila contains four CsrA homologs, of which one (*lpg0781*) was identified as able to complement a *csrA* deletion in *E. coli* (Fettes et al., 2001). The roles of the other CsrA homologs are currently unknown. In *L. pneumophila,* CsrA is responsible for the repression of post-exponential traits during exponential growth, including pigmentation, motility, and cell shortening (Fettes et al., 2001; Molofsky and Swanson, 2003; Forsbach-Birk et al., 2004). Moreover, CsrA is required for intracellular growth in both mammalian macrophages and *A. castellanii* (Molofsky and Swanson, 2003; Forsbach-Birk et al., 2004). Recently, it was shown that CsrA directly repressed the expression of *ylfA/legC7, ylfB/legC2,* and *vipA*, which encode Icm/Dot effectors (Rasis and Segal, 2009b). Regulation of CsrA expression seems to be dependant on PmrA, another well-known virulence regulator (Rasis and Segal, 2009b).

The *L. pneumophila* genome encodes homologs of the BarA/UvrY TCS named LetA/LetS. This system was first identified as a positive regulator of flagellin expression (Hammer et al., 2002). Although a *letA* mutant still replicates in mammalian macrophages, it is defective for replication in *A. castellanii* (Gal-Mor and Segal, 2003; Lynch et al., 2003). Subsequently, LetA was shown to regulate expression of a number of virulence factors, including Mip, IcmR, IcmT, DotA, and the Icm/Dot effector RalF (Gal-Mor and Segal, 2003; Shi et al., 2006).

Based on these results, the consensus model is that during exponential phase, CsrA represses expression of post-exponential phase genes, either by inhibiting mRNA translation, or by modulating their stability. During post-exponential phase, the LetA/LetS TCS, supposedly by inducing expression of CsrB homologs, inhibits the activity of CsrA, allowing expression of post-exponential traits (pigmentation, cytotoxicity, and motility). Computer predictions of CsrB homologs in several bacterial species identified two candidate CsrB homologs in *L. pneumophila*, based on the identification of intergenic regions enriched for the GGA motif (Kulkarni et al., 2006). These two sRNAs were named RsmY and RsmZ (**Table 1**), based on their short size, which more closely resemble the sRNAs involved in the RsmA (CsrA) system of *P. aeruginosa* (Lapouge et al., 2008). It was shown that: (i) LetA specifically binds upstream of *rsmY* and *rsmZ* and that the LetA/LetS TCS controls their expression; (ii) expression of *rsmY* and *rsmZ* in *E. coli* results in a similar phenotype as over-expression of *csrB* and *csrC* and; (iii) RsmY and RsmZ bind CsrA, confirming that RsmY and RsmZ are the missing link in the LetA/S–CsrA regulatory pathway (Hovel-Miner et al., 2009; Rasis and Segal, 2009b; Sahr et al., 2009; **Figure 1E**). Deletion of either *rsmY* or *rsmZ* has little impact on virulence, but deletion of both strongly impaired replication in both mammalian macrophages and *A. castellanii* (Sahr et al., 2009). It was also shown that increased expression of *rsmY* and *rsmZ* during post-exponential phase requires RpoS, probably due to the regulation of *letS* expression by RpoS (Hovel-Miner et al., 2009; Rasis and Segal, 2009b). Reduced expression of CsrA leads to an increase in *rpoS* expression, which suggests the existence of a positive feedback loop (Forsbach-Birk et al., 2004). However, deletion of *rsmYZ*, which should mimic over-expression of CsrA, also resulted in increased expression of *rpoS* (Sahr et al., 2009). Therefore, the interplay between LetS, RsmYZ, CsrA, and RpoS remains unclear and will require further investigation (**Figure 3**).

Interestingly, RpoS and LetA, two major regulators of virulence-related traits in *L. pneumophila* positively regulate expression of *hfq* during exponential growth (McNealy et al., 2005). Whether or not Hfq, in turn, affects RsmY and RsmZ function or stability is currently unknown (**Figure 3**). In *P. aeruginosa*, Hfq binds to and affects the stability of RsmY (Sonnleitner et al., 2008). Also, the LqsR/LqsS TCS is regulated by the CsrA system, which is similar to what was shown for *V. cholerae* (Lenz et al., 2005; Tiaden et al., 2007; Sahr et al., 2009).

Microarray studies revealed that no genes were significantly affected by the deletion of either *letA*, *letS,* or *rsmYZ* during exponential growth in rich broth, in agreement with the current working model in which CsrA is active during exponential phase and that the LetA/LetS/RsmYZ part of the regulatory cascade is silent (Sahr et al., 2009). However, during the post-exponential phase of

growth, many genes were negatively affected by deletion of either *letA* or *letS* or both *rsmYZ*, including a number of Icm/Dot effectors (RalF, SidC, SdeA, SdeC, SidF, and SdhB) (Sahr et al., 2009). Independently, it was shown that RsmY and RsmZ relieve the CsrA-mediated repression of the expression of *ylfA/legC7*, *ylfB/legC2,* and *vipA* (Rasis and Segal, 2009b). However, expression of flagellar genes was largely RsmYZ independent but negatively affected by deletion of either *letA* or *letS* (Sahr et al., 2009). However, since CsrA affects mRNA translation, over-expression of RsmY and RsmZ could result in a stronger phenotype at the protein level. Interestingly several genes positively affected by LetA/S and RsmYZ were predicted to encode GGDEF and/or EAL domains, including *lpg0156* (*cdgS4*) and *lpg2132* (*cdgs20*) (Sahr et al., 2009; Levi et al., 2010), suggesting that there may be crosstalk between the CsrA system and the cyclic di-GMP system (**Figure 3**) as it was shown in *E. coli* (Jonas et al., 2008). Interestingly, wild-type bacteria that over-express *cdgs20* are defective for intracellular multiplication (Levi et al., 2010).

THE RNA POLYMERASE/6S RNA SYSTEM

The 6S RNA of *E. coli* was first identified and sequenced 40 years ago (Hindley, 1967; Brownlee, 1971). However, its function remained elusive until the year 2000 when Wassarman and Storz (2000) showed that 6S RNA binds to the σ^{70} and the β/β′ subunits of RNAP and inhibits transcription of the *rsd* gene from its σ^{70}-dependant promoter. Later, it was shown that, in laboratory *E. coli* strains, deletion of the 6S RNA gene, *ssrS,* renders cells more resistant to high pH and less able to compete against wild-type bacteria for survival in deep stationary phase (Trotochaud and Wassarman, 2004, 2006).

In bacteria, functional RNAP holoenzyme consists of the core subunits $\beta/\beta'\alpha_2\omega$, which associate with a σ subunit that provides promoter specificity. In *E. coli*, the σ^{70}-RNAP holoenzyme ($E\sigma^{70}$) is responsible for bulk transcription during exponential phase. During stationary phase, the σ^S subunit preferentially associates with the $\beta/\beta'\alpha_2\omega$ subunits of RNAP to allow transcription of stationary phase genes. The general consensus for the role of 6S RNA's regulatory effect is based on its preferential binding to $E\sigma^{70}$, compared to $E\sigma^S$, and the observation that binding of 6S RNA to $E\sigma^{70}$ inhibits its binding to DNA promoters (**Figure 1E**). Thus, in the presence of 6S RNA, $E\sigma^{70}$ is sequestered, promoting the formation of other holoenzymes, such as $E\sigma^S$, that are able to activate transcription from their specific promoters (Wassarman, 2007). Later, it was shown that σ^{70}-dependant promoters negatively affected by the presence of 6S RNA contained a weak -35 element and an extended -10 element (Cavanagh et al., 2008). Thus, 6S RNA may function as a competitor for the binding of $E\sigma^{70}$ to a specific subset of promoters.

Following bioinformatic prediction of sRNAs in *L. pneumophila*, one sRNA showed very high expression during the post-exponential phase of growth, similar to *E. coli* 6S RNA (Wassarman and Storz, 2000). Its predicted structure was highly similar to the published consensus structure of the widely distributed 6S RNA (Barrick et al., 2005; Trotochaud and Wassarman, 2005). All the previously identified conserved features of 6S RNA homologs were present in the *L. pneumophila* 6S RNA candidate, including: (i) a 22-nt closing stem with two small bulges; (ii) a central bulge composed of 14 nt on the 5′ strand and 13 nt on the 3′ strand of low %GC content; (iii) two G–C base pairs surrounding the central bulge; and (iv) a terminal loop comprising four small bulges resembling the consensus terminal loop of the γ-proteobacteria lineage of 6S RNAs. Co-immunoprecipitation studies revealed that the *L. pneumophila* 6S RNA candidate physically associate with RNAP (Faucher et al., 2010). Therefore, the gene encoding this sRNA was named *ssrS* in accordance with the published nomenclature recommendations (Barrick et al., 2005).

Deletion of the *ssrS* gene reduced intracellular growth in human macrophages and in *A. castellanii* by 10-fold despite no difference in Icm/Dot translocation activity or cytotoxicity (Faucher et al., 2010). Also, the 6S RNA deficient strain was unable to compete against the wild-type strain during intracellular growth but grew equally well in AYE broth. Thus, it seems that in *L. pneumophila* 6S RNA is important for optimal expression of genes related to intracellular growth (**Figure 3**). In order to further dissect the effects of 6S RNA on gene expression, microarray analysis was used to monitor global gene expression patterns during the post-exponential phase of growth, when the 6S RNA is most abundant. When the *ssrS* deletion mutant strain was compared to the wild-type it was observed that *L. pneumophila* 6S RNA negatively affects expression of six genes and promotes transcription of 127 genes during post-exponential phase of growth, including those encoding: a subset of Icm/Dot effectors (VipA, LegC5, SdeC, SdbC), small molecule transporters, DNA repair enzymes as well as genes involved in fatty acid metabolism, amino acid metabolism, and carbohydrate metabolism. This was somewhat in contradiction with the consensus understanding of 6S RNA being mainly an inhibitor of transcription from σ^{70}-dependant promoters. However, a recent study revealed that 6S RNA is also an activator of transcription in *E. coli*, where it negatively affects transcription of 148 genes and positively affects expression of 125 genes (Neusser et al., 2010). In this study, genes affected by 6S RNA contain promoters that are specific for a variety of σ subunits, including σ^S, σ^{32}, and σ^{54}. Accordingly, 6S RNA seems to bind also to $E\sigma^S$, although with much less affinity than for $E\sigma^{70}$ (Gildehaus et al., 2007). Therefore, it seems that 6S RNA regulation is not as clear-cut as first conceived and these results suggest that many variations on a common theme may exist in different bacterial species. Factors that could influence 6S RNA regulation in *L. pneumophila* include distinctive usage of the different σ subunits, strength of the promoters present in the genome and overall regulatory organization.

In *E. coli*, RNAP can use 6S RNA as a template to generate 14–24 nt long *de novo* RNA molecules, named pRNA, originating from the central bulge on the 5′ strand (Wassarman and Saecker, 2006; Gildehaus et al., 2007). However, transcription from 6S RNA only occurs after a sudden increase in the NTP pool, for example when bacteria in post-exponential phase are diluted with fresh medium. Transcription from 6S RNA leads to the dissociation of 6S RNA from $E\sigma^{70}$, which is then free to transcribe genes again. This also causes destabilization of 6S RNA, due to increased access of nucleases to unbound 6S RNA or recognition of the 6S RNA–pRNA duplex by RNases (Wassarman and Saecker, 2006). Therefore, synthesis of pRNA seems to be a way to "reset" this regulatory system. Synthesis of pRNA probably also occurs in other bacteria as well, including *L. pneumophila*, but at present direct evidence for this is lacking.

Some bacterial species contain two or more 6S RNA homologs, such as *Bacillus subtilis* and *Clostridium* (Barrick et al., 2005; Trotochaud and Wassarman, 2005). A second 6S RNA homolog, named 6S2 RNA, was recently identified in the *L. pneumophila* genome (Weissenmayer et al., 2011). Surprisingly, the authors could detect transcription from the opposite strand encoding 6S2 RNA and suggest that its expression is regulated by a cis-acting sRNA. The 6S2 RNA is expressed in E and PE phase at a similar level, but the antisense transcript is only expressed in E phase, which could inhibit 6S2 function during E phase and therefore effectively result in functional 6S2 RNA expression only during PE phase. That would result in a situation similar to the 6S RNA of *E. coli* and the 6S RNA of *L. pneumophila* that are only highly expressed in PE phase. The role of 6S2 RNA is currently unknown and it would be interesting to investigate the phenotype of a mutant defective in both 6S RNA and 6S2 RNA.

THE CRISPR IMMUNITY SYSTEM

The CRISPR loci encode a sRNA-based immunity system against viruses and other invading DNA (Horvath and Barrangou, 2010). It consists of a leader sequence followed by several non-contiguous direct repeats separated by pieces of variable sequences called spacers. The spacer is a sequence of DNA (21–72 bp) originating from invading viral or plasmid DNA that has been integrated in the bacterial genome. Following transcription of the CRISPR loci, the multi-repeat, multi-spacer RNA is processed by CRISPR-associated protein (Cas) into small units consisting of a spacer flanked by two partial repeats, called crRNA. Those crRNA provide specificity to the system by guiding the Cas interference machinery to the invading nucleic acids that match its sequence. Therefore, the spacers are remnants of past viral infections or plasmid invasions and can be viewed as a form of acquired immunity. New spacers can be added at the leader end of the CRISPR loci.

In *L. pneumophila*, CRISPR loci have been identified in the Lens, Alcoy, and Paris strains, but not in Philadelphia-1 (D'Auria et al., 2010). The Lens strain possess two CRISPR loci, one on the chromosome, the other on a plasmid. The Alcoy and Lens CRISPR systems are almost identical, composed of three Cas genes (*cas1*, *cas3*, and *csy4*) and 55 or 52 repeats, respectively, of 27 bp with one bp difference between the two strains. The Paris locus are not related to the Alcoy/Lens loci and is composed of *cas1, cas2,* and *cas4* and contains 34 repeats of 37 bp. BLAST analysis of the spacer sequences did not identify any homologous sequences in the GenBank database. It is noteworthy that four bacteriophages of *L. pneumophila* have been identified from environmental water samples, but their sequences are unknown (Lammertyn et al., 2008). There is currently no evidence of any implication of the CRISPR system in regulation of virulence-related traits in *L. pneumophila*. However, in *P. aeruginosa*, the CRISPR system is needed for bacteriophage-mediated inhibition of biofilm formation and swarming motility following lysogenic infection with bacteriophage DMS3 (Zegans et al., 2009). This suggests that the combination of lysogenic infection and the presence of an active CRISPR system may have an impact on the regulation of group behavior traits. Whether or not this is relevant in the context of host infection by bacterial pathogens still needs to be determined.

RELIEF OF STALLED RIBOSOMES BY tmRNA

Stalling of ribosomes on a mRNA occurs when the translation machinery reaches the end of of the transcript without encountering a stop codon. This is a consequence of co-transcriptional translation that occurs in bacteria and the translation of mRNA that are being degraded from the 3′ end. Stalling of the ribosome prevents its release from the mRNA and can cause decay of the active ribosome pool. Moreover, generation of incomplete proteins can be toxic to the cells. Therefore, a system is needed to release the ribosome and target the incomplete protein for degradation. This function is performed by the tmRNA that is universally conserved in the bacterial kingdom (reviewed in Keiler, 2007; **Table 1**). The name tmRNA comes from the two functions performed by this sRNA. It acts as a tRNA and is charged with alanine and it acts as an mRNA, encoding a short peptide tag, which targets a protein for degradation. The current model of tmRNA-mediated rescue of stalled ribosome includes two proteins: SmpB and EF-Tu. A complex formed from alanyl–tmRNA–SmpB–EF-Tu enters the A-site of the stalled ribosome. The nascent protein is transferred to the alanyl–tmRNA. The complex then moves to the P-site and the ribosome translates the short peptide tag encoded on the tmRNA, resulting in tagging of the protein and release of the mRNA. Deletion of tmRNA usually results in strong phenotypes such as a marked reduction in growth rate and lethality (Keiler, 2007). In the intracellular pathogen *Salmonella*, deletion of tmRNA or the *smpB* gene results in severe reduction in survival capacity and pathogenesis in mouse macrophages (Julio et al., 2000; Ansong et al., 2009). The effect of the deletion of tmRNA in *L. pneumophila* is currently unknown but SmpB may be essential for axenic growth since a *smpB* deletion mutant could not be constructed (Charpentier et al., 2008).

A NOTE ABOUT 5′ AND 3′ UNTRANSLATED REGIONS OF mRNA

In addition to their coding sequences, mRNAs have two distinct regions that can perform regulatory functions: the 5′UTR and the 3′UTR (Gripenland et al., 2010). Both regions can vary greatly in length from only a few, to several hundred bases. Some 5′UTR can adopt different structural states depending on conditions inside cells, including temperature (e.g., thermosensor), pH, and the presence of specific metabolites (**Figure 1A**). Such 5′UTR are called riboswitches. One of the best-known riboswitches regulates transcription of the *prfA* gene, a major virulence regulator of *Listeria monocytogenes*. At low temperatures, the *prfA* 5′UTR adopts a structural state that masks the RBS and thus prevents translation. In contrast, at 37°C, the 5′UTR structure changes, exposing the RBS and allowing translation of the PrfA protein and expression of virulence determinants (Johansson et al., 2002). No riboswitches have been identified in *L. pneumophila* as yet. However, temperature is known to affect biofilm formation by *L. pneumophila* (Piao et al., 2006). Moreover, optimal growth at high and low temperature requires specific stress response proteins: ClpP and RNase R respectively (Charpentier et al., 2008; Li et al., 2010). Therefore, one may speculate that RNA thermosensors could be involved in *L. pneumophila* gene regulation to promote growth at extreme temperatures and to form biofilms.

The small nucleotide cyclic di-GMP regulates many biological processes in bacteria, including biofilm formation, motility, and virulence (Hengge, 2009). Cyclic di-GMP is produced from

two guanosine-5′-triphosphate molecules by diguanylate cyclases (DGC, containing a GGDEF domain) and degraded selectively by phosphosdiesterases containing either EAL or HD-GYP domains (Hengge, 2009). Therefore, the quantities and activities of DGC and EAL/HD-GYP enzymes determine the net intracellular concentration of cyclic di-GMP, which may be an integration point for many different signals. Consequently, the mechanism(s) of gene regulation by cyclic di-GMP has been the subject of intense investigation. A new riboswitch class that regulates gene expression by binding to the second messenger cyclic di-GMP was described and found in many different bacterial species (Sudarsan et al., 2008). Recently, our lab provided evidence that the cyclic di-GMP signaling pathway of *L. pneumophila* is involved in the regulation of intracellular growth and flagellin synthesis (Levi et al., 2010). Given the large number of DGC and EAL/HD-GYP enzymes present in *L. pneumophila* genome, it is tempting to speculate that an, as yet, unidentified riboswitch may play a role in cyclic di-GMP regulatory pathways in *L. pneumophila*. However, no riboswitch has been identified in *L. pneumophila* as yet and it would therefore be interesting to performed a systematic search to identify possible candidate.

In eukaryotes, 3′UTRs are important for the control of translation (Sonenberg and Hinnebusch, 2009). The importance of 3′UTR for bacterial gene regulation is currently unclear but probably underestimated. Long overlapping 3′UTRs were identified in *L. monocytogenes* and in *B. subtilis* (Rasmussen et al., 2009; Toledo-Arana et al., 2009). Such 3′UTRs could affect the stability of convergent genes by a mechanism similar to cis-encoded base-pairing sRNAs (see below). Whole-genome tiling array experiments were used to find transcriptionally active regions in *B. subtilis,* which identified a group of genes with long (~200 nt) homologous 3′UTR (Rasmussen et al., 2009). Structure predictions revealed that those 3′UTR fold into a highly stable Y-shaped double-stranded structure ending with a very short single-stranded tail. The author suggested that such structures could target the mRNA to a location in the cells were the protein is needed (i.e., the membrane) or prevent access of RNAses to the 3′ end of the transcript. Stable structures at the 3′ end of mRNAs block the activities of most 3′-exoribonucleases. RNase R is able to degrade double-stranded RNA molecules but needs a single-stranded tail of at least 10 nt (Vincent and Deutscher, 2006). In *L. pneumophila,* six actively transcribed 3′UTRs were identified (**Table 1**), ranging from 66 to 180 bases (Faucher et al., 2010). Whether or not they are involved in gene regulation requires clarification. Interestingly, the predicted structure of *gltX-3′UTR* is similar to the Y-shape structure reported in *B. subtilis* homologous 3′UTRs.

THE NEXT STEP: TARGET IDENTIFICATION AND CHARACTERIZATION OF *L. PNEUMOPHILA* sRNAs

Now that a number of actively transcribed sRNAs have been identified in *L. pneumophila,* further research should focus on the determination of their functions and their specific targets. First of all, it is important to define what a true target is. Essentially, a true target is a mRNA or a protein that physically interacts with the sRNA and whose function, stability or translation is affected by this interaction (Vogel and Wagner, 2007). The inferred targets of cis-encoded base-pairing sRNAs are obvious, they are the mRNA encoded on the complementary strand. However, even in this case, molecular evidence is needed to establish the link between the two molecules and the effect of the sRNA on the target mRNA. For trans-encoded base-pairing sRNAs, there are *a priori* no indications of what the target might be. As a start, it could be useful to use a bioinformatic approach to generate a list of putative targets that can then be tested experimentally. Target prediction usually relies on the estimation of optimal hybridization scores between sRNA and mRNA targets and often includes the effects of stable secondary structures. Many web servers are available for genome-wide prediction of mRNA targets, including, but not limited to sRNATarget (Cao et al., 2009) and TargetRNA (Tjaden et al., 2006).

Target prediction could also be used in conjunction with experimental genome-wide approaches such as transcriptional profiling. Comparison of the transcription profile of a mutant strain or an over-expresser strain to the wild-type strain can highlight putative targets (Papenfort et al., 2008). One has to keep in mind that any observed effects on transcript expression could be indirect, when, for example, a transcriptional regulator is the true target. Since the effect of some sRNAs can only be seen at the protein level, the effect of a sRNA is not necessarily observable at the steady-state RNA level. Comparison at the proteome level, by 2D gel analysis, could be more informative, but because of detection limitation, poorly expressed proteins are usually missed. Comparison of the sRNA deletion mutant, the over-expresser strain and the wild-type strain by SDS-PAGE and Coomassie staining may be sufficient to suggest a putative target. Then, a protein of interest can be identified by mass spectrometry analysis. The target of the GlmY sRNA, a polycistronic mRNA encoding *glmUS*, was identified with this strategy (Urban et al., 2007).

A more direct approach to find the mRNA target of trans-encoded sRNA is to use the sRNA as a bait to fish out the target. In the case of a sRNA that interact with Hfq, the sRNA–Hfq complex can be preloaded into an affinity purification column and incubated with extracted mRNA. After washing, the eluted mRNA are converted to cDNA and identified by sequencing or by microarray analysis. Such method was used to identify the target of the *E. coli* RydC sRNA, an ATP-binding cassette permease (Antal et al., 2005). Alternatively, a sRNA could be tagged with biotin, bound to streptavidin–coated magnetic beads and incubated with extracted mRNA. Identification of the captured mRNA could be performed as explained above. This method has been used to identify two targets, *ompA* and *ompC* mRNA, of the RseX sRNA of *E. coli* (Douchin et al., 2006).

The identification of protein targets of protein-binding sRNAs is somewhat similar to what was described above for mRNA-binding sRNAs. However, in this case, secondary structures are often very well conserved, which is illustrated by 6S RNA and the CsrB homologs, and therefore structure predictions could serve as a guide. Then proteomic studies could be undertaken or more direct approaches, such as the streptavidin-binding aptamer tag described above could be used (Windbichler et al., 2008). Said et al. (2009) have performed a systematic analysis of the use of different aptamers and configurations to identify protein targets of sRNA.

CONCLUDING REMARKS

Increasing evidence points to important and broad implications of sRNAs in the regulation of life cycles, stress responses and virulence properties of several pathogenic bacteria (Papenfort and Vogel, 2010). This is evident in *L. pneumophila* where three sRNAs, 6S RNA, RsmY, and RsmZ, are already known as major determinants of virulence regulation. However, this is probably only the tip of the iceberg and it is likely that other sRNAs are involved in regulation of virulence and other traits such as biofilm formation and the responses to environmental stresses. In the near future, important goals for characterizing the specific roles of sRNA in *L. pneumophila* biology are the identification of sRNA targets and determining the phenotypes of mutant that are defective in the production of individual and multiple sRNA species.

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Legionella pneumophila transcriptome during intracellular multiplication in human macrophages

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Legionella pneumophila is the causative agent of Legionnaires' disease, an acute pulmonary infection. *L. pneumophila* is able to infect and multiply in both phagocytic protozoa, such as *Acanthamoeba castellanii*, and mammalian professional phagocytes. The best-known *L. pneumophila* virulence determinant is the Icm/Dot type IVB secretion system, which is used to translocate more than 150 effector proteins into host cells. While the transcriptional response of *Legionella* to the intracellular environment of *A. castellanii* has been investigated, much less is known about the *Legionella* transcriptional response inside human macrophages. In this study, the transcriptome of *L. pneumophila* was monitored during exponential and post-exponential phase in rich AYE broth as well as during infection of human cultured macrophages. This was accomplished with microarrays and an RNA amplification procedure called selective capture of transcribed sequences to detect small amounts of mRNA from low numbers of intracellular bacteria. Among the genes induced intracellularly are those involved in amino acid biosynthetic pathways leading to L-arginine, L-histidine, and L-proline as well as many transport systems involved in amino acid and iron uptake. Genes involved in catabolism of glycerol are also induced during intracellular growth, suggesting that glycerol could be used as a carbon source. The genes encoding the Icm/Dot system are not differentially expressed inside cells compared to control bacteria grown in rich broth, but the genes encoding several translocated effectors are strongly induced. Moreover, we used the transcriptome data to predict previously unrecognized Icm/Dot effector genes based on their expression pattern and confirmed translocation for three candidates. This study provides a comprehensive view of how *L. pneumophila* responds to the human macrophage intracellular environment.

Keywords: THP-1, microarray, SCOTS, Icm/Dot effectors, iron

INTRODUCTION

Legionella pneumophila is a human opportunistic pathogen and the causative agent of Legionnaires' disease, a pulmonary infection acquired by inhaling contaminated aerosols (Fraser et al., 1977). Once in the lung, *L. pneumophila* infects and multiplies in alveolar macrophages. Legionellosis accounts for 2–15% of community acquired pneumonia cases that require hospitalization (Marston et al., 1994). In most cases healthy individuals clear *L. pneumophila* from their lungs but immune compromised patients or individuals with sustained lung damage often suffer complications. Nosocomial infection with *L. pneumophila* is usually more severe and the fatality rate even with effective antibiotic therapy is close to 50% (Carratala et al., 1994). In the environment, *L. pneumophila* is found in many natural and man made water systems where it infects amebae and other protozoa.

To successfully infect and grow inside host cells, *L. pneumophila* circumvents normal endocytic trafficking pathways and inhibits phagosome maturation, including acidification and fusion with lysosomes. This results in a permissive replication niche called the *Legionella* containing vacuole (LCV; reviewed in Franco et al., 2009). The LCV is characterized by recruitment of early secretory vesicles, mitochondria, and membrane vesicles derived from the golgi and the endoplasmic reticulum (Roy and Tilney, 2002; Molofsky and Swanson, 2004; Shin and Roy, 2008; Hubber and Roy, 2010).

The Icm/Dot type IVB secretion system (TFBSS) of *L. pneumophila* is essential for intracellular growth of the bacteria in all known hosts. It translocates ~200 diverse effector proteins, which often contain eukaryotic-like domains, into host cells (Segal and Shuman, 1998; Segal et al., 1998; Vogel et al., 1998; Cazalet et al., 2004; Chien et al., 2004; de Felipe et al., 2005; Burstein et al., 2009; Huang et al., 2011). In the absence of a functional Icm/Dot TFBSS, effectors are not translocated and the bacteria are unable to avoid intracellular degradation. There appears to be a high degree of functional redundancy among the effectors, because deletion of a single effector gene, or even groups of related genes usually has no or a very limited impact on the fate of the LCV (Ninio and Roy, 2007). This apparent functional redundancy has made the characterization of the effectors difficult.

It has been proposed that *L. pneumophila* adopts a biphasic lifestyle consisting of the replicative phase inside the LCV and an extracellular transmissive phase (Molofsky and Swanson, 2004). The replicative phase is characterized by exponential growth, no motility, and repression of transmissive traits. At the transition to the transmissive phase the bacteria become motile and more cytotoxic. The current assumption is that the *L. pneumophila* replicative and transmissive phases are equivalent to exponential and post-exponential growth in broth (Molofsky and Swanson, 2004).

In this study we used microarray technology to monitor global gene expression changes among several different conditions, to gain further insight into the processes occurring during intracellular growth of *L. pneumophila* inside human macrophages and to directly evaluate the relationship between the patterns of gene expression during growth in axenic medium and during growth in macrophages. At early times following macrophage infection at low multiplicity of infection (MOI), extracted RNA is largely of eukaryotic origin, which interferes with, and reduces the hybridization signal on microarrays. Due to this limitation other studies of gene expression have been limited to infections at non-physiologic MOI. To circumvent this limitation, we used a previously described method called selective capture of transcribed sequences (SCOTS), to enrich bacterial transcripts and discard host cell transcripts and rRNA (Graham and Clark-Curtiss, 1999; Faucher et al., 2006).

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Legionella pneumophila strain JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* strain Philadelphia-1 was used. Media and antibiotics were used as previously described (Chen et al., 2004). For liquid culture, AYE broth was inoculated with a culture grown overnight to a final OD_{600} of 0.1 and incubated at 37°C with vigorous shaking. Exponential (E) phase bacteria were harvested at an OD_{600} of 0.7–0.8 and post-exponential (PE) phase bacteria were harvested approximately 6 h after the cessation of growth.

INFECTION OF CULTURED HUMAN MACROPHAGES

The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in Advanced RPMI (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). A stock culture of the cells was maintained as monocyte-like, non-adherent cells at 37°C in an atmosphere containing 5% (v/v) CO_2. For macrophage infection, cells were seeded at 2×10^7 cells in 10 cm culture dishes and were differentiated by addition of 10^{-7} M phorbol 12-myristate 13-acetate for 48 h (Sigma). Before infection, macrophages were treated with antibodies raised against the major outer membrane protein (MOMP) of *L. pneumophila* for 30 min (Charpentier et al., 2009). Bacteria were grown overnight shaking in AYE at 37°C and were then added to the cell monolayer at a MOI of 1, and centrifuged for 5 min at $800 \times g$ to synchronize bacterial uptake. After incubation for 2 h at 37°C, the infected cells were washed three times with phosphate buffered saline (PBS) pH 7.4 and fresh complete RPMI medium containing 100 μg ml^{-1} gentamicin was added to each well. After incubation for 1 h at 37°C, cells were washed three times with PBS and the cells were either harvested (T0) or incubated with fresh complete RPMI medium for a further 6 h (T6) or 18 h (T18). At each time point, cells were scraped into 2 ml PBS and 100 μl were removed to determine the number of colony forming units (CFU) by plating dilutions on AYE plates. Samples were then centrifuged for 5 min at $1000 \times g$ and the pellet was lysed in TRIzol (Invitrogen) and stored at −80°C.

RNA EXTRACTION

RNA was isolated using TRIzol reagents as described by the manufacturer (Invitrogen). The RNA was subsequently treated with DNase I (Invitrogen) for 1 h at 37°C. The DNase was then inactivated by incubation at 75°C for 5 min and after acid phenol–chloroform (Ambion) extraction the RNA was precipitated with NaAc–ethanol. Purity and quantity of RNA was determined by spectrophotometry.

SELECTIVE CAPTURE OF TRANSCRIBED SEQUENCES

Each RNA sample was converted to cDNA in five independent reverse-transcription reactions. Briefly, 5 μg of RNA was converted to first strand cDNA by random priming, using primer RB1-RNA (**Table 1**) containing a defined 5′ end and random non amer at the 3′ end, with Superscript II (Invitrogen), according to the manufacturer's instruction. A second strand of cDNA was synthesized using Klenow fragment (New England Biolab) according to the manufacturer's instruction. Bacterial transcripts were then separated from host cDNA by SCOTS, a selective hybridization to bacterial genomic DNA (gDNA) as described previously (Graham and Clark-Curtiss, 1999; Daigle et al., 2001; Faucher et al., 2006). Briefly, denatured, biotinylated, and sonicated *L. pneumophila* gDNA fragments (0.3 μg) were mixed with 5 μg of sonicated ribosomal DNA (from plasmid pSF6) to pre-block rRNA encoding regions on the gDNA. After hybridization at 60°C for 30 min, total cDNA (1 μg) was added, and hybridization was allowed to proceed for 22 h at 60°C. Bacterial cDNA that was hybridized to biotinylated gDNA was then captured by binding hybrids to streptavidin-coated magnetic beads (Promega) according to manufacturer's instructions. Captured cDNA was eluted, precipitated, and amplified by PCR using primer RB1-PCR (**Table 1**). For each condition, three rounds of capture were performed.

MICROARRAY PROCEDURE

The whole genome microarray of *L. pneumophila* has been previously described (Hovel-Miner et al., 2009). Two micrograms of cDNA from each condition/replicate was labeled independently by PCR using amino-allyl dUTP and RB1 primer. Bacterial gDNA was used as the reference channel on each slide to allow comparison of each time point and of different samples (Talaat et al., 2002). Five micrograms of gDNA was labeled with amino-allyl dUTP using Klenow fragment and random primers (Invitrogen) at 37°C for 18 h (Faucher et al., 2006). DNA was subsequently coupled to the succinimidyl ester fluorescent dye AlexaFluor 546 (for cDNA) or AlexaFluor 647 (for gDNA) (Invitrogen) following the manufacturer's protocols. Hybridization and data acquisition were performed as previously described (Hovel-Miner et al., 2009). Local background was removed from spot signal intensity and normalization was carried out by calculating the fraction over the total signal intensity in both channels as previously described (Faucher et al., 2006). Signal levels that were lower than background in experiments and controls were filtered out. A total of 12 cDNA to reference ratios were recorded for each time point. Statistical analysis between test and control conditions was performed using an unpaired one-tailed Student's *t*-test. Genes were considered differentially expressed if they demonstrated a ratio to control value of ±2-fold with a $p < 0.001$. The microarray data have been submitted to the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE23029 and GSE23032.

QUANTITATIVE REAL-TIME PCR

RNA extraction was performed as described above. cDNA was synthesized in triplicate using Superscript II (Invitrogen) with random hexamers (Invitrogen), according to the manufacturer's

Table 1 | Primers used in this study.

Primer name	Sequence	Use
RB1-RNA	CGGGATCCAGCTTCTCACGCANNNNNNNNN	SCOTS
RB1-PCR	CGGGATCCAGCTTCTCACGCA	SCOTS
rrnB-F	AACTGAAGAGTTTGATCCTGGCTCAG	Cloning
rrnB-R	ACCCTGGCGATGACCTACTTTC	Cloning
16S-F	AGAGATGCATTAGTGCCTTCGGGA	qPCR
16S-R	ACTAAGGATAAGGGTTGCGCTCGT	qPCR
icmQ-F	CGCTAGTCAGGCCAAGTTAAAAG	qPCR
icmQ-R	TCCTGCTGACCACTCCTTAAGG	qPCR
cspA-F	GCCCGGATGTATTTGCTCACT	qPCR
cspA-R	TGCTCCTTGCGTCACAATGA	qPCR
lpg0491-F	CAACCAAGCGATAGAAGCTTTAATC	qPCR
lpg0491-R	CCTTGTGCCCCATCCATAAG	qPCR
lpg0494-F	GCCACCGGTAAAGGGAATG	qPCR
lpg0494-R	GAGGTGCAAGTGCCTTAATCG	qPCR
ceg29-F	CTTGGTGCCTGGAATGATTTATG	qPCR
ceg29-R	CGGTTTGCTGATGGATTAAGG	qPCR
cegC1-F	TGCCTAAACGGTATGACCGCATCA	qPCR
cegC1-R	GGCATATGCACCAAACCACCGAAT	qPCR
lpg0941-F	TTCTGCCTCTGTAACTCTCTGGCA	qPCR
lpg0941-R	TTTCTGCCGGGTCTTCTTTCAGGA	qPCR
dotA-F	CTCTACTCTACCTTTGGCTTCCTC	qPCR
dotA-R	CTGAGATGGATAGGTGGTAGTC	qPCR
sidF-F	ATTGTTCGCGAGGGTATGAAAGCG	qPCR
sidF-R	TCTTTCCAAGACAGACTCTCGCGT	qPCR
Lpg2145-F	ATCCGATTAAGGTTGTTATCTTCACG	qPCR
Lpg2145-R	GATGTGATTTTTTTTCCAGCAAGTG	qPCR
lpg1959KpnIFw	CGATGGTACCATGTTAGTTTCCAATACAAT	TEM-fusion
lpg1959XbaIRv	CGATTCTAGAAATGGATACCCTATGATTATT	TEM-fusion
lpg1961KpnIFw	CGATGGTACCTTGTTATGCGAGAGTTTCAT	TEM-fusion
lpg1961XbaIRv	CGATTCTAGAGTATGGTTTTTCCCCATACT	TEM-fusion
lpg2827KpnIFw	CGATGGTACCGTGGATATGGATTTTTGCAAATACTATCAG	TEM-fusion
lpg2827XbaIRv	CGATTCTAGAATAAAAAATCTCAGCCATCATGCATCGTGC	TEM-fusion
lpg2828KpnIFw	CGATGGTACCATGAAAATTAGTGAATTAAA	TEM-fusion
lpg2828XbaIRv	CGATTCTAGAATTTCTTAGTAAAGGATAGGG	TEM-fusion

instructions. For each sample, a no reverse-transcriptase reaction served as a no template control (NTC). qPCR was performed using the Applied Biosystems StepOne Plus 96 well RT-PCR system with Power Syber green PCR master mix following the manufacturer's instructions (Applied Biosystems). Primers are described in **Table 1**. For each qPCR run, the calculated threshold cycle (C_t) was normalized to the C_t of the internal control 16S rRNA amplified from the corresponding samples and the fold-change was calculated as previously described (Livak and Schmittgen, 2001).

SOUTHERN BLOT

The PCR DIG labeling mix (Roche Molecular Biochemicals) was used to produce Digoxigenin-labeled cDNA probes according to the manufacturer's instructions. gDNA was extracted with the Wizard kit (Promega) and digested with *Hin*cII (NEB). The DNA was transferred to a positively charged membrane. Membranes were prehybridized and subsequently incubated at 42°C with the digoxigenin-labeled specific probe in 50% (v/v) formamide, 5× SSC; 2% (w/v) blocking reagent (Roche Molecular Biochemicals), 0.1% (w/v) *N*-laurylsarcosine, 0.02% (w/v) SDS. The blots were hybridized overnight and subjected to stringency washes in 0.1× SSC and 0.1% (w/v) SDS. Hybridization signals were detected with a DIG Luminescent Detection Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

β-LACTAMASE (TEM) FUSION TRANSLOCATION ASSAY

The putative effector genes (lpg1959, lpg1961, lpg2827, lpg2828) were PCR amplified and cloned in frame with the beta-lactamase gene at the *Kpn*I/*Xba*I sites of pXDC61 (de Felipe et al., 2008). The resulting plasmids were introduced into KS79 (JR32Δ*comR*) or KS79 *dotA*::Tn903dII*lacZ* by transformation. All primers, strains, and plasmids used are listed in **Tables 1 and 2**. The TEM-translocation assay was performed as previously described (de Felipe et al., 2008) with the difference that the MOI was lowered from 50 to 20. Images of cells after the translocation were obtained by epifluorescence on individual

Table 2 | Strains used in this study.

Name	Description and/or genotype	References
STRAINS		
JR32	Philadelphia-1; Sm^r; $r^- m^+$	Sadosky et al. (1993)
KS79	JR32 Δ*comR*	de Felipe et al. (2008)
LELA3118	JR32 *dotA*::Tn*903*dII*lacZ*	Sadosky et al. (1993)
SPF48	KS79 pXDC61-FabI	This study
SPF49	KS79 pXDC61-RalF	This study
SPF50	KS79 pXDC61-LepA	This study
SPF55	LELA3118 pXDC61-FabI	This study
SFP56	LELA3118 pXDC61-RalF	This study
SPF57	LELA3118 pXDC61-LepA	This study
PLASMIDS		
pSF6	*rrnB* in pGEMT-easy	This study
pMMB207C	Derivative of IncQ plasmid RSF1010; Cm^r; Δ*mobA*	Charpentier et al. (2008)
pXDC61	pMMB207c *Ptac-TEM1*	de Felipe et al. (2008)
pXDC61-FabI	pMMB207c *Ptac-TEM1-fabI*	de Felipe et al. (2008)
pXDC61-LepA	pMMB207c *Ptac-TEM1-lepA*	de Felipe et al. (2008)
pCAM49	pMMB207c *Ptac-TEM1-lpg1959*	This study
pCAM50	pMMB207c *Ptac-TEM1-lpg1961*	This study
pCAM51	pMMB207c *Ptac-TEM1-lpg2827*	This study
pCAM52	pMMB207c *Ptac-TEM1-lpg2828*	This study

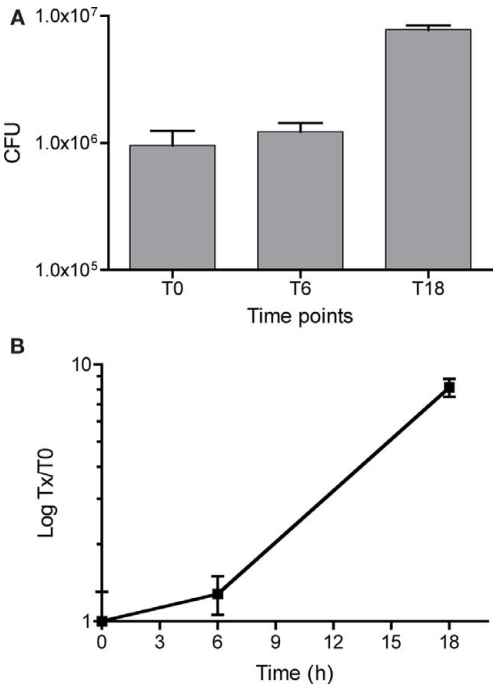

FIGURE 1 | Kinetics of THP-1 macrophage infection by *Legionella pneumophila*. (A) Ten million human cultured THP-1 macrophages were infected with *L. pneumophila* at a MOI of 1. At each time point, cells were washed three times and a fraction was resuspended in distilled water to release intracellular bacteria. Shown is the number of colony forming units (CFU) present inside the totality of cells. **(B)** CFU was normalized against the number of CFU at T0 to show change in intracellular multiplication.

assay wells (data not shown). For immunoblots bacteria used for the TEM-translocation assay were resuspended in 1× laemmli buffer and boiled. Whole cell lysate corresponding to 2×10^6 bacteria was loaded per lane. Immunoblotting was carried out using rabbit polyclonal antibodies directed against β-lactamase (anti-TEM). Detection was performed with secondary antibodies conjugated to horseradish peroxidase (1:5000, Goat Anti-Rabbit-HRP conjugated, Pierce) before development with supersignal chemiluminescent substrate (Pierce).

RESULTS

INFECTION MODEL, EFFECT OF SCOTS, AND VALIDATION OF THE METHOD

The aim of this work was to study the gene expression profile of *L. pneumophila* during infection of macrophages infected at a MOI of 1. SCOTS is a method that allows amplification of small amounts of bacterial RNA from infected host cells, while discarding host cell transcripts and ribosomal RNA (Faucher et al., 2006). Macrophage-like cells derived from the human THP-1 monocyte cell line were infected with *L. pneumophila* opsonized with antibodies raised against the *L. pneumophila* MOMP, which increases the efficiency of bacterial entry into host cells (Charpentier et al., 2009). After 2 h of infection, the macrophages were washed and treated with gentamicin for 1 h to kill extracellular bacteria, and cells were washed three times, and fresh medium was added. Samples for the first time point (T0) were collected after the gentamicin treatment. Samples were also collected after 6 h (T6) and 18 h (T18). Only 1×10^6 CFU were recovered at T0, indicating that approximately 1 in 20 macrophages were infected by *L. pneumophila* (**Figure 1A**). At T6, the number of bacteria increased and by T18, a 10-fold increase in the number of intracellular bacteria was observed (**Figure 1B**) and some of the cells were detached from the cell monolayer (data not shown). T0 can be considered an early time point of infection where the bacteria are still adapting to the intracellular environment. At T6 the bacteria are actively growing and by T18 the bacteria have reached their maximum number and start to lyse the host cells.

Following isolation of total RNA of the infected cells, the RNA was converted to cDNA with reverse-transcriptase and processed using the SCOTS method as described in the Section "Materials and Methods." The effect of SCOTS on the cDNA pool was visualized by Southern blotting (**Figure 2A**). Bacterial cDNA not treated by SCOTS is almost exclusively of prokaryotic ribosomal origin (**Figure 2A** lane 1 and 5). The diversity of the bacterial cDNA increases with the number of SCOTS rounds performed while the amount of cDNA of ribosomal origin decreases (**Figure 2A** lanes 1 through 4) as previously described (Graham and Clark-Curtiss, 1999). Therefore, samples from all conditions, including growth in AYE broth to E or PE phase, were treated with three consecutive rounds of SCOTS and the resulting cDNA was labeled and used to hybridize to the microarray slides. As an internal reference, labeled *L. pneumophila* gDNA was also hybridized to the microarrays (Talaat et al., 2002). For each condition studied, three independent biological replicates and two technical replicates were analyzed, resulting in six replicates for each condition. The background values

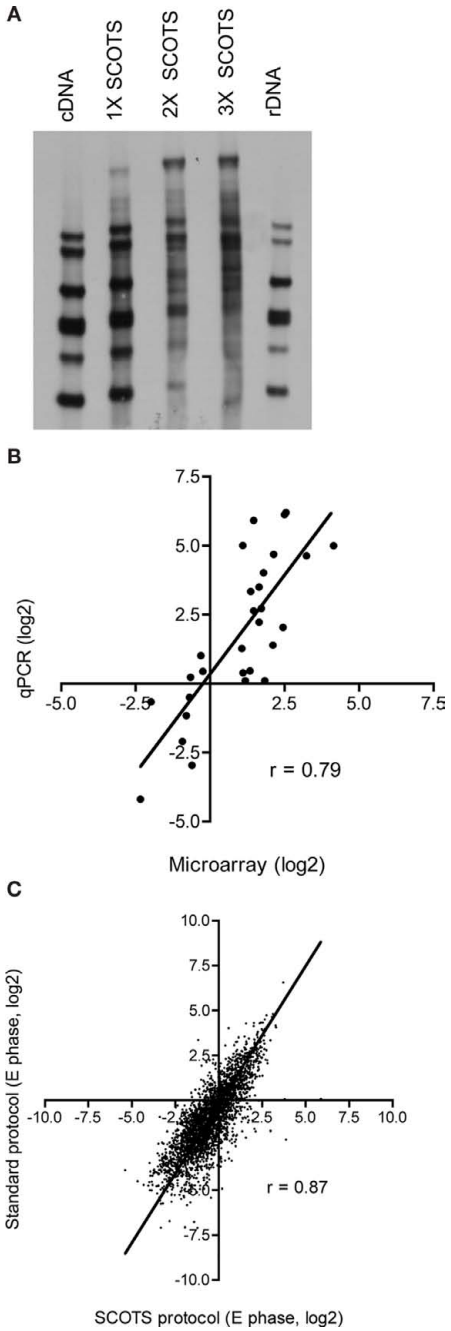

FIGURE 2 | Effects of SCOTS on the cDNA population. (A) Southern blot of *L. pneumophila* gDNA digested with *Hin*cII and hybridized with labeled cDNA from the T0 time point obtained before (lane 1) and after the first (lane 2), the second (lane 3), and the third (lane 4) round of SCOTS. Lane 5 was hybridized with labeled rDNA. **(B)** qPCR was used to validate the expression profiles obtained by microarray for eight genes in all the conditions. **(C)** Comparison of microarray data obtained when using the SCOTS amplification method and when using a standard microarray protocol. Shown is the normalized signal intensity obtained from E phase growth in rich AYE broth. See text for details.

were subtracted, the data were normalized by calculating the contribution of each spot to the total signal intensity and the ratio to the gDNA signal was recorded. A one-tailed Student's *t*-test was used for statistical analysis and the ratio between test conditions (PE, T0, T6, and T18) and the control conditions (E phase or T0) was calculated. Despite the very small number of bacteria present during infection, SCOTS allowed us to obtain easily measurable microarray signals.

We used qPCR to validate the expression patterns of eight genes (**Figure 2B**) and the correlation between the microarray values and the qPCR values was 0.79 with a slope of 1.4. Next, we compared the expression patterns obtained by SCOTS to those obtained when a standard microarray protocol was used. RNA from axenically grown bacteria in E phase was treated using a standard microarray protocol where the cDNA is labeled during the reverse-transcription reaction as previously published (Faucher et al., 2010). The resulting cDNA was hybridized to the microarray slides as described above and data analysis was carried out the same way as for the SCOTS treated samples. The normalized signal intensities of the transcriptome in E phase obtained by SCOTS and by the standard microarray protocol were compared (**Figure 2C**). The correlation of the two datasets is 0.87, which indicates that SCOTS treatment of samples does not introduce significant bias on the gene expression data, in agreement with previous reports (Faucher et al., 2006; Poirier et al., 2008).

COMPARISON OF GENE EXPRESSION PATTERNS BETWEEN GROWTH IN THP-1 CELLS AND GROWTH IN BROTH

The normalized signal intensities for each gene were subjected to hierarchical clustering which revealed that there are some similarities, but also striking differences between PE phase and intracellular growth (T0, T6, T18, **Figure 3A**). To quantify these differences on a global level, a correlation matrix analysis of the five conditions was performed using Bioconductor (**Figure 3B**; Gentleman et al., 2004). The correlation between PE and any intracellular condition was approximately 0.6, indicating that PE phase is quite different than intracellular growth in human cultured macrophages. Interestingly, the correlation between E phase and *in vivo* time points was higher for early time points (0.77 and 0.70 for T0 and T6 respectively) than the later time point (0.64 for T18). The T0 pattern was more similar to T6 (0.86) than to T18 (0.80) and T6 was very similar to T18 (0.94). These observations suggest that the pattern of gene expression gradually changes as the infection proceeds from T0 to T18 and that T6 represents a mixture between genes differentially expressed at T0 and T18.

SOME GENES ARE HIGHLY EXPRESSED IN MOST CONDITIONS

Hierarchical clustering of signal intensities reveals a cluster of genes that are highly expressed in most conditions tested (**Figure 3A**, red line). A value of 1 (blue–green) means that the normalized signal intensity of the cDNA was equal to the normalized signal intensity of the gDNA used as a reference. This cluster contains genes involved in basic cell functions such as transcription (*rpoA*, *rpoB*, *rpoC*, and the sigma factor *rpoD*), translation (ribosomal genes, tRNA genes, and elongation factors), replication (*dnaB*, *dnaG*, and topoisomerase genes such as *gyrA*, *gyrB*, *parC*), and cell division (*minC*, *minD*, and *ftsY*). In addition, some genes known to be involved in virulence, such as the macrophage infectivity potentiator (*mip*), the regulator *letA* and a number of Icm/Dot genes (*icmC*, *icmH*, *icmO*, *icmR*, and *icmS*) as well as some Icm/Dot translocated effectors (*legA15*, *lem3*, *lem21*, *legA14*, *ceg19*, and *sidB*) are present

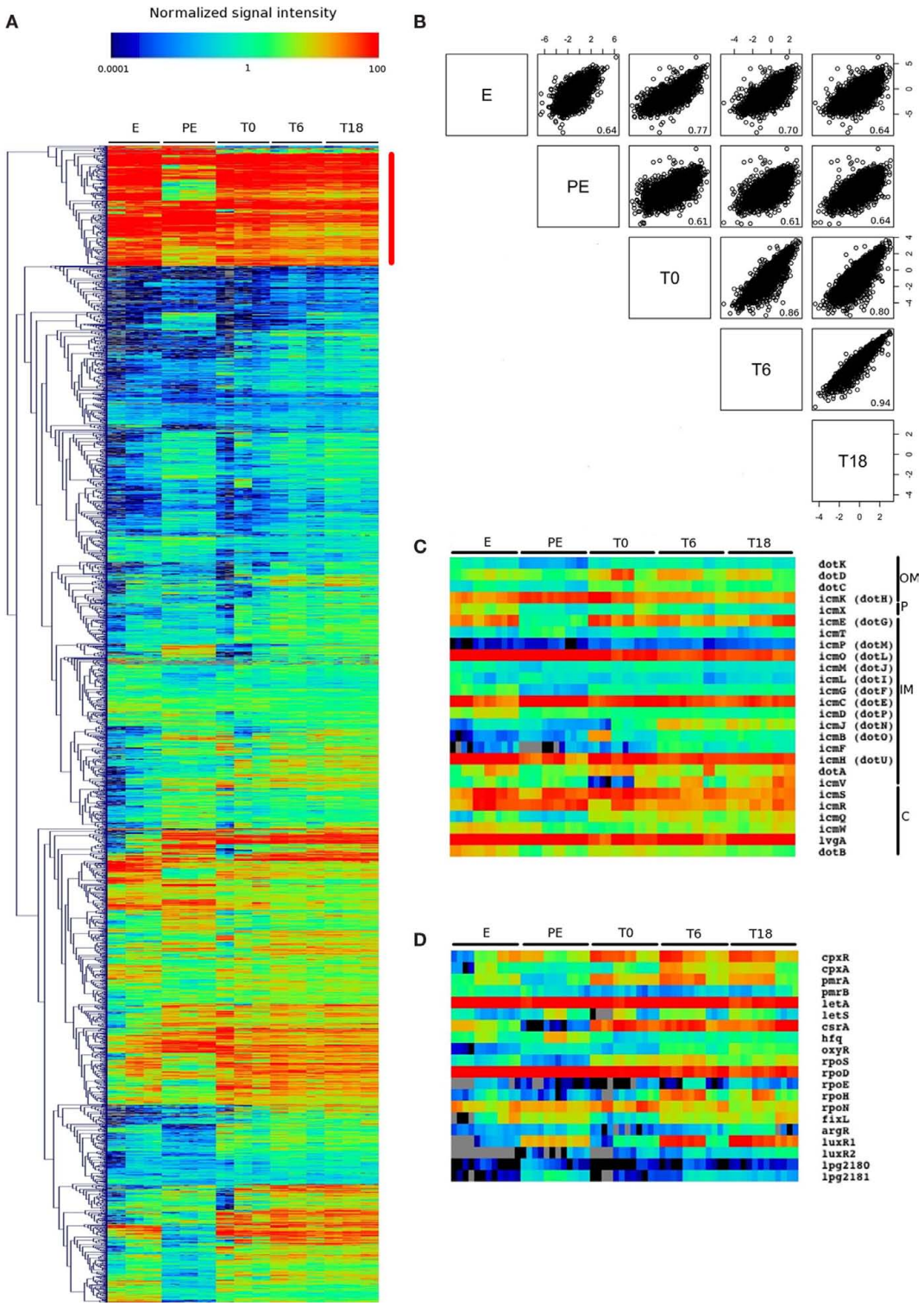

FIGURE 3 | Analysis of the normalized signal intensities for each condition reveals similarity between *in vivo* conditions. (A) Hierarchical clustering of the normalized signal intensity of each replicate for each condition. The red line marks a cluster of genes highly expressed in all conditions tested. **(B)** The Bioconductor package was used to generate paired correlation matrix showing the degree of similarity between conditions. The correlation value is displayed in the lower right part of each graph. The *x* and *y* axis represent the median of the log2 transform of the normalized signal intensity. **(C)** Normalized signal intensities for genes encoding proteins of the Icm/Dot Type IVB secretion system. Putative location of each gene products is shown on the left side of the annotation: OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm. **(D)** Normalized signal intensities for genes encoding known and putative regulators.

in this cluster of genes. The Icm/Dot secretion system is essential for virulence and almost all of the *icm/dot* genes show high signal intensities with low variability between all conditions (**Figure 3C**). Most well-known virulence regulators are also expressed at similar levels during infection (**Figure 3D**), including *cpxR*, *cpxA*, *pmrA*, *pmrB*, *letA*, and *letS*.

GENE EXPRESSION PATTERNS DURING GROWTH IN MACROPHAGES COMPARED TO EXPONENTIAL GROWTH IN AYE BROTH

The transcriptome of *L. pneumophila* during infection of THP-1 macrophage-like cells at T0, T6, and T18 post-infection, and during PE phase in broth were compared to that of bacteria in E phase and subjected to hierarchical clustering (**Figure 4A**; **Table S1** in Supplementary Material). For clarity, a transcript with a higher or lower steady-state level in the test condition (PE, T0, T6, T18) compared to the control (E) is considered induced or repressed, respectively. Globally there were 1956 genes (65.6%) that show significant changes of expression ($-2 > \log2 > 2$, $P < 0.001$) in at least one of the test conditions. Three hundred thirty-four genes were induced at all three time points in THP-1 cells and 110 were repressed at all three time points (**Figure 4B**). There were also a number of genes that were either induced or repressed in one specific condition (**Figure 4B**). **Table 3** displays the genes with the highest level of induction or repression during intracellular growth. Interestingly, 8 of the 10 most highly induced genes have no assigned function, suggesting that novel virulence strategies could be used by *L. pneumophila* to infect host cells. The most highly induced gene during intracellular growth is an Icm/Dot effector of unknown function (lpg2527). Also highly induced intracellularly is a gene encoding a putative glutamine ABC transporter (lpg0491), which is encoded next to the *argR* gene (lpg0490). The ArgR arginine repressor is required for maximal growth of *L. pneumophila* in its ameba host *Acanthamoeba castellanii* (Hovel-Miner et al., 2009).

Based on the hierarchical clustering, four different groups of genes were defined (**Figure 4A**): (i) genes that were strongly induced inside cells (237 genes, group #1), (ii) genes induced at all three time points in THP-1 cells (171 genes, group #2), (iii) genes that were induced inside cells but repressed or unchanged in PE phase (224 genes, group #3), and (iv) genes that were repressed at all time points (169 genes, group #4). The complete list of genes present in these groups is shown in **Table S3** in Supplementary Material. Group #1 and #2 contain 61 genes encoding Icm/Dot effectors, many genes involved in flagella production, a number of Lvh Type IV secretion system genes and genes of unknown or putative function. Group #3 contains 23 genes encoding Icm/Dot effectors that are only induced intracellularly, including SidF and SdhA, which are known to inhibit macrophage apoptosis following infection (Laguna et al., 2006; Banga et al., 2007). The virulence factor RtxA, which is involved in attachment of *L. pneumophila* to host cells (Cirillo et al., 2000, 2001) is also present in group #3 and its expression increases over the course of infection. Group #4 includes two Icm/Dot effectors (MavG and MavM) and a number of genes involved in translation, such as ribosomal subunits, tRNAs and elongation factors (EF-TU, EF-G, EF-P) and genes involved in transcription, including RNA polymerase subunits (*rpoA*, *rpoB*, and *rpoC*), and the σ^{70} gene *rpoD*.

To obtain a broad overview of the data, genes with significant changes in expression were clustered based on the genome annotation and their known or predicted function (**Figures 4C and 5**). The number of induced genes involved in motility (flagella), transport (ABC transporters, permeases, multidrug efflux pumps, Type II, and Lvh/Lvr secretion system), and detoxification/adaptation increases during the course of infection (**Figure 4C**). Of the 176 genes involved in amino acid metabolism 20% were induced during intracellular growth.

NUTRITION OF *L. PNEUMOPHILA* DURING INTRACELLULAR MULTIPLICATION IN HUMAN MACROPHAGES

Of all the genes involved in the metabolism of amino acids, lipids, carbohydrates, nucleotides, cofactors, and vitamins a larger proportion was induced than repressed inside cells, regardless of the time post-infection (**Figures 4C and 5**). The pathway tool Omics Viewer from the BioCyc Database was used to analyze trends in expression of genes involved in catabolism and anabolism pathways (Paley and Karp, 2006). The most highly induced pathway inside human macrophages was the one leading to thiamine synthesis (**Figure 6**). *L. pneumophila* has been shown to rely on amino acids as a carbon and nitrogen source (Tesh et al., 1983). Many pathways involved in the synthesis of amino acids (L-histidine, L-arginine, L-aspartate, L-lysine, and L-proline) are induced during growth inside macrophages (**Figure 6**). In addition, amino acid transporters and oligopeptide transporters are also highly induced during growth in macrophages. The degradation pathways for L-lysine, L-arginine, L-histidine, L-threonine, L-glutamine, and L-glutamate were all induced inside cells. In contrast, genes involved in translation and the tRNA-charging pathway are repressed during intracellular growth (**Figure 5**). Taken together these observations suggest that *L. pneumophila* can acquire amino acids from the host but the induction of synthesis pathways for certain amino acids and the repression of translation and tRNA-charging suggests that *L. pneumophila* has limited access to certain amino acids inside the host cell. However, this should be taken cautiously, since the intracellular transcriptome was compared to exponential growth in rich broth, in which all the amino acids, sugars, and cofactors, essential or not, for *Legionella* growth are likely supplied in unlimited quantity. Whether or not these pathways are essential for intracellular growth remains to be elucidated. However, it is known that L-arginine biosynthesis is not essential for growth inside a protozoan host (Hovel-Miner et al., 2010), but its role during infection of mammalian cells has not been investigated. Interestingly, genes required for glycerol catabolism (*lpg1414* and *glpD*) are induced intracellularly (**Figure 6**). However most of the genes involved in glycolysis were not differentially regulated compared to growth in rich broth. We also noted the induction of *lpg1607* and *lpg0466* that are predicted to encode enzymes that would mediate oxaloacetate production, from phospho-enolpyruvate and pyruvate respectively, which can then be used in the TCA cycle or for the production of L-aspartate and L-lysine (**Figure 6**).

Legionella pneumophila requires relatively large amounts of iron to grow in broth and inside host cells and has many systems to acquire sufficient amounts (Cianciotto, 2007). Iron transport systems were induced during intracellular growth (**Figure 7A**), including genes involved in legiobactin production (*lbtAB*), fer-

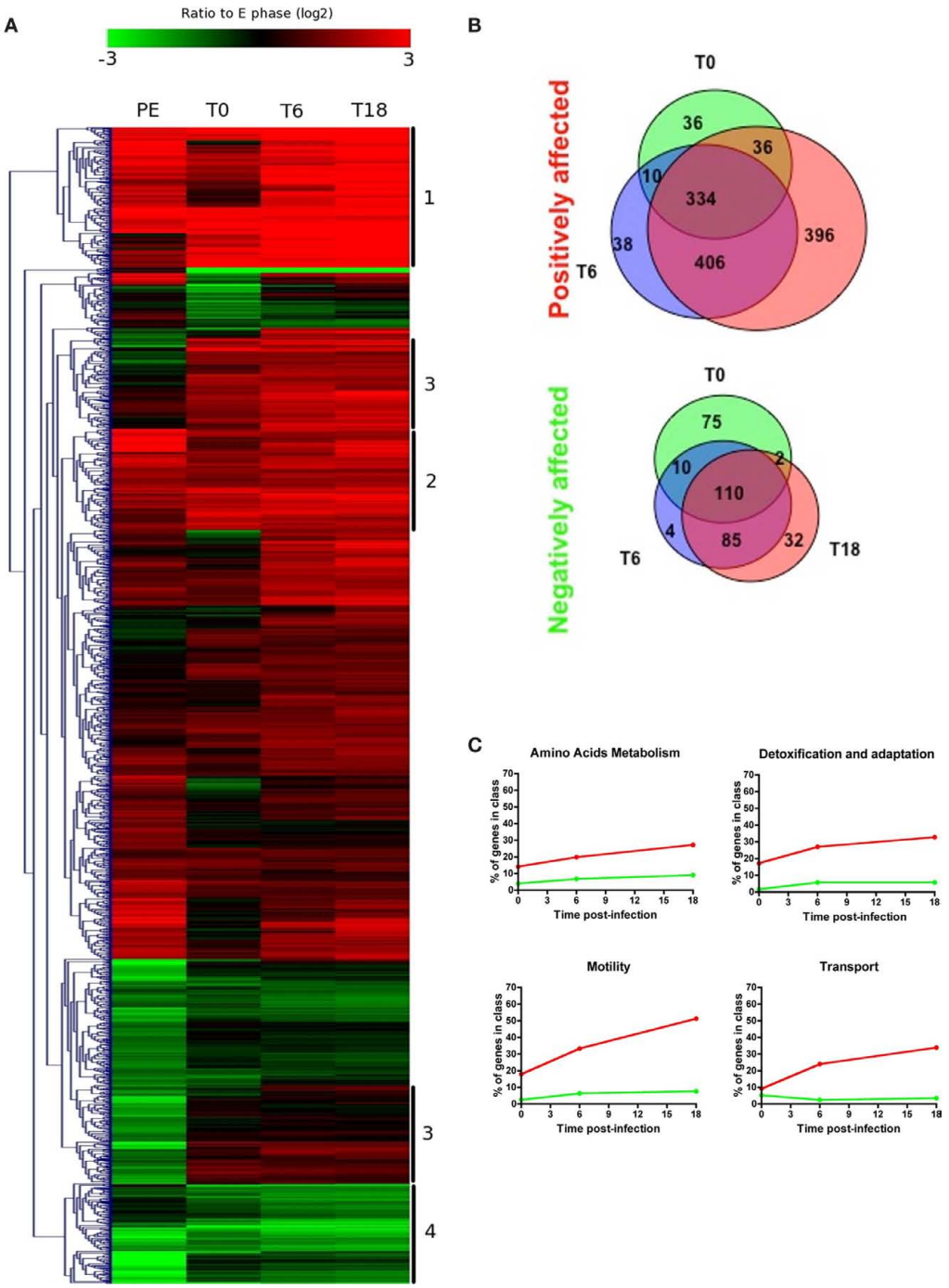

FIGURE 4 | Genes differentially expressed compared to the E phase control. **(A)** Hierarchical clustering of the relative expression of *L. pneumophila* genes during intracellular multiplication in macrophages and during PE phase in rich AYE broth compared to exponential growth in broth. Only genes with significant change in expression ($-2 > \log2 > 2$, $P < 0.001$) in at least one condition are shown. **(B)** The number of genes positively or negatively affected during intracellular growth compared to E phase is displayed in Venn diagrams. **(C)** Genes with significant changes in expression were grouped according to their published COG class. The percentage of genes that change positively (red line) or negatively (green line) during intracellular growth, compared to the E phase control, is shown for selected COG classes. Refer to **Figure 5** for the results of other COG classes.

rous iron uptake (*feoAB*), and iron acquisition by an unknown mechanism (*iraAB*). Of note, *lbtB* is one of the most highly induced genes during infection (**Table 3**). The two genes *pvcA* and *pvcB*, which encode proteins similar to the pyoverdine synthesis genes of *Pseudomonas aeruginosa*, are also induced during intracellular growth even though these two proteins do not seem to be involved

Table 3 | The 10 most highly induced and repressed genes during intracellular growth, compared to E phase.

Lpg #	Product	Gene	PE[1]	T0[1]	T6[1]	T18[1]
lpg2527	Contains coiled coil domain, Icm/Dot effector		2.6	3.1	5.2	6.7
lpg0166	Hypothetical (integral membrane protein)		2.6	5.9	5.8	6.4
lpg0152	Acetyltransferase, GNAT family, putative		3.8	4.0	6.0	6.0
lpg1636	Acetyltransferase, GNAT family, putative		5.4	5.1	3.7	5.9
lpg1987	Phosphohistidine phosphatase, putative		2.6	2.8	4.8	5.7
lpg1670	ORF		3.6	2.2	4.9	5.6
lpg1454	Multidrug efflux protein, putative		2.5	4.9	5.4	5.5
lpg0491	Glutamine ABC transporter, putative		2.4	2.3	4.4	5.3
lpg1625	Small ORF (130aa), Icm/Dot effector	*lem12*	–0.1	4.6	4.5	5.3
lpg1324	Major facilitator family transporter	*lbtB*	3.1	3.9	5.4	5.2
lpg1420	Cytidylate kinase	*cmk*	–2.3	–2.1	–2.5	–2.8
lpg0347	50S ribosomal protein L30/(L7E)	*rpmD*	–2.9	–2.0	–2.6	–2.9
lpg2292	tRNA-Gly		–1.6	–2.8	–2.7	–3.0
lpg2752	tRNA-Ile		–0.7	–2.8	–3.4	–3.2
lpg2902	Conserved hypothetical protein		–0.8	–3.5	–3.2	–3.3
lpg0303	tRNA-Ala		0.2	–3.2	–3.8	–3.6
lpg0307	Hypothetical protein		0.4	–3.9	–4.5	–4.2
lpg0306	ORF		0.2	–4.1	–4.5	–4.3
lpg0305	ORF		–0.1	–4.8	–5.4	–5.1
lpg0308	Cell wall associated hydrolase, pseudogene		0.4	–5.2	–5.5	–5.2

[1]*The expression value compared to E phase is shown as a log2 transform.*

in iron assimilation in *L. pneumophila* (Allard et al., 2006). A recent study showed that *pvcA* and *pvcB* are highly induced in *L. pneumophila* within biofilms and the authors suggest that they might be involved in resistance to oxidative stress generated by an overload of ferrous iron (Hindre et al., 2008).

EXPRESSION OF THE GENES FOR THE Icm/Dot SECRETION SYSTEM AND ITS SUBSTRATES

Most of the 27 *icm/dot* genes show high signal intensities in all conditions (**Figure 3C**) and were not differentially expressed during intracellular growth compared to the E phase control (**Table S1** in Supplementary Material). Only five *icm/dot* genes (*icmQ*, *icmB*, *icmJ*, *icmF*, and *icmV*) were somewhat induced at later time points during intracellular growth compared to the E phase control. This suggests that the secretion machinery is likely always present and ready to translocate effectors when needed. The normalized signal intensities of the genes encoding 191 Icm/Dot effectors were analyzed by hierarchical clustering (**Figure 8A**). Most Icm/Dot effector genes show good signal intensity (around or above 1) in all or a subset of the conditions tested. Interestingly, the *sidM/drrA*, *vipA*, *legC7*, *lepA*, and *lepB* genes were not differentially expressed and all show strong signal intensities in all of the conditions tested and are therefore likely produced and usable during intracellular growth in human macrophages. One hundred and three (64%) of the 191 genes encoding Icm/Dot effectors were significantly induced at T18 compared to E phase (**Figure 8B**), including genes encoding some well-characterized effectors (*ralF*, *sidH*) and two effector genes are among the most highly induced genes (lpg2527 and *lem*12, **Table 3**). The only known effector genes that are repressed inside host cells are *lem3*, *mavG*, *mavK*, *mavM*, and *mavP*. Many effector genes are expressed throughout the infection process, including *ralF*, *lepA*, *lepB*, *sidM/drrA*, *vipA*, and *legC7*, whereas others are more highly expressed during the initial phase (*sidF*) or at the end of infection (*ceg17*, *legK2*, *lirB*, *lem26*, *lem5*).

We wanted to examine the patterns of gene expression in order to look for additional genes that encode effectors. In the past, Icm/Dot effectors were identified using a variety of bioinformatic and experimental approaches. In total these approaches led to the identification of around 200 effectors. In general, the effectors are scattered across the *L. pneumophila* genome, but there are four areas of the genome that contain clusters of effector genes (Burstein et al., 2009). As an example, 17 effectors are grouped together in the region bordered by *lpg1933* and *lpg1978*. In order to identify candidate effector genes based on gene expression patterns, the genome was manually scanned for regions enriched in genes encoding known Icm/Dot effectors and genes of unknown function with similar expression patterns as the neighboring effector genes. Two regions were identified, *lpg1958–lpg1966* and *lpg2826–lpg2831*, containing four putative effectors (*lpg1959*, *lpg1961*, *lpg2827*, and *lpg2828*). In order to test the products of these genes for their ability to be translocated by the Icm/Dot TFBSS, they were fused to the TEM-1 β-lactamase and translocation of the hybrid proteins into J774 macrophages was measured as previously described (de Felipe et al., 2008). Using this approach we found two new Icm/Dot effector proteins (*lpg1959*, *lpg1961*) encoded within the previously identified cluster of effector genes (*lpg1933–lpg1978*) and one new effector gene (*lpg2828*) within the other region located between *lpg2826* and *lpg2831* (**Figure 9A**). Lpg2827 appeared not to be translocated. Expression of the four β-lactamase fusion proteins in *Legionella* was analyzed by western blotting using an anti-TEM polyclonal antibody (**Figure 9B**). Three of the four proteins (TEM-Lpg1961, TEM-Lpg2827, and TEM-Lpg2828) are stably expressed,

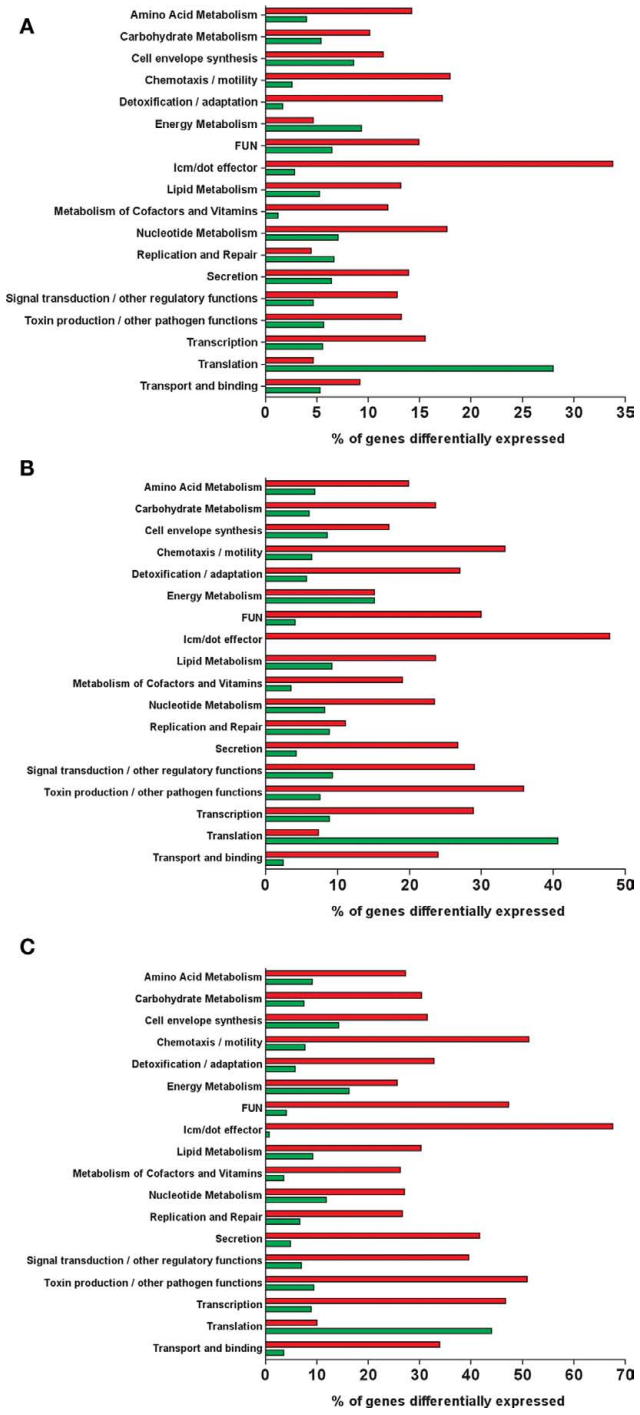

FIGURE 5 | Fraction of genes in orthologous groups deferentially expressed during infection. Number of genes significantly induced (red bar) or repressed (green bar) intracellularly at T0 **(A)**, T6 **(B)**, and T18 **(C)** compared to the E phase control.

but the third protein (TEM-Lpg1959) appears to be unstable, which may explain the low level of translocation observed. The expression pattern of these newly identified effector genes closely resembles the pattern of known effectors (**Figure 8B**).

EXPRESSION OF REGULATORS

Several two-component systems, including PmrA/PmrB (Zusman et al., 2007), CpxR/CpxA (Altman and Segal, 2008), and LetA/LetS (Hammer et al., 2002), are known to be involved in the regulation of virulence factors of *L. pneumophila*. The *cpxR* and *cpxA* genes are significantly induced at T6 and T18 (**Figure 7C**). Although the *pmrA* gene encoding the response regulator component is not differentially expressed inside cells, the *pmrB* gene encoding the cognate sensor kinase is significantly repressed at early time points (T0 and T6, **Figure 7C**), suggesting that a negative feedback loop acts on the expression of *pmrB* early in infection, which is released at later time points to activate the system. In contrast, the gene encoding the LetS sensor is induced inside cells, but the gene encoding its cognate transcription activator, LetA is repressed. The LetA/LetS system controls the expression of two small RNAs (RsmY and RsmZ), which, in turn, control the activity of CsrA, a global regulator that represses the expression of post-exponential traits during exponential growth (Molofsky and Swanson, 2003; Forsbach-Birk et al., 2004; Rasis and Segal, 2009; Sahr et al., 2009). Inside host cells, CsrA was reported to control transmissive traits to allow intracellular multiplication. In accordance with this, CsrA is highly expressed at all time points inside host cells. A number of other two-component systems and regulators are strongly induced inside host cells, including *oxyR*, *fixL*, the putative two-component system *lpg2180/lpg2181* and the two *luxR* homologs (**Figure 7C**).

Sigma factors also regulate gene expression in response to stress or other environmental signals. While the *rpoD* gene encoding the vegetative σ^{70} is repressed during growth inside THP-1 cells compared to the E phase control, the *rpoS* gene encoding σ^{S} is strongly induced (**Figure 7C**). RpoS (σ^{S}) has been shown to regulate a number of known virulence factors such as the Icm/Dot effectors (Hovel-Miner et al., 2009) and is required for intracellular multiplication in ameba and primary macrophages (Hales and Shuman, 1999; Abu-Zant et al., 2006). Other sigma factor genes are also induced inside human macrophages including *rpoH*, which is strongly induced at T6 and T18 inside human macrophages.

LEGIONELLA RESPONSE TO HOST ANTIMICROBIAL SYSTEMS

Phagocytes use a variety of strategies to kill bacteria, which include: (i) acidification of the phagosome, (ii) production of reactive oxygen and nitrogen species (ROS and NOS), and (iii) production of antimicrobial peptides (Flannagan et al., 2009). *L. pneumophila* has evolved several ways to alter host cell responses after infection, but the mechanisms remain unclear in most cases. The Icm/Dot effectors play a central role in altering host cell responses. Inhibition of acidification has recently been shown to be mediated by the SidK effector (Xu et al., 2010), which is induced at all time points inside cells (**Figure 8B**). It has been shown that infection of macrophages with *L. pneumophila* prevents the formation of ROS (Harada et al., 2007), which may explain why genes involved in oxidative stress adaptation such as *sodB*, *sodC*, *katG*, *katB*, *aphC*, and *aphD* were not induced during intracellular growth (**Figure 7D**). *Legionella* expresses a number of proteases and peptidases during intracellular growth, which could be a countermeasure against antimicrobial peptides produced by the host cell. Even though the protein Rcp has been reported to have a role in resistance against antimicrobial peptides in macrophages (Robey et al., 2001), the *rcp* gene was not

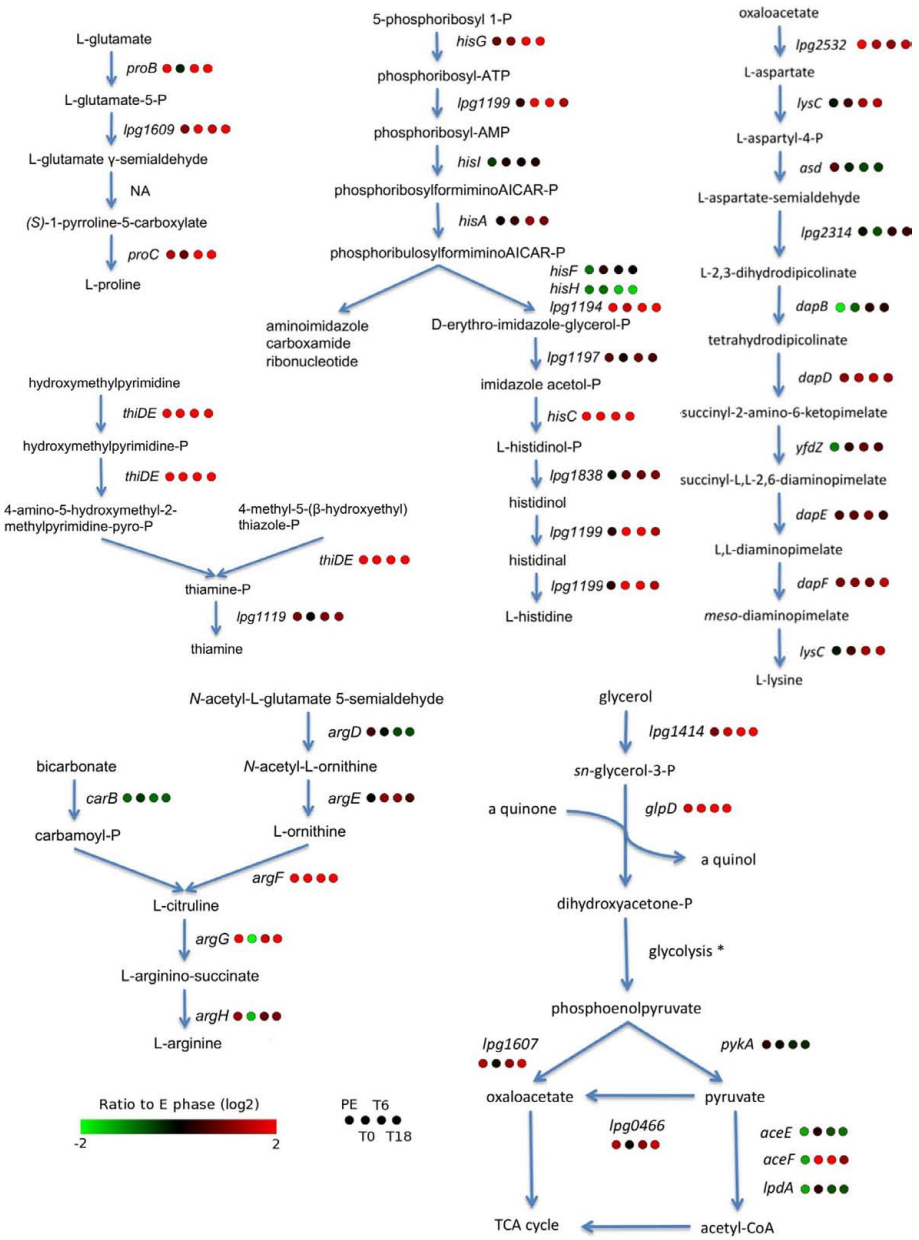

FIGURE 6 | Some metabolic pathways are induced during intracellular growth. The synthesis pathways were drawn according to the Biocyc database (http://biocyc.org). A series of four circles next to each gene display its relative expression value compared to the E phase control. NA, There is no known or predicted gene mediating this reaction in *L. pneumophila*; *glycolysis genes are not differentially expressed.

induced inside cells compared to E phase (**Figure 7D**). Similarly, *lag-1*, which encodes an O-acetyltransferase involved in lipopolysaccharide modification (Luck et al., 2001), is not induced inside cells.

GENES DIFFERENTIALLY EXPRESSED DURING INTRACELLULAR GROWTH

To get a better view of the genes differentially expressed during the intracellular stages of infection, T0 was used as the control condition and compared to T6 and T18 (**Figure 10A**). As a result of this comparison, 667 (22%) genes with a significant change in expression at later time points compared to T0 were identified, 290 of which were induced at both T6 and T18 (**Figure 10B**). Hierarchical clustering of the data shows that the gene expression patterns at T6 and T18 are very similar (**Figure 10A**). This is not surprising since the correlation between the normalized signal intensity of T6 and T18 was 0.94 (**Figure 3B**). The genes can be grouped in three clusters based on their expression: (i) genes that were strongly induced (around eightfold) compared to T0 (group #1, 39 genes), (ii) genes repressed compared to T0 (group #2, 12 genes), and (iii) genes induced *in vivo* but repressed in PE phase (group #3, 83 genes). The complete list of genes present in these groups is shown in **Table S4** in Supplementary Material. Most of the genes contained in group #1 have no known or putative function (33 out of 39 genes). The remaining six genes are

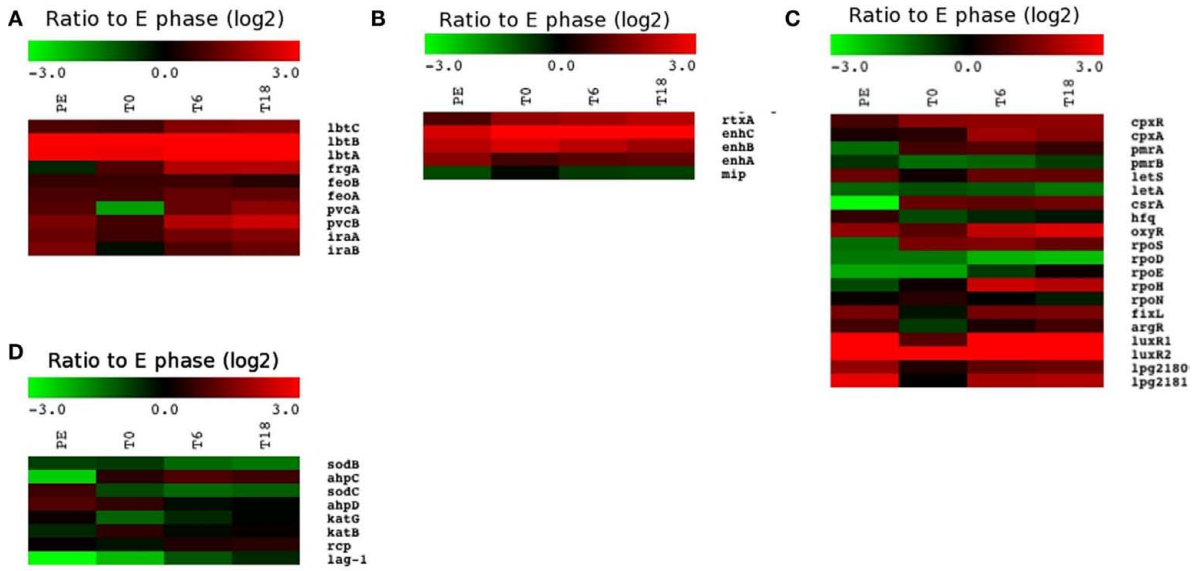

FIGURE 7 | Relative expression of genes involved in various functions. Heat map of the expression ratio to E phase of **(A)** genes involved in iron acquisition, **(B)** known virulence factors other than the Icm/Dot system, **(C)** genes encoding regulators, and **(D)** genes encoding proteins involved in defense mechanisms against oxidative stress and antimicrobial peptides.

the Icm/Dot effectors *lirB* and *lpg2527*, a gene involved in arginine synthesis (*argG*), a gene involved in proline synthesis (*proB*), the pyoverdine synthesis gene *pvcA* and a gene involved in tRNA modification (*gidA*). Group #2 contains two Icm/Dot effectors (*cegC1* and *lem25*) and many genes of unknown or putative function. Group #3 contains 6 Icm/Dot effector genes (*ravA*, lpg1751, *legLC8*, *sidM/drrA*, *lirE*, and *lem26*) and 62 genes of unknown or putative function. In addition a number of transcriptional regulators, such as *oruR* and the sigma factor *rpoH* are found in this cluster.

Overall 50/191 (35%) of Icm/Dot effector genes were differentially expressed at T6 or T18 compared to T0 (**Figure 10C**), 13 of which were not induced when compared to E phase (*vipF*, *ceg9*, *wipB*, *lem4*, *lem6*, *vpdB*, *lpg1751*, *lem15*, *lirD*, *lirE*, *legC2*, *sidM/drrA*, and *lem26)*. Other genes induced at later time points compared to T0 include flagella biosynthesis genes (*flgA*, *flhB*, *motA*, and *motB*), amino acid and peptide transporters, arginine synthesis genes (*argG* and *argF*), lipid A modification (*waaM*), and genes of unknown function (298 genes). Unlike what was observed when E phase was used as the control condition, whole metabolism pathways were not induced when T6 and T18 were compared to T0. However, some genes involved in amino acid synthesis (*proB*, *argG*, and *argH*) were induced at T6 or T18 compared to T0 and were not induced at T0 compared to E phase. The differences in gene expression patterns that are obtained when using E phase and T0 as the control conditions are likely reflective of the vast re-organization of gene expression that occurs when the bacteria transition from growing exponentially in rich media to the intracellular environment, compared to the gradual changes in gene expression that occur as intracellular growth proceeds.

DISCUSSION

In this study, we analyzed the gene expression profile of *L. pneumophila* during multiplication inside human macrophage-like cells shortly after infection is established (T0) and at 6 and 18 h later (T6 and T18) and compared this profile to the profile obtained during exponential and post-exponential growth in rich media. To circumvent the inherent problem of low levels of bacterial RNA during intracellular growth conditions at low multiplicities of infection, we used a method called SCOTS (Graham and Clark-Curtiss, 1999; Faucher et al., 2006) to successfully remove host cell transcripts and amplify bacterial transcripts. Additionally, this method discards bacterial ribosomal RNA (**Figure 2A**) resulting in improved hybridization signals. The data obtained from the microarrays were validated by qPCR performed on eight randomly chosen genes (**Figure 2B**) and by comparison of expression patterns obtained using the SCOTS protocol and a standard microarray protocol (**Figure 2C**) for exponentially growing bacteria. Globally, expression of 65.6% of the *L. pneumophila* genome is affected during intracellular growth when compared to exponential growth in rich media (**Figure 4A**). The number of genes induced increases as the infection proceeds, with only a few genes with decreased expression levels (**Figure 4B**) suggesting that additional functions may be required as intracellular growth proceeds. Some genes whose expression level increases during the latter stages of intracellular growth may be induced prematurely in preparation for stresses that appear later in infection, or in preparation for host cell lysis and escape. This phenomenon of anticipation, although not investigated in *L. pneumophila* has been recently observed in *Escherichia coli* (Mitchell et al., 2009).

Comparison of the gene expression profiles of *L. pneumophila* growing inside host cells to bacteria in E or PE phase in broth revealed some striking differences between the growth conditions. The current assumption is that early stages of infection and intracellular growth can be compared to E phase and later stages of infection and transmission are comparable to PE phase (Molofsky and Swanson, 2004). Our analysis shows that there are significant differences between intracellular growth and PE phase even if a

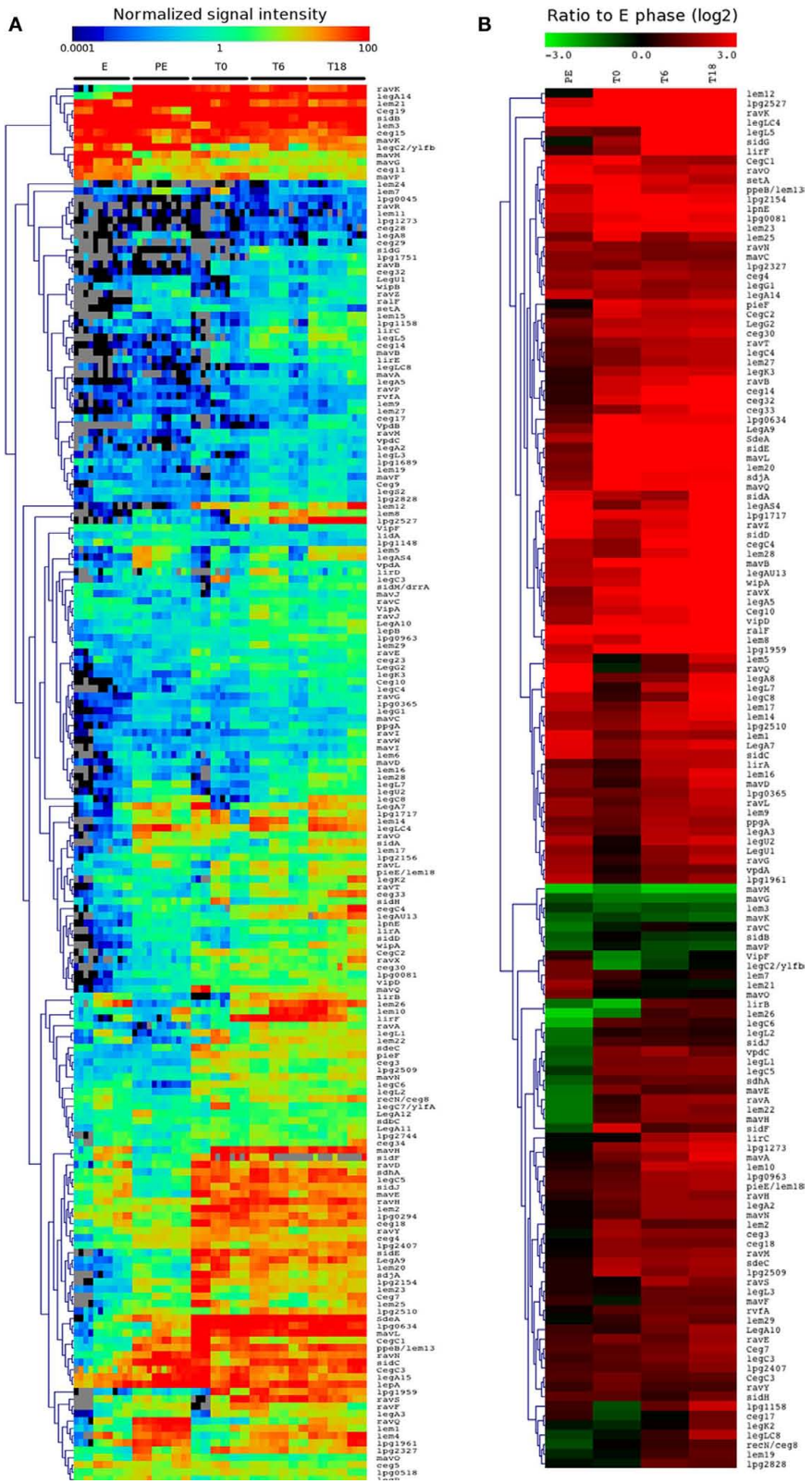

FIGURE 8 | lcm/Dot effectors are differentially expressed inside human cells. (A) Hierarchical clustering of the normalized signal intensity of the genes encoding lcm/Dot effectors for each replicate and each condition. **(B)** Hierarchical clustering of the expression ratio of the genes encoding lcm/Dot effectors compared to the E phase control. Only genes with significant change in expression ($-2 > \log2 > 2$, $P < 0.001$) in at least one condition are shown.

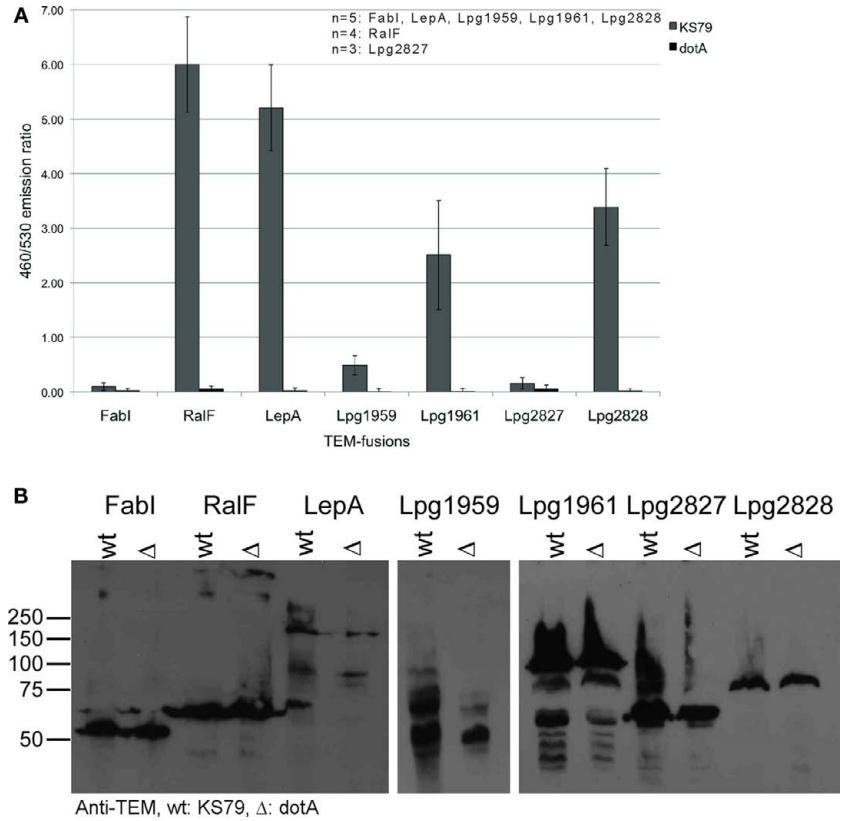

FIGURE 9 | Identification of new Icm/Dot effectors. (A) Translocation of the TEM-effector fusions leads to the cleavage of CCF4/AM. Translocation was determined for each TEM-effector fusion by measuring the ratio of cleaved (460 nm) to uncleaved (530 nm) CCF4/AM in wild type KS79 or KS79 *dotA* (Type IV secretion deficient). FabI serves as negative control; RalF and LepA are known *Legionella* effector and serves as positive control. The number of biological replicates analyzed is shown. **(B)** Immunoblot on whole cell lysate using an anti-TEM rabbit polyclonal antibody showing expression of the TEM-effector fusions. Moleular weight of fusion proteins: TEM-FabI: 59.3 kDa, TEM-RalF: 73 kDa, TEM-LepA: 161.9 kDa, TEM-lpg1959: 106 kDa, TEM-lpg1961: 88 kDa, TEM-lpg2827: 66.7 kDa, TEM-lpg2828: 78.5 kDa.

late stage in infection (T18) is compared to PE phase (**Figure 3B**). This argues that processes that occur in PE phase are not necessarily representative of what happens inside host cells. However, the early stages of intracellular growth and exponential growth in broth are at least partially comparable (**Figure 3B**). Still, growth in rich media and intracellular growth have clearly distinct effects on the *L. pneumophila* transcriptome.

Legionella pneumophila relies on amino acids as a carbon and nitrogen source, when grown in broth and inside host cells (Tesh et al., 1983). Many genes involved in amino acid transport and degradation were induced during intracellular growth (**Figure 6**). Induction of amino acid transport genes was also observed during intracellular growth of *Yersinia pestis*, *Salmonella typhimurium*, *Salmonella typhi*, *Shigella flexneri*, and *Bacillus anthracis* (Eriksson et al., 2003; Lucchini et al., 2005; Faucher et al., 2006; Bergman et al., 2007; Fukuto et al., 2010). Moreover, many amino acid transporters were identified as essential for intracellular growth of *L. monocytogenes* (Schauer et al., 2010). Therefore, amino acid acquisition from the host during intracellular multiplication seems to be a general strategy and one could speculates that a fraction of the virulence strategies employed by intracellular pathogens is aimed at the modification of the host cell process in order to supply essential amino acids and other nutrients to the vacuole. The Icm/Dot mediated modification of the LCV and acquisition of vesicles coming from the endoplasmic reticulum, containing polypeptides, is a striking example of that (reviewed in Hubber and Roy, 2010).

Genes involved in the biosynthesis of thiamine, L-arginine, L-aspartate, L-lysine, and L-histidine were induced as well during *Legionella* intracellular growth, suggesting that the concentration of these metabolites are lower inside cells than during exponential growth in rich broth. It is unclear if the intermediates needed for the synthesis of thiamine, L-arginine, and L-histidine are provided directly by the host or if *L. pneumophila* encodes as yet unidentified enzymes that could provide them from other molecules. Induction of L-arginine biosynthesis genes and aspartate-family biosynthesis genes was also observed during intracellular infection of macrophages by *B. anthracis* and by *Y. pestis*, respectively (Bergman et al., 2007; Fukuto et al., 2010).

Interestingly, glycerol catabolism seems to be induced during intracellular growth and suggests that *L. pneumophila* could use this carbon source inside mammalian macrophages. The metabolism of *Listeria monocytogenes* during intracellular growth in murine cultured macrophages was previously investigated by

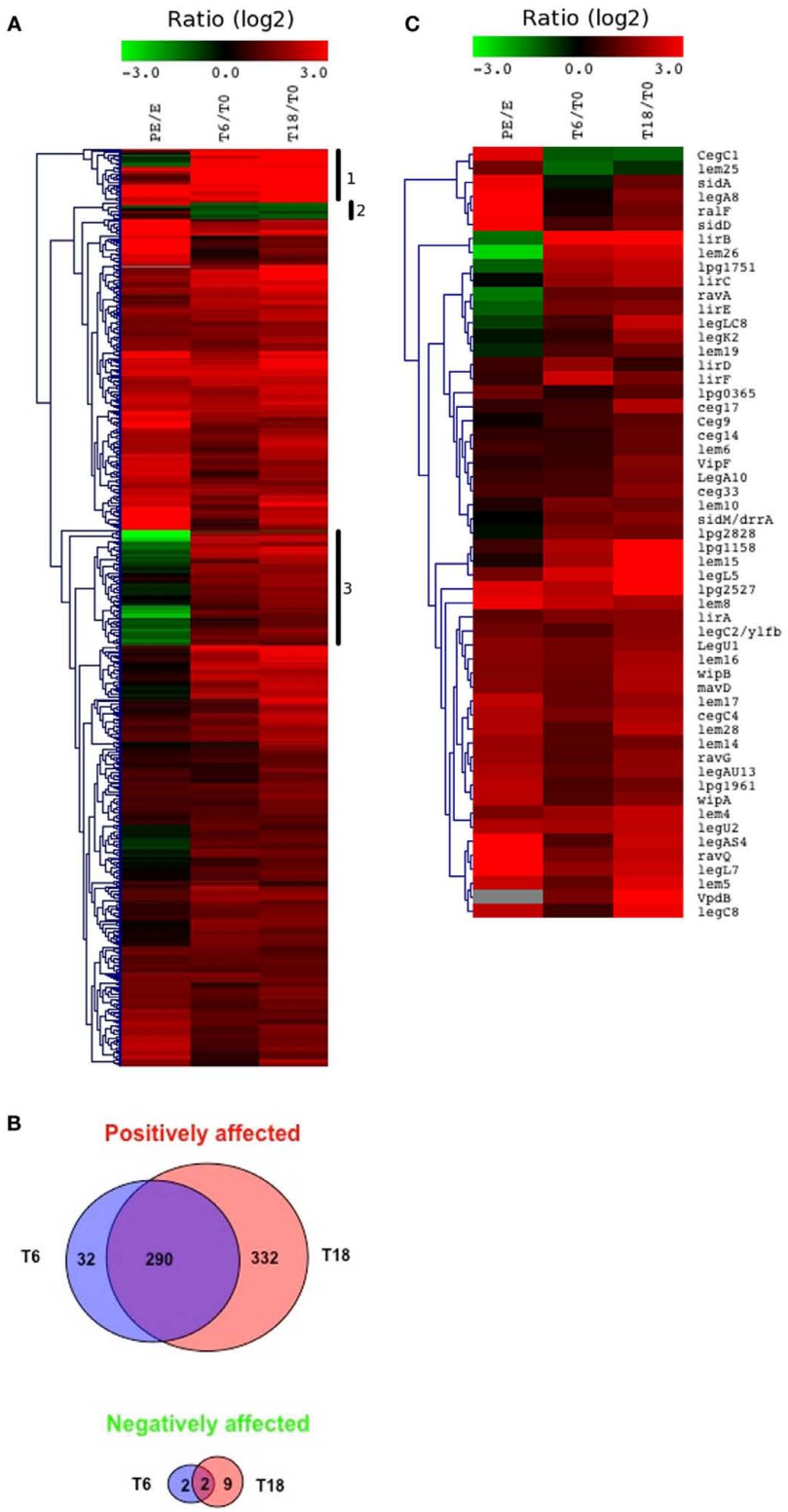

FIGURE 10 | Identification of genes differentially expressed compared to the T0 control. (A) Hierarchical clustering of the transcriptome of *L. pneumophila* during intracellular multiplication in THP-1 macrophages compared to intracellular growth at T0. PE phase compared to E phase is shown as a reference. Only genes with significant change in expression (–2 > log2 > 2, $P < 0.001$) in at least one condition are shown. **(B)** The number of genes positively or negatively affected during intracellular growth compared to T0 is displayed in Venn diagrams. **(C)** Heat map of the Icm/Dot effectors differentially expressed compared to T0. PE phase compared to E phase is shown as a reference.

using ^{13}C-isotopologue profiling (Eylert et al., 2008). This study showed that *L. monocytogenes* acquires a significant proportion of its amino acids from the host. Moreover, it shows that a C_3-metabolite, probably glycerol, serves as a carbon source during intracellular growth of *L. monocytogenes* and that L-aspartate is synthesized from oxaloacetate derived from the carboxylation of pyruvate. In addition, a *L. monocytogenes* deletion mutant of glycerol-3-phosphate dehydrogenase (*glpD*) shows reduced intracellular growth (Schauer et al., 2010). Therefore, one could postulate that *L. pneumophila* also uses glycerol as a carbon source and that carboxylation of pyruvate by Lpg0466 or phospho-*enol*-pyruvate by Lpg1607 provide oxaloacetate that could then be use for the biosynthesis of L-aspartate and L-lysine (**Figure 6**), but it will need further investigation. Interestingly, in *L. monocytogenes*, a deletion mutant of pyruvate carboxylase is defective for intracellular growth (Schar et al., 2010).

Based on our results (**Figure 8**), the effector proteins which are secreted by the Icm/Dot TFBSS can be organized into several distinct groups, based on relative expression to the E phase control: (i) effectors induced during intracellular growth (64% of all effectors), (ii) effectors repressed inside cells (2.6% of all effectors) and effectors not differentially regulated (33.4% of all effectors). Some Icm/Dot effector genes were not differentially expressed when E phase was used as the control condition, but when expression at T6 and T18 was compared to T0, 13 effector genes were differentially expressed, including *legC2*, *sidM/drrA*, and others (see above). Some of these effectors appear to be expressed during growth in broth (**Figure 8A**), which precludes detecting their induction during intracellular growth when the transcription levels were compared to E phase.

Comparison to T0 reveals that the expression of these effectors increases as the infection proceeds and suggests that their function is required at later stages in infection. Consistent with this observation, LegC2 was found to localize only to large structures that resemble mature LCV (de Felipe et al., 2008). Expression of some Icm/Dot effector genes decreases over time (*cegC1* and *lem25*) suggesting that these effectors are required during the initial stages of infection (**Figure 10C**). Taken together, these results indicated that the effectors are regulated independently of the Icm/Dot secretion system and their expression is probably linked to the stage in infection where they are relevant.

Previously, some effectors were identified by searching for genes encoding proteins containing eukaryotic-like domains in the different *Legionella* genomes available (Chien et al., 2004; de Felipe et al., 2005; Bruggemann et al., 2006; Burstein et al., 2009). Here we show that effectors can also be identified based on microarray data by comparing the expression patterns of genes of unknown function to the patterns of closely linked, known effector genes. The three new effectors identified here are located in two large clusters containing several other known effectors and are regulated similarly to the other effector genes in the cluster. These results show that microarray data and the organization of effector genes can be used to predict the identity of novel effector genes.

We also compared our data to the previously published transcriptome of *L. pneumophila* growing inside its protozoan host *A. castellanii* (Bruggemann et al., 2006; Jules and Buchrieser, 2007). It is important to keep in mind that this study of Bruggemann et al. (2006) and the present study used different infection protocols (MOI, infection medium, time points). Also, in the case of the *A. castellanii* study, the intracellular time points (8, 11, and 14 h post-infection) were analyzed two-by-two, in order to unravel how genes expression evolved during intracellular growth, whereas we compare the intracellular transcriptome (T0, T6, and T18) to exponential growth in broth, in order to identify infection-related changes in gene expression. We also analyzed how gene expression evolved during infection but in contrast to Bruggemann et al. (2006) we used a very early time point (T0) as the control. Therefore, comparison between the data or the conclusion of both studies should be done cautiously. In other words, differences between these data sets are likely due to differences in the experimental design and these two studies should be seen as complementary. For example, glycerol catabolism, although induced inside THP-1 cells compared to exponential growth, is not differentially regulated in *A. castellanii* between intracellular time points. However, when the THP-1 dataset was analyzed for differential expression during intracellular multiplication (T6/T0 and T18/T0), glycerol catabolism is not differentially expressed, because it is highly expressed at T0. **Table S5** in Supplementary Material contains the complete data sets of *L. pneumophila* growing inside *A. castellanii* (8 vs 14, 11 vs 14, and 8 vs 11) and the complete data sets of *L. pneumophila* growing inside THP-1 macrophages (T0/E, T6/E, T18/E and T6/T0, T18/T0). Nonetheless, some of the similarities and the differences will be mentioned hereafter but should be taken cautiously. Many genes involved in amino acid transport and degradation (**Figure 6**) as well as genes predicted to encode myo-inositol catabolism pathways were induced in both protozoan and mammalian host. In addition, protein synthesis machinery was repressed in both the protozoan and the mammalian host. Many known virulence factors are induced in both host cells, including *enhA*, *enhB*, *enhC*, and *rtxA* (**Figure 7B**). However, inside protozoa, *L. pneumophila* does not seem to induce expression of genes involved in amino acid synthesis pathways, contrary to what was observed during growth inside macrophages. The Entner–Doudoroff pathway, which was induced in *A. castellanii* was not induced during growth in human macrophages. Strikingly, we did not observe differential regulation of the *icm/dot* genes during growth in human macrophages contrary to what was observed inside *A. castellanii* even when we compare the late time points (T6, T18) to T0. Induction of genes encoding many Icm/Dot effectors was observed in both cases (for example RalF) but some genes were not induced in *A. castellanii* (for example LegC4, LegL5, and the three new effectors identified: Lpg1959, Lpg1961, and Lpg2828).

In conclusion, we have analyzed the transcriptome of *L. pneumophila* during infection of human tissue culture macrophages. The use of SCOTS to enrich bacterial transcript allowed us to use a low MOI and to study time points where the number of bacteria would not have yielded sufficient levels of RNA for standard microarray protocols. Acquisition of amino acids and biosynthesis of L-arginine, L-histidine, L-aspartate, and L-lysine were induced during intracellular multiplication. Interestingly, glycerol catabolism was also induced, suggesting that inside cells, *Legionella* not only acquires carbon from amino acids, but also from glycerol. The

high proportion of Icm/Dot effectors induced during infection, together with the lack of induction of a stress response enforces the idea that during *L. pneumophila* infection, the Icm/Dot system is the major mediator of virulence.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/cellular_and_infection_microbiology/10.3389/fmicb.2011.00060/abstract/

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Secrets of a successful pathogen: *Legionella* resistance to progression along the autophagic pathway

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To proliferate within phagocytes, *Legionella pneumophila* relies on Type IV secretion to modulate host cellular pathways. Autophagy is an evolutionarily conserved degradative pathway that captures and transfers a variety of microbes to lysosomes. Biogenesis of *L. pneumophila*-containing vacuoles and autophagosomes share several features, including endoplasmic reticulum (ER)-derived membranes, contributions by the host GTPases Rab1, Arf1 and Sar1, and a final destiny in lysosomes. We discuss morphological, molecular genetic, and immunological data that support the model that, although A/J mouse macrophages efficiently engulf *L. pneumophila* within autophagosomal membranes, the Type IV effector proteins DrrA/SidM, LidA, and RalF prolong association with the ER. By inhibiting immediately delivery to lysosomes, the bacteria persist in immature autophagosomal vacuoles for a period sufficient to differentiate into an acid-resistant, replicative form. Subsequent secretion of the Type IV effector LepB releases the block to autophagosome maturation, and the adapted progeny continue to replicate within autophagolysosomes. Accordingly, *L. pneumophila* can be exploited as a genetic tool to analyze the recruitment and function of the macrophage autophagy pathway.

Keywords: *Legionella pneumophila*, Type IV secretion system, autophagy, vacuole maturation, Rab conversion

INTRODUCTION

Legionella pneumophila is an accidental respiratory pathogen that can cause pneumonia in people whose immune defenses are compromised. The natural hosts of *L. pneumophila* are different species of protozoa that are abundant in aquatic environments (Lau and Ashbolt, 2009). *L. pneumophila* thrives in natural ecosystems such as ponds, rivers and moist soil, and also in man-made water systems, including cooling towers, whirlpools, and vegetable misters. Although protozoa routinely ingest bacteria as a food source, they can be parasitized by some species of *Legionella*. Evolutionary pressure to survive and replicate in professional phagocytes of water and soil has led to the emergence of virulence traits that also equip *L. pneumophila* to proliferate in a similar eukaryotic host, the macrophage. Protozoa and macrophages possess similar anti-microbial defenses, such as production of reactive oxygen and nitrogen species and delivery of invading microbes to the acidic, hydrolytic lysosomes via phagocytosis. Indeed, prior growth in ameba augments subsequent replication in both macrophages and mouse models of infection (Cirillo et al., 1994, 1999; Neumeister et al., 2000).

Upon inhalation within contaminated aerosols, *L. pneumophila* are phagocytosed by alveolar macrophages. However, the nascent *L. pneumophila* phagosome avoids the endocytic pathway and instead forms a unique replication vacuole that interacts with particular organelles, including mitochondria and endoplasmic reticulum (ER; Horwitz, 1983a, 1983b; Swanson and Isberg, 1995). After a few rounds of replication in permissive A/J mouse macrophages, the *L. pneumophila* vacuole acquires lysosomal components, and the progeny continue to replicate in a phagolysosomal compartment (Sturgill-Koszycki and Swanson, 2000). Its mode of entry and replication in host cells require a Type IV secretion system named *d*efect in *o*rganelle *t*rafficking/*i*ntracellular *m*ultiplication (Dot/Icm; Hilbi et al., 2001; Bandyopadhyay et al., 2004, 2007; Hubber and Roy, 2010). To establish a replication niche, intracellular *L. pneumophila* exploit Type IV secretion to deliver to the host cytosol a large number of effectors predicted to modulate cellular pathways that are highly conserved in ameba and macrophages (Ensminger and Isberg, 2009). Here we focus on interactions between *L. pneumophila* and the autophagy pathway, an alternate route to the lysosomes of macrophages and amebae.

Autophagy is best known as a catabolic process in which cellular cytoplasm and organelles are degraded as a means to cope with starvation. More than 30 autophagy (Atg) genes in yeast and at least 20 in mammals regulate autophagosome formation and maturation (Mehrpour et al., 2010). Autophagy begins when a double-membraned structure called an isolation membrane, or phagophore, forms around cytoplasm or organelles destined for degradation. The phagophore expands and closes on itself to form a double-membraned vacuole, or autophagosome. In a series of tightly controlled events, the phagophore fuses with vesicles from the endocytic pathway. Maturation is complete when the autophagosome merges with lysosomal vacuoles to form an autophagolysosome, wherein contents of the vacuole are degraded. In addition to its long-established role as a non-selective response to starvation and more recent recognition as a selective mechanism for disposal of damaged organelles or misfolded proteins marked by ubiquitin (Pankiv et al., 2007; Kirkin et al., 2009; Thurston

et al., 2009), autophagy is also recruited by the innate and adaptive immune systems (Levine et al., 2011).

AUTOPHAGY, AN INNATE DEFENSE MECHANISM AGAINST INTRACELLULAR PATHOGENS

By capturing cytosolic invaders and delivering them to lysosomes, autophagy acts as a barrier against a variety of microbes. When *Streptococcus pyogenes*, *Salmonella enterica*, *Listeria monocytogenes*, or *Mycobacterium tuberculosis* damage or escape from their phagosomes, some of the microbes are ubiquitinated, recognized by the autophagic surveillance system and trafficked to lysosomes for degradation (Nakagawa et al., 2004; Perrin et al., 2004; Birmingham et al., 2006; Thurston et al., 2009; Yoshikawa et al., 2009; Zheng et al., 2009; Ponpuak et al., 2010). A recent study identified another pathway to capture cargo for autophagy: the cytoskeletal protein Septin traps *Shigella flexneri* within a meshwork that targets the intracellular bacterium for autophagic degradation (Mostowy et al., 2010).

Bacterial pathogens that reside in endosomal compartments also face death by autophagy, and some of the host regulatory factors have been identified. For example, IFN-γ stimulated cells deliver vacuoles containing *M. tuberculosis* to lysosomes via autophagy (Gutierrez et al., 2004). Similarly, *Chlamydia trachomatis* inclusion vacuoles, which typically evade lysosomes, are routed to the autophagic pathway upon IFN-γ activation. This alteration in trafficking is mediated by the host immunity related GTPase (IRG), Irga6 (Al-Younes et al., 2004; Al-Zeer et al., 2009). Infection by the parasite *Toxoplasma gondii* is also controlled by autophagy *in vivo* and *in vitro*. CD40 signaling recruits the autophagy pathway to capture the intracellular parasites, evident from the exacerbated ocular and brain pathology displayed by CD40−/− mice, which are susceptible to chronic toxoplasmosis (Andrade et al., 2006; Ling et al., 2006). *S. enterica* serovar Typhimurium is captured within autophagosomal membranes derived from the ER by a mechanism that requires Rab1 (Huang et al., 2011), a host GTPase that will be discussed in more detail below.

Selective pressure to circumvent autophagic killing has led to emergence of virulence traits that equip pathogens to survive and replicate in host cells. *Coxiella burnetii* is an intracellular pathogen that proliferates in spacious vacuoles that eventually fuse with lysosomes. For efficient replication, *C. burnetii* requires induction of autophagy and inhibition of apoptotic cell death (Beron et al., 2002; Gutierrez et al., 2005; Romano et al., 2007). *Francisella tularensis* has a remarkable trafficking pattern inside mouse bone marrow-derived macrophages. Soon after infection, *F. tularensis* breaks out of its phagosome and replicates in the cytoplasm. At later stages of infection, *F. tularensis* resides in vacuoles with features of autophagosomes. After reentering the endocytic pathway via autophagy, *F. tularensis* can be exocytosed from the cell (Checroun et al., 2006).

Several lines of evidence have also pointed to autophagy as a strategy for host cells to combat *L. pneumophila* infection. First, *Dictyostelium discoideum* that lack the autophagy protein Atg9 are more permissive for infection by *L. pneumophila* (Tung et al., 2010). Likewise, when expression of Atg5 by A/J mouse peritoneal macrophages is reduced by siRNA, the yield of *L. pneumophila* increases throughout the 48-h infection period (Matsuda et al., 2009). Conversely, *L. pneumophila* replication is inhibited when autophagy is induced by treating A/J macrophages with 2-deoxy-d-glucose (Matsuda et al., 2009), a non-hydrolyzable analog of glucose that inhibits glycolysis (Wick et al., 1957). In addition, as discussed in more detail below, the *L. pneumophila* vacuole traffics along the autophagic pathway more rapidly in restrictive C57Bl/6 mouse macrophages compared with permissive A/J *naip5* mutant mouse macrophages (Amer and Swanson, 2005). Next we review morphological, molecular, and immunological data, drawn primarily from studies of *L. pneumophila* trafficking in A/J mouse macrophages, that are consistent with a model in which *L. pneumophila* utilizes effectors of the Dot/Icm Type IV secretion system to stall progression of its autophagosomal vacuole to overcome this innate defense against intracellular infection.

ER CONTRIBUTES TO BIOGENESIS OF *L. PNEUMOPHILA* VACUOLES AND AUTOPHAGOSOMES

The biogenesis of the *L. pneumophila* replication vacuole is remarkably similar to autophagosome formation. First, the two vacuoles can receive membrane from the same source, the ER. Elegant electron microscopic studies first by Horwitz and later by Swanson and Tilney and their colleagues describe *L. pneumophila* vacuole biogenesis (Horwitz, 1983b; Swanson and Isberg, 1995; Tilney et al., 2001). Immediately after infection, the *L. pneumophila* phagosomal membrane resembles the host plasma membrane. Within minutes of uptake by U937 human monocytic cells, osmophilic hair-like projections connect vesicles to the cytoplasmic face of the *L. pneumophila* phagosome. These vesicles appear to fuse with the phagosomal membrane and with each other to form a double-membraned vacuole, whose thickness is typical of ER. Within the first hours of infection, ribosomes are attached to the cytoplasmic face of the vacuole, which now resembles rough ER. Furthermore, fluorescence microscopy studies of macrophages derived from bone marrow of A/J mice demonstrate that *L. pneumophila* vacuoles co-localize with a variety of ER markers, including the luminal proteins BiP, glucose-6-phosphatase, and protein disulfide isomerase; the ER membrane proteins calnexin and Sec22b; and yellow fluorescent protein coupled to the ER localization signal KDEL (Swanson and Isberg, 1995; Kagan and Roy, 2002; Derre and Isberg, 2004a; Robinson and Roy, 2006; Arasaki and Roy, 2010).

Several recent reports provide compelling morphological evidence that the ER is also one source of autophagosomal membranes (Axe et al., 2008; Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009). Autophagosomes originate at phosphatidylinositol-3-phosphate (PI3P)-enriched sections of ER called omegasomes. In addition, autophagosomes are connected to the ER by narrow extensions, and their membranes are a similar thickness (5–7 nm; Arstila and Trump, 1968; Yla-Anttila et al., 2009). Furthermore, immunoelectron microscopy studies of the phagophore membranes identify ER marker proteins and cisternae (Hayashi-Nishino et al., 2009). Thus, in addition to the plasma membrane (Ravikumar et al., 2010) and mitochondria (Hailey et al., 2010), the ER can contribute membrane for autophagosome biogenesis.

Initiation and elongation of the phagophore is coordinated by a cascade of Atg proteins, including two ubiquitin-like conjugation systems (Geng and Klionsky, 2008; Mehrpour et al., 2010).

Once Atg7 activates Atg12, then Atg5 is conjugated to Atg12. Atg16L1 is recruited to the growing autophagosome where it forms a multimeric complex with Atg5 and Atg12. Next, cytosolic Atg8, also known as LC3, is cleaved and conjugated to phosphatidylethanolamine in the autophagosomal membrane. Conjugation of LC3 to phosphatidylethanolamine depends on Atg7 and the Atg5–Atg12:Atg16L1 complex. As the phagophore closes to form an autophagosome, Atg16L1 dissociates. Like autophagosomes, newly formed *L. pneumophila* vacuoles sequentially acquire and then lose Atg7 and Atg8/LC3 (Amer and Swanson, 2005).

During starvation-induced autophagy, the sudden demand for membrane may be readily met by membranous folds of the ER. Similar to *L. pneumophila*, the intracellular eukaryotic parasite *T. gondii* resides in a protective vacuole that interacts intimately with the ER (Melo and de Souza, 1997) and that can also be a target of autophagy (Andrade et al., 2006; Ling et al., 2006). Accordingly, we propose that, in response to cytosolic infection, the autophagy machinery recruits the ER to sequester invading microbes.

L. PNEUMOPHILA VACUOLES AND AUTOPHAGOSOMES MERGE WITH THE LYSOSOMES

In the initial stages of infection, the *L. pneumophila* vacuole does not fuse with lysosomes (Horwitz, 1983b). The vacuole is isolated from the endocytic pathway, as the compartment is inaccessible to exogenous soluble and lipid probes (Joshi et al., 2001). Nutrient cues likely trigger differentiation of intracellular *L. pneumophila* to the replicative phase (Sauer et al., 2005; Wieland et al., 2005). Since only post-exponential phase *L. pneumophila* express the virulence factors that inhibit phagosome–lysosome fusion (Byrne and Swanson, 1998; Fernandez-Moreira et al., 2006), the differentiation state of the bacteria impacts the fate of the pathogen's vacuole. Beginning ∼8 h after infection of A/J mouse macrophages, lysosomal markers co-localize with the *L. pneumophila* vacuole (Sturgill-Koszycki and Swanson, 2000). Although *L. pneumophila* can replicate at neutral pH in broth and human monocytic cells (Wieland et al., 2004), in A/J macrophages fusion with the lysosomes actually promotes bacterial replication, since pharmacological inhibition of lysosome acidification and phagosome maturation by bafilomycin A inhibits *L. pneumophila* growth. The period when lysosome fusion is stalled likely allows the bacteria to differentiate to an acid-resistant form (Sturgill-Koszycki and Swanson, 2000). The lysosomal bacteria continue to replicate in A/J macrophages for an additional 10–15 h, until the cell lyses to release progeny that are primed for infection (Byrne and Swanson, 1998).

The fate of a newly formed autophagosome is similar to that of the *L. pneumophila* replication vacuole. Once formed from membranes of the ER, autophagosomes quickly fuse with early endosomes and late endosomes, generating vacuoles referred to as amphisomes (Liou et al., 1997; Berg et al., 1998; Swanson et al., 2009). These vacuoles acidify and traffic along the microtubular network toward the perinuclear region, where they merge with lysosomes (Kimura et al., 2008).

From the origin of their membrane to their final destination, the *L. pneumophila* vacuole and the autophagosome share many morphological features. However, differences exist between the two vacuoles. First, unlike *L. pneumophila* replication vacuoles, autophagosomes do not accumulate ribosomes on their surface. Second, in macrophages derived from the bone marrow of A/J *naip5* mutant mice, non-selective autophagosomes induced by starvation or pharmacologically mature at a faster rate than *L. pneumophila* vacuoles (Amer and Swanson, 2005). We speculate that the capacity of particular Type IV effector proteins, discussed below, to prolong interactions with the ER slows maturation of the *L. pneumophila* autophagosomal vacuole, enabling ribosomes to accumulate.

Genetic analysis of the contribution of Atg proteins to biogenesis of *L. pneumophila* replication vacuoles is one approach to test the impact of autophagy on the pathogen's fate. Otto et al. (2004) analyzed the yield of *L. pneumophila* over an 8-day period in wild-type *D. discoideum* or a number of autophagy mutants. The host factors analyzed were Atg1 and Atg6, proteins that contribute to the initial stages of autophagosome formation, and Atg5, Atg7, and Atg8, components of ubiquitin-like conjugation systems that mediate autophagosome elongation (Chen and Klionsky, 2011). Since the mutant ameba supported *L. pneumophila* replication to levels similar to wild-type, *L. pneumophila* can replicate independently of several factors that promote autophagosome biogenesis. Whether the bacteria resided in ER-derived. lysosomal, or other compartments when *L. pneumophila* replication was first evident >48 h after infection of the *atg* mutant *D. discoideum* was not analyzed. Subsequent molecular genetic studies in the *D. discoideum* model reported that the autophagy protein Atg9 equips amebae to restrict infection by *L. pneumophila* (Tung et al., 2010). Kinetic studies using markers for ER and the endosomal pathway in wild-type and mutant *D. discoideum* phagocytes are needed to determine when the *L. pneumophila* vacuole intersects the autophagy pathway, whether the pathogen delays autophagosome maturation, and the composition of vacuoles that support bacterial replication in these environmental host cells.

A SUBSET OF GTPASES DIRECT ER RECRUITMENT BY BOTH *L. PNEUMOPHILA* VACUOLES AND AUTOPHAGOSOMES

Rab proteins are small GTP-binding proteins that regulate vesicle trafficking. Rab proteins cycle between a cytosolic, inactive GDP-bound state and a membrane-associated, active GTP-bound state (Barr and Lambright, 2010). Cycling between the two states is catalyzed by guanine exchange factors (GEFs), proteins that exchange GDP for GTP, and by GTPase activating proteins (GAPs) that stimulate GTP hydrolysis to inactivate the Rab protein. Additionally, Rab proteins bind SNARE and SNARE-associated proteins, soluble effector proteins that mediate membrane fusion. As vesicles progress along either the secretory and endosomal pathways, Rab proteins dedicated to each distinct compartment are sequentially recruited and then displaced: this so-called "Rab conversion" is critical for maturation of the organelles (Rink et al., 2005).

Legionella pneumophila replication vacuoles and autophagosomes each associate with Rab1, a GTPase of the early secretory pathway that regulates fusion between vesicles exiting the ER and the cis-Golgi (Stenmark, 2009). Immediately after uptake by A/J mouse macrophages or U937 human monocytic cells, Rab1 co-localizes with the *L. pneumophila* phagosome; by 4 h, it cycles off the vacuole (Derre and Isberg, 2004b; Kagan et al., 2004). Rab1 promotes bacterial replication, since transfection of COS1 and

CHO cells with an inactive form of Rab1 reduces the yield of *L. pneumophila*.

Several *L. pneumophila* effectors that regulate Rab1 activity have been identified, highlighting the significance of this host protein for *L. pneumophila* trafficking. For example, two *L. pneumophila* Type IV secreted proteins, DrrA and LidA, increase the pool of Rab1–GTP, whereas a third, LepB, stimulates GTP hydrolysis to generate Rab1–GDP (Murata et al., 2006; Ingmundson et al., 2007; Machner and Isberg, 2007; Brombacher et al., 2009; Muller et al., 2010). The capacity of this trio of *L. pneumophila* effectors to modulate trafficking in the host secretory pathway was verified when their ectopic expression disrupted the Golgi network of CHO and COS1 cells (Derre and Isberg, 2005; Machner and Isberg, 2007). LidA may play additional roles because it also interacts with Rab6 and Rab8, and the effector is expressed throughout the bacterial replication period (Conover et al., 2003; Machner and Isberg, 2007).

Time course studies of co-localization of the *L. pneumophila* effectors that modulate Rab1 suggest that the pathogen vacuole retains secretory vesicles for a defined period. Both DrrA and LidA are detected on *L. pneumophila* vacuoles shortly after uptake by primary mouse macrophages (Conover et al., 2003; Ingmundson et al., 2007). In contrast, LepB decorates the *L. pneumophila* vacuole at later times, reaching a high plateau 9 h after infection of A/J mouse macrophages (Ingmundson et al., 2007). Single cell analysis underscored that as LepB accumulates, host Rab1 and bacterial DrrA/SidM disassociate from the *L. pneumophila* vacuole, becoming undetectable by 4 h. The continued presence of DrrA and LidA during the initial stage of *L. pneumophila* vacuole maturation is predicted to ensure persistent Rab1 activation and prolong recruitment of ER vesicles. By blocking Rab conversion, retention of active Rab1 by DrrA and LidA may stall *L. pneumophila* vacuole maturation by inhibiting association of downstream Rab GTPases such as Rab5 and Rab7, which facilitate fusion with early and late endosomes, respectively. Subsequent secretion by *L. pneumophila* of the effector LepB catalyzes Rab1 to cycle to its inactive form. As a consequence, Rab conversion is predicted to proceed, thereby relieving the block to autophagosome maturation. By this stage of the infection of A/J macrophages, the intracellular bacteria have differentiated to a replicative form that is acid-resistant and equipped to exploit lysosomes as a replication niche (Sturgill-Koszycki and Swanson, 2000).

Consistent with the contribution of ER membrane to autophagosome biogenesis, Rab1 co-localizes with LC3 (Atg8) on autophagosomes generated by starvation of CHO cells (Zoppino et al., 2010). Rab1-positive autophagosomes do not acquire cathepsin D or degradative capacity, indicating that these vesicles represent an early stage of autophagosome maturation. Moreover, over-expression of Rab1 stimulates autophagosome biogenesis, as judged by localization and processing of LC3. Conversely, reducing Rab1 expression by siRNA reduces autophagy (Zoppino et al., 2010). Using similar approaches, Huang et al. (2011) documented that Rab1 contributes to autophagosome formation, clearance of ubiquitinated protein aggregates, and sequestration and degradation of *Salmonella typhimurium*. In summary, Rab1 association with *L. pneumophila* vacuoles and with autophagosomes is a critical step in biogenesis of both of these ER-derived vacuoles. By secreting effectors that trap active Rab1, *L. pneumophila* is predicted to stall maturation, providing the time needed for the pathogen to differentiate to a state that can tolerate and exploit an acidic, hydrolytic autophagolysosomal compartment of A/J macrophages.

A second GTPase in the early secretory pathway that has been implicated in the biogenesis of both *L. pneumophila* replication vacuoles and autophagosomes is Sar1. CHO FcgRII cells that express a dominant negative form of Sar1 fail to tether ER vesicles to the *L. pneumophila* phagosome, and their bacterial yield 11 h after infection is reduced (Kagan and Roy, 2002). Likewise, CHO cells that express either a dominant negative form of Sar1 or reduced amounts of wild-type Sar1 protein contain fewer autophagosomes (Zoppino et al., 2010). That Sar1 promotes formation of *L. pneumophila* replication vacuoles and autophagosomes lends further strength to the model that *L. pneumophila* is captured by autophagy, but the pathogen utilizes Type IV secretion to stall this host defense pathway, securing time for the intracellular bacterium to adapt to its replication niche.

Arf1 is a third host GTPase known to regulate vesicular traffic in the secretory pathway that contributes to formation of not only *L. pneumophila* replication vacuoles but also autophagosomes. *L. pneumophila*-infected CHO FcgRII cells that express a dominant negative form of Arf1 contain a reduced number of intracellular bacteria 11 h after infection (Kagan and Roy, 2002). The *L. pneumophila* Type IV secretion effector protein RalF acts as a GEF that promotes Arf1 association with the bacterial vacuole (Nagai et al., 2002). However, since RalF mutants do not exhibit the intracellular growth defect observed in cells whose Arf1 function is impaired, other bacterial factors likely regulate the GTPase activity. Recent experiments in yeast determined that Arf1 also contributes to autophagosome biogenesis. In particular, genetic analysis identified this GTPase, as well as the Arf1 GEF protein Sec7, as critical for the Atg8/LC3 processing that promotes autophagosome biogenesis (van der Vaart et al., 2010). Thus, Arf1 is one of three GTPases that function in the secretory pathway and contribute to biogenesis of both *L. pneumophila* replication vacuoles and autophagosomes.

POLYUBIQUITINATED PROTEINS ARE ASSOCIATED WITH AUTOPHAGOSOMES AND *L. PNEUMOPHILA* VACUOLES

Since polyubiquitinated proteins are cargo for autophagosomes and also surround *L. pneumophila* vacuoles, they provide more clues to understanding the pathogen's fate. Ubiquitination is an evolutionarily conserved mechanism that tags proteins for degradation by host proteasomes or lysosomes (Clague and Urbe, 2010). Ubiquitin also targets proteins and cytosolic bacteria for selective autophagy. The cytoplasmic adaptor proteins p62, NBR1, or NDP52 bind either ubiquitinated protein aggregates or intracellular *S. typhimurium, L. monocytogenes*, or *M. tuberculosis*, marking them as cargo for selective autophagy (Pankiv et al., 2007; Kirkin et al., 2009; Thurston et al., 2009; Yoshikawa et al., 2009; Ponpuak et al., 2010). For example, *S. typhimurium* that enter the cytosol become ubiquitinated and are degraded by autophagy, and p62 and NDP52 are required for efficient killing of the cytosolic bacteria (Zheng et al., 2009).

Shortly after ingestion by A/J mouse macrophages, U937 human monocytic cells or *Acanthamoeba polyphaga*, the *L. pneumophila* vacuole is studded with ubiquitinated proteins, which persist during the bacterial replication period (Dorer et al., 2006; Ivanov and Roy, 2009; Price et al., 2009). A number of proteins translocated by the Dot/Icm Type IV secretion system contain F-box and U-box motifs, hallmarks of E3 ubiquitin ligases, which transfer ubiquitin to proteins to be degraded (Cazalet et al., 2004; Al-Khodor et al., 2008; Kubori et al., 2010). At least two of the five known F-box containing proteins of *L. pneumophila* exhibit E3 ubiquitin ligase activity and interact with host proteins (Ensminger and Isberg, 2010; Lomma et al., 2010). For example, AnkB (LegAU13) induces ubiquitinated proteins to accumulate on the bacterial vacuole; it also enhances intracellular of growth of one strain of *L. pneumophila*, but not others (Al-Khodor et al., 2008; Ivanov and Roy, 2009; Ensminger and Isberg, 2010). LubX is a U-box containing E3 ubiquitin ligase that negatively regulates the bacterial Dot/Icm effector SidH by ubiquitinylation and also increases survival of infected *D. melanogaster* (Kubori et al., 2010). However, LubX is not required for *L. pneumophila* replication inside murine macrophages or protozoan cells (Kubori et al., 2008). Yet, bacterial replication is attenuated in cells that express reduced amounts of the host proteins Clk1, a substrate of LubX, and Cdc48/p97, a chaperone predicted to enhance translocation of ubiquitinated Type IV secretion substrates (Dorer et al., 2006; Kubori et al., 2008).

At present it is unclear whether ubiquitination of the *L. pneumophila* vacuole is driven by the pathogen, or the host. The protein modification may aid translocation of Type IV secretion substrates, or modulate maturation of the vacuole. Certain host Atg enzymes catalyze ubiquitin-like reactions during autophagosome biogenesis. However, since none of the Atg proteins have the structure or the HECT- or RING-type motifs typical of E3 ligases (Geng and Klionsky, 2008), it seems unlikely that any of the known *L. pneumophila* E3 enzymes, which do contain these motifs, act directly as mimics of Atg proteins. An alternative hypothesis that remains to be tested is that, by tagging vacuoles modified or damaged by Type IV secretion with ubiquitin, the host cell directs *L. pneumophila* to the selective autophagic pathway, perhaps via the adaptor proteins NDP52 or p62 (Thurston et al., 2009; Ponpuak et al., 2010). Consistent with the idea that ubiquitination of the pathogen vacuole is advantageous to the host is the unexpected observation that the F-box bacterial effector AnkB reduces, rather than increases, endogenous ubiquitination of one host target protein (Lomma et al., 2010).

MATURATION OF THE *L. PNEUMOPHILA* VACUOLE IS GOVERNED BY THE INNATE IMMUNE RESPONSE

The innate intracellular immune receptor Naip5 plays a critical role in resistance to *L. pneumophila* infection (Diez et al., 2003; Wright et al., 2003; Derre and Isberg, 2004b; Zamboni et al., 2006). Naip5 is a NOD-like receptor (NLR) protein postulated to detect cytosolic contamination of flagellin delivered to the cytoplasm, perhaps via the Dot/Icm system. Recognition of flagellin by Naip5 initiates host immune responses that control *L. pneumophila* infection in C57Bl6 macrophages (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Fortier et al., 2007). Interestingly, in A/J macrophages, which have reduced Naip5 function, maturation of the *L. pneumophila* vacuole is sluggish, and the pathogen replicates to high numbers (Amer and Swanson, 2005). For example, bone marrow-derived macrophages from A/J mice display maximum co-localization of Atg7 with *L. pneumophila* phagosomes within minutes, and then the association diminishes by 2 h. LC3 becomes visible on the phagosome at 4 h; by 6 h, 50% of *L. pneumophila* phagosomes co-localize with LC3. In contrast, in restrictive C57Bl6 macrophages Atg7 and LC3 association with the bacterial vacuole is rapid: by 1 h, some phagosomes first acquire, and then shed, both Atg7 and Atg8 (Amer and Swanson, 2005); by 2 h more vacuoles have merged with lysosomes (Fortier et al., 2007); by 48 h, intracellular bacteria are degraded (Molofsky et al., 2006). Whether Naip5 protein equips mouse macrophages to restrict *L. pneumophila* infection by promoting a rapid autophagic response to cytosolic contamination remains to be tested directly in isogenic mouse strains. We next propose a model that incorporates these interesting mouse genetics observations in the context of the morphological and molecular genetic studies of the biogenesis of *L. pneumophila* vacuoles and autophagosomes.

L. PNEUMOPHILA VACUOLES RESIST MATURATION ALONG AUTOPHAGIC PATHWAY

We postulate that, when confronted by autophagy as a host defense, *L. pneumophila* retards autophagosome maturation to establish a productive infection in professional phagocytes. The morphological and molecular similarities between biogenesis of *L. pneumophila* replication vacuoles and autophagosomes, as well as the capacity of Naip5 to restrict infection of mouse and human macrophages by *L. pneumophila* and increase delivery of the pathogen to lysosomes (Diez et al., 2003; Wright et al., 2003; Derre and Isberg, 2004b; Amer et al., 2006; Molofsky et al., 2006; Fortier et al., 2007; Vinzing et al., 2008) are consistent with the following working model (**Figure 1**). After uptake by cells, *L. pneumophila* effectors along with contaminating flagellin are transported by Type IV secretion to the host cytoplasm. Recognition of flagellin by Naip5 triggers the autophagic defense mechanism, which relies on the GTPases Rab1, Arf1, and Sar1 to deliver secretory vesicles from the ER to envelop the pathogen's vacuole. To inhibit rapid maturation into a toxic, acidic autophagolysosome, *L. pneumophila* immediately delivers Type IV effectors to retain ER components. In particular, DrrA and LidA secreted by the pathogen activate and retain Rab1. Likewise, the bacterial effector RalF activates Arf1. Retention of active Rab1 and Arf1 prevents their replacement by a distinct set of Rab proteins needed to recruit vesicles from the endosomal pathway. Thus, *L. pneumophila* stalls in an ER-derived vacuole that resembles an immature autophagosome. After several hours in permissive A/J macrophages, *L. pneumophila* expresses a new class of effectors, including LepB, that release Rab1 from the vacuole (Ingmundson et al., 2007). As a consequence, the vacuole resumes maturation to form an autophagolysosome. Presumably, a deliberate, measured pause within an ER-derived immature autophagosome provides *L. pneumophila* time sufficient to induce acid resistance and other traits critical to exploit lysosomes as a replication niche (Sturgill-Koszycki and Swanson, 2000). For example, *L. pneumophila* can use as a nutrient source short peptides (Sauer et al., 2005; Wieland et al., 2005), which are likely abundant in lysosomes. Thus, we speculate that residence in the lysosomal compartment provides a constant supply of

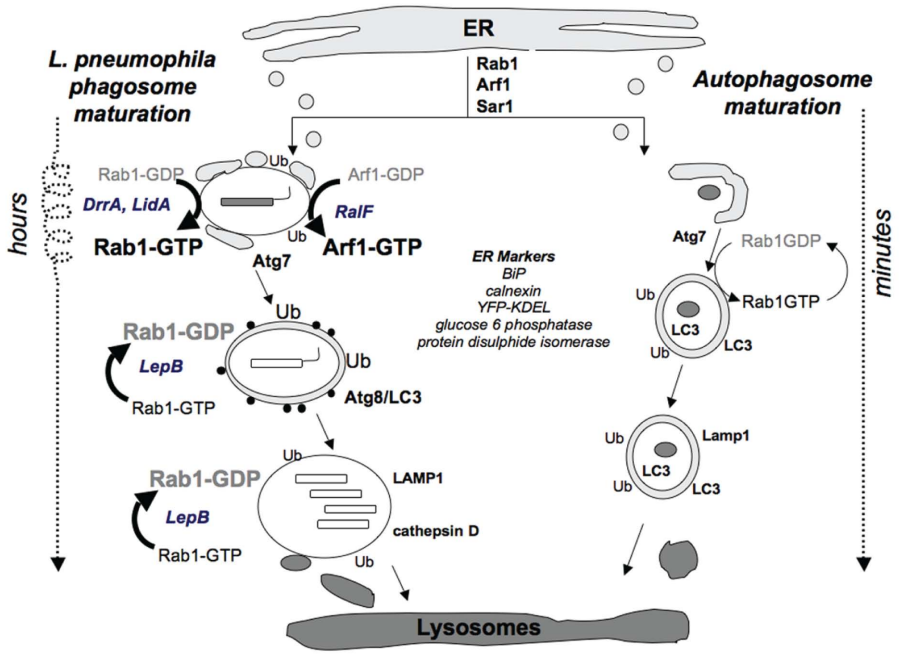

FIGURE 1 | Model for stalled maturation of the *L. pneumophila* vacuole in the autophagic pathway of permissive phagocytes. ER serves as a membrane source for both *L. pneumophila* vacuoles and autophagosomes. Early secretory vesicles (gray circles) from the ER are recruited to both organelles in a Rab1-, Arf1-, and Sar1-dependent manner. The double-membraned, LC3 positive, autophagosome sequentially fuses with vesicles from the early endocytic pathway and finally with lysosomes, where its cargo is degraded. Maturation of *L. pneumophila* is stalled at an early stage when bacterial proteins (italics) DrrA and LidA persistently activate Rab1 and RalF activates Arf1. Thus, the vacuole acquires ER markers BiP, glucose-6-phosphate, calnexin, protein disulfide isomerase, YFP–KDEL, and ribosomes (filled circles). After several hours, *L. pneumophila* secretes LepB, an effector that inactivates Rab1, releasing it from the membrane. Subsequently, the immature autophagosomal vacuole matures to an autophagolysosome, which accumulates the lysosomal proteins cathepsin D and LAMP1. The deliberate pause coordinated by Type IV secretion effectors enables the pathogen to differentiate into an acid-resistant, replicative form that exploits lysosomes as a replication niche.

nutrients and vacuolar membrane to support bacterial replication in A/J macrophages.

Our model predicts that, in response to cytosolic flagellin, the NLR protein Naip5 stimulates macrophage autophagy as a barrier to cytosolic infection. A precedent for NLR-mediated induction of autophagy is the discovery that NOD1 and NOD2 physically interact with Atg16L1 and promote autophagy of intracellular *S. flexneri* and *S. typhimurium* (Cooney et al., 2010; Travassos et al., 2010). In particular, we postulate that the wild-type level of Naip5 protein expressed by restrictive C57Bl6 macrophages triggers a robust autophagic response (Amer and Swanson, 2005) that is sufficient to overcome the pathogen's effector proteins and deliver the vacuole to lysosomes. In contrast, the partial Naip5 function of A/J macrophages elicits a sluggish autophagic response, and the *L. pneumophila* effectors successfully stall autophagosome maturation. Our model also predicts either that autophagosomes mature more slowly in human macrophages and amebae, which are permissive for infection, compared to mouse phagocytes, which are not. Alternatively, the bacterial effectors DrrA, LidA, and RalF may bind and activate the Rab1 and Arf1 proteins of human and amebae more efficiently than the mouse substrates. Thus, we postulate that the outcome of *L. pneumophila* infection of environmental or mammalian phagocytes is dictated by a competition: how efficiently host autophagy delivers the microbe to lysosomes versus How effectively the pathogen arsenal unleashed by Type IV secretion stalls autophagosome maturation.

CONCLUSION

A large number of effectors released by *L. pneumophila* have eukaryotic-like motifs and functions (Cazalet et al., 2004; Lurie-Weinberger et al., 2010). Remarkably, a number of these proteins equip the pathogen to modulate the activity of host GTPases, first to stall, and then later resume, the exchange of vesicles from the host secretory pathway. By this strategy, *L. pneumophila* delays its immediate delivery to anti-microbial autophagolysosomes.

Autophagy is modulated by at least two other pathogens to replicate inside cells, *C. burnetii* and *F. tularensis*. The *C. burnetii* phagosome associates with LC3 within minutes, and this association is maintained for up to 48 h in CHO cells (Gutierrez et al., 2005; Romano et al., 2007). Notably, cathepsin D, a lysosomal enzyme, is acquired at a slower rate by phagosomes containing live *C. burnetii* compared with inactivated bacteria (Romano et al., 2007). The authors propose that association of *C. burnetii* with autophagosomes delays their delivery with lysosomes, perhaps providing time for infectious *C. burnetii* to differentiate into their replicative cell type. Later in infection, maturation of the *C. burnetii* phagosome depends on association with Rab1 and fusion with vesicles from the early secretory pathway, possibly

to acquire nutrients and membrane for the expanding vacuole (Campoy et al., 2011).

Similar to *L. pneumophila* and *C. burnetii*, delay in progression along the autophagic pathway has been suggested as a successful strategy of *F. tularensis*. Peripheral blood monocytes infected with *F. tularensis* down regulate key autophagy genes, as shown by microarray analysis; yet, morphological evidence demonstrates *F. tularensis* within autophagosomal vacuoles later in infection (Checroun et al., 2006; Butchar et al., 2008). Perhaps by inhibiting expression of autophagy genes, *F. tularensis* secures the time needed to differentiate to a form that can resist the harsh environment of autophagolysosomes (Cremer et al., 2009). Moreover, *F. tularensis* may hijack exocytosis of lysosomes to egress out of the cell. In summary, the three pathogens *L. pneumophila*, *C. burnetii*, and *F. tularensis* appear to manipulate the autophagic system to meet their particular nutritional, membrane expansion, and exit requirements. Accordingly, pharmaceutical induction of autophagy may be an effective strategy to combat infection by these and other intracellular pathogens.

Two of the outstanding questions in the autophagy field are how autophagosomes select their cargo and how their maturation is regulated. As a genetically tractable BSL2 microbe, *L. pneumophila* is an attractive tool to study autophagic processes, which remain challenging to track. Indeed, observations regarding the contribution of secretory pathway membranes and GTPases to autophagosome-like vacuoles were made first by *Legionella* experimentalists and later by the autophagy field. By exploiting *L. pneumophila* as a molecular genetic probe, scientists can gain insight to the mechanisms that regulate formation and maturation of autophagosomes.

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Striking a balance: modulation of host cell death pathways by *Legionella pneumophila*

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Programmed cell death is considered the ultimate solution for the host to eliminate infected cells, leading to the abolishment of the niche for microbial replication and the ablation of infection. Thus, it is not surprising that successful pathogens have evolved diverse strategies to reprogram the cell death pathways for their proliferation. Using effector proteins translocated by the Dot/Icm type IV secretion system, the facultative intracellular pathogen *Legionella pneumophila* manipulates multiple host cellular processes to create a niche within host cells to support its replication. Investigation in the past decade has established that in mammalian cells this bacterium actively modulates two host cell death pathways, namely the canonical apoptotic pathway controlled by the mitochondrion and the pyroptotic pathway controlled by the Nod-like receptor Naip5 and the Ipaf inflammasome. In this review, I will discuss the recent progress in understanding the mechanisms the bacterium employs to interfere with these host cell death pathways and how such modulation contribute to the intracellular life cycle of the pathogen.

Keywords: apoptosis, caspase, Bcl-2 protein, infection, effector, type IV secretion

INTRODUCTION

For intracellular pathogens whose proliferation requires nutritional supplies from the host cell cytosol, the death of the cell before the completion of a productive infection is disastrous. Furthermore, cells actively executing the apoptotic processes often secrete chemical signals or display specific molecules on their surface so that phagocytes can recognize and engulf them, leading to the termination of infection. Even if such engulfment did not occur, pathogens released prior to mature infection often are not primed for the second round infection and can be recognized and destroyed more easily by the immune system. Therefore, it is not unexpected to learn that hijacking host cell death pathways constitutes an important pathogenic strategy for almost all well adapted intracellular pathogens.

As detailed in the several excellent articles of this review series, *Legionella pneumophila* is a facultative intracellular pathogen that uses similar strategies to replicate in phylogenetically distant eukaryotic cells, ranging from amebae to human alveolar macrophages. Within these evolutionarily distant host cells, the *L. pneumophila*-containing vacuole (LCV) undertakes a unique maturation pathway characterized by the evasion of endocytic fusion and the interception of membrane trafficking vesicles originating from the endoplasmic reticulum (ER; Isberg et al., 2009; Hubber and Roy, 2010). As the bacterium begins to multiply, active acquisition of membrane materials from the ER-derived vesicles compensates the expansion of the LCV. The result of such remodeling is the formation of a compartment morphologically and cell biologically resembling the ER (Isberg et al., 2009). Whereas the interaction with the host membrane trafficking pathways is probably the best understood process during intracellular *L. pneumophila* growth, it is becoming clear that modulation of several other pathways, including lipid metabolism, autophagy, ubiquitination, and host cell death, is also critical for successful infection (Hubber and Roy, 2010). To accomplish this feat, *L. pneumophila* delivers more than 200 bacterial proteins via the Dot/Icm type IV secretion system into host cells where they engage in distinct host pathways to facilitate the biogenesis of the LCV permissive for bacterial replication (Ensminger and Isberg, 2009). Here, I will discuss recent progress in the interplays between *L. pneumophila* and the host cell death pathways and how such interplays contribute to successful bacterial infection in mammalian cells.

HOST CELL DEATH PATHWAYS AND THEIR REGULATION

In mammalian cells, programmed cell death is divided into at least four categories: apoptosis, pyroptosis, necrosis, and necroptosis (Fink and Cookson, 2005; Vandenabeele et al., 2010). Apoptosis is the best characterized programmed cell death mode; it plays critical roles in development, maintaining tissue homeostasis, shaping the immune repertoire, and restricting the progress of infections (Danial and Korsmeyer, 2004). This cell death mode can be initiated by two distinct but partially overlapping pathways: the extrinsic, receptor-mediated pathway and the intrinsic mitochondrial pathway (Salvesen and Riedl, 2008). In both cases, biochemical cascades triggered by extracellular ligands or intracellular damage led to the activation of caspases, which are a family of cysteine-dependent aspartate-specific proteases. These enzymes mediate most of the apoptotic program and some of them can be blocked by inhibitor of apoptotic proteins (IAPs; Scott et al., 2005). The mitochondrion is the central controlling site for the intrinsic apoptotic pathway because it harbors cytochrome *c* and second mitochondrion-derived activator of caspase (SMAC or DIABLO).

The release of cytochrome *c* into the cytosol leads to the assembly of a supramolecular complex known as the apoptosome, which initiates the caspase activation cascade (Riedl and Salvesen, 2007). In parallel, SMAC neutralizes the caspase-inhibitory activity of XIAP, thereby indirectly contributing to the maximal activation of the caspase cascade (Riedl and Salvesen, 2007). The release of

these two apoptosis-initiating molecules is caused by perturbation of the integrity of the outer mitochondrial membrane (OMM), which is delicately regulated by members of the Bcl-2 protein family. Based on their roles in controlling apoptosis, Bcl-2 family proteins can be divided into two subsets: pro- and anti-apoptotic molecules. Members of this protein family can form homo- as well as heterodimers (Chipuk et al., 2010). Indeed, the formation of heterodimers between pro- and anti-apoptotic members, a process that alters the cellular ratios between these two subsets of proteins, determines at least in part the susceptibility of cells to a death signal (Cory and Adams, 2002). Members of the Bcl-2 family share two common features. First, they possess up to four conserved Bcl-2 homology (BH) domains, designated BH1, BH2, BH3, and BH4 (Chipuk et al., 2010); However, a number of pro-apoptotic Bcl-2 family proteins, such as Bid, Bim, Bad, and BNIP3 contain only the BH3 domain and are classified as the "BH3-only" subfamily (Chipuk et al., 2010). Second, most members of this protein family contain a carboxy-terminal hydrophobic domain, which in many cases is critical for their biological activities by membrane insertion and membrane remodeling (Lomonosova and Chinnadurai, 2008). Upon sensing cell stress caused by various insults such as DNA damage, cytokine deprivation, or infection, these BH3-only proteins trigger the insertion of the two pro-apoptotic, pore-forming proteins BAX and/or BAK, into the OMM (Chinnadurai et al., 2008), causing the release of the cytochrome *c* and SMAC. Members of the pro-survival proteins, including Bcl-2, BCL-X_L, and MCL1, inhibit apoptosis by directly sequestering BAK, BAX, and BH3-only proteins to prevent permeabilization of OMM (Chipuk et al., 2010).

REGULATION OF HOST CELL DEATH PATHWAYS BY *L. PNEUMOPHILA*

Apoptosis plays an important role in the defense against pathogens on the level of both the reaction of an individual host cell to an invading microorganism and the reacting immune system (Creagh et al., 2003). Accordingly, successful pathogens have evolved different but often equally effective mechanisms to manipulate host cell death pathways to benefit their proliferation. Such manipulation can be achieved by targeting the activity of one or more host proteins critical in each step of the apoptotic pathways. For example, many viruses code for proteins that specifically inhibit apoptosis of infected cells by directly interacting with pro-apoptotic members of the BH3-only proteins (Roulston et al., 1999; Everett and McFadden, 2002). Similarly, some obligate intracellular bacterial pathogens such as *Chlamydia trachomatis* and *Rickettsia rickettsii* actively inhibit apoptosis (Clifton et al., 1998; Fan et al., 1998). Of particular interest is that *C. trachomatis* prevents infected cells from undergoing apoptosis by specifically degrading members of the pro-death BH3-only proteins (Dong et al., 2005), probably by the *Chlamydia* protease-like factor (CPAF; Pirbhai et al., 2006).

Accumulating evidence indicates that *L. pneumophila* is able to manipulate host cell death pathways by targeting regulatory molecules with diverse mechanisms at different points of the signaling cascade. Earlier studies suggest that *L. pneumophila* actively induces apoptosis of infected cells via the activation of the executioner caspase, caspase-3 (Gao and Abu Kwaik, 1999a,b; Molmeret et al., 2004). In permissive cell lines such as U937 or human peripheral blood monocytes, infection by *L. pneumophila* caused high level apoptosis within the first 3 h of bacterial uptake, and, in some cases, apoptotic cells reached 100% (Gao and Abu Kwaik, 1999b). However, a later study found that despite vigorous bacterial replication over a time span of 13 h, the proportion of apoptotic cells did not significantly increase in permissive macrophages, suggesting that in permissive host cells, *L. pneumophila* actively inhibits infected cells from undergoing apoptosis (Derre and Isberg, 2004). This notion was validated by the discovery of SdhA and SidF, two Dot/Icm substrates involved in the inhibition of host cell death. Mouse bone marrow macrophages infected with a *sdhA* mutant became apoptotic, displaying increased nuclear degradation, mitochondrial disruption, membrane permeability, and caspase activation, indicating a role for SdhA in inhibiting host cell death (Laguna et al., 2006). Interestingly, the requirement for SdhA is cell-type specific because the growth defect was less severe in the ameba *Dictyostelium discoideum* or the more permissive U937 cell-derived macrophages (Laguna et al., 2006). SdhA appears to be multifunctional because it also plays a key role in the suppression of type I interferons (IFN) response in macrophages infected by *L. pneumophila* (Monroe et al., 2009). Although less severe, cells infected by mutants lacking SidF also exhibited higher levels of apoptosis, which led to marginal but detectable defects in intracellular growth (Banga et al., 2007). SidF interacts and inhibits the cell death-inducing activity of BNIP3 and Bcl-rambo, two non-canonical pro-apoptotic members of Bcl-2 protein family (Banga et al., 2007; **Figure 1**). Both mitochondrial proteins, BNIP3 and Bcl-rambo appears to induce apoptosis by different mechanisms. BNIP3 appear to play a role in cellular response to stress (Chinnadurai et al., 2008). On the other hand, Bcl-rambo induces cell apoptosis specifically blocked by the caspase inhibitors, IAPs (Kataoka et al., 2001). Thus, it is attempting to speculate that SidF plays a role in making infected cells less sensitive to stress caused by *L. pneumophila* infection. Nevertheless, the activities of SdhA and SidF indicate that *L. pneumophila* inhibits host apoptosis by directly targeting host proteins involved in controlling the cell death pathways at the mitochondrion (**Figure 1**).

Another layer of cell death inhibition mechanisms utilized by *L. pneumophila* is revealed by experiments designed to examine host gene expression profiles in response to low dose bacterial challenge (Abu-Zant et al., 2007; Losick et al., 2010). Several groups of genes known to be directly or indirectly involved in regulating host cell death were significantly induced, including stress response genes such as heat shock protein genes *s* and pro-survival members of the Bcl-2 protein family (Losick and Isberg, 2006; Abu-Zant et al., 2007). Interestingly, the most striking induction was observed in a collection of anti-apoptotic genes positively regulated at a transcriptional level by the regulator nuclear factor-κB (NF-κB; Losick and Isberg, 2006; Abu-Zant et al., 2007; **Figure 1**). Consistently, in human and permissive mouse macrophages, infection by wild type *L. pneumophila* led to nuclear translocation of NF-κB (Losick and Isberg, 2006; Abu-Zant et al., 2007). Thus, *L. pneumophila* is able to inhibit mammalian cell death by increasing the levels of anti-apoptotic proteins at transcriptional level. Two lines of evidence indicate that the induction of these anti-apoptotic genes is important for productive *L. pneumophila* infection. First, bacterial challenge of macrophages lacking one such anti-apoptotic gene, the plasminogen activator inhibitor-2 (PAI-2), led to significantly

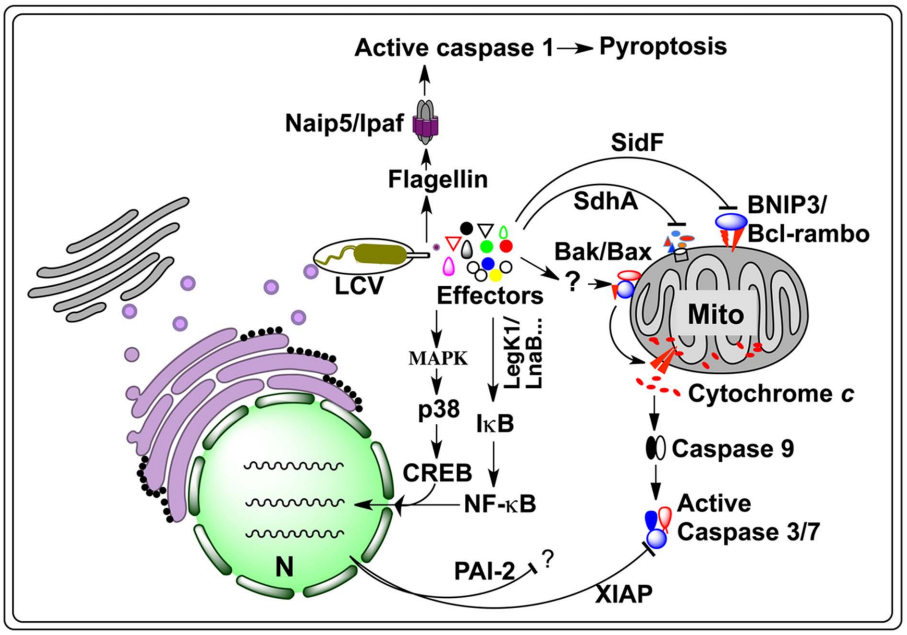

FIGURE 1 | Host cell death pathways targeted by *L. pneumophila*. Internalized *L. pneumophila* translocates a large number of effectors into host cytosol via the Dot/Icm type IV secretion system. A yet unidentified set of effectors trigger an imbalance between the pro-death and pro-survival members of the Bcl-2 protein family, leading to the insertion of Bax/Bak into the mitochondrial out membrane, thus the release of cytochrome *c* and subsequent activation of the caspases 3 and 7. Another set of effectors, including LegK1 and LnaB, activate NF-κB, most likely by initiating the kinase cascade that ultimately causes phosphorylation and subsequent degradation of IκB, the inhibitor of NF-κB, leading to nucleus translocation of NF-κB and the induction of anti-apoptotic genes such as *Xiap* and *Pai-2*, whose product inhibits cell death by targeting caspases 3 and 7. Activation of the MAP kinase pathway by another set of unknown effectors can lead to similar effects. A third set of proteins such as SidF and SdhA, which inhibit host cell death by targeting pro-apoptotic proteins BNIP3 and Bcl-rambo or by unknown mechanisms. In non-permissive mouse macrophages, flagellin reached the cytosol probably via the Dot/Icm transporter is sensed by the NLR receptor Naip5, which together with Ipaf and the inflammasome activates caspase-1, leading to pyroptotic cell death. CREB, cAMP response element-binding protein; LCV, *Legionella* containing vacuole; MAPK, mitogen-activated protein kinases; Mito, mitochondrion; N, nucleus; Naip5, NLR family, apoptosis inhibitory protein 5; PAI-2, plasminogen activator inhibitor-2; XIAP, X-linked inhibitor of apoptosis protein.

higher levels of apoptosis and reduction in bacterial replication (Losick and Isberg, 2006). Second, inhibition of nuclear translocation of NF-κB by genetic or pharmaceutical agents caused extensive cell death upon low dose bacterial challenge (Losick and Isberg, 2006). These anti-apoptotic proteins arrest host cell death by various mechanisms. Proteins like Bcl-2 can interact and inhibit the activity of several pro-death BH3-only proteins (Chipuk et al., 2010). On the other hand, IAPs such as XIAP can directly neutralize the activity of caspase-3 and -7 (Scott et al., 2005).

Interestingly, the observed NF-κB activation is involved in a signaling pathway independent of the Toll-like receptor (TLR) adaptor MyD88 and the cytoplasmic sensor Nod1, but is absolutely dependent upon the Dot/Icm secretion system (Losick and Isberg, 2006). These observations suggest the existence of Dot/Icm substrates capable of activating NF-κB. Consistent with this notion, LegK1 and LnaB, two Dot/Icm substrates with such activity have been identified in screenings using NF-κB responsive reporters and constructs that direct the expression of individual bacterial genes (Ge et al., 2009; Losick et al., 2010; **Figure 1**). Whereas the biochemical mechanisms of LnaB is unknown, LegK1, appears to directly target IκBα and other IκB family of inhibitors including p100 in the non-canonical NF-κB pathway by phosphorylation (Ge et al., 2009). Similar to most characterized Dot/Icm substrates, deletion of *legK1* or *lnaB* resulted in little or only partial reduction in NF-κB activation. Consistently, such mutants did not exhibit defects in intracellular bacterial growth (Ge et al., 2009; Losick et al., 2010). Several reasons can account for the lack of growth defect phenotypes of these mutants. First, proteins such as SdhA and SidF that target host cell death proteins can provide the protection in these mutants. Second, other yet unidentified effectors may contribute to the activation of NF-κB during the infection by these mutants. This is very likely because functional redundancy has been observed in effectors targeting other cellular pathways important for *L. pneumophila* intracellular growth (Isberg et al., 2009). Third, as documented in a recent report, NF-κB activated by a non-canonical mechanism may also contribute to the induction of anti-apoptotic genes. In the study, Fontana et al. (2011) found that in conjunction with classic pathogen associated molecular pattern (PAMP) molecules, inhibition of host protein synthesis by several Dot/Icm substrates led to prolonged activation of NF-κB, thus strong induction of a set of inflammatory cytokines such as interleukin (IL)-23 and granulocyte–macrophage colony stimulating factor. Such activation was achieved by the failure to resynthesize IκB, the short-lived inhibitor of the NF-κB transcription factor in the presence of these protein synthesis inhibitors (Fontana et al., 2011). Finally, some substrates of the Dot/Icm transporter also potently activate the MAP kinase pathway, which could also lead to induction of the anti-apoptotic genes such as *pai-2* (Shin et al., 2008). Given the

high level of conservation in the MAPK pathway in eukaryotes, it is not unexpected that the activation of this pathway also occurs in the ameba *D. discoideum* (Li et al., 2009). Clearly, inhibition of host cell apoptosis by *L. pneumophila* is achieved by collective activities of various bacterial proteins; these proteins either directly or indirectly, reprogram the various cell death pathways to ensure maximal bacterial replication.

In contrast to the many lines of evidence directly supporting active inhibition of host cell death by *L. pneumophila*, evidence pointing to the induction of apoptosis by this bacterium mostly is indirect. The apoptotic phenotypes associated with host cells infected with mutants lacking one or more cell death inhibiting Dot/Icm substrates such as SdhA and SidF suggested the existence of effectors capable of inducing cell death (Laguna et al., 2006; Banga et al., 2007). Apoptosis induced by *L. pneumophila* is extremely apparent in specialized phagocytes such as dendritic cells. In these cells, infection by *L. pneumophila* induced a caspase-3-dependent apoptotic pathway that aborted intracellular bacterial replication in the early phase of infection (Nogueira et al., 2009; **Figure 1**). Interestingly, dendritic cells from mice deficient in Bak and Bax ($Bak^{-/-}$ $Bax^{-/-}$) or mice overexpressing the pro-survival protein Bcl-2 are able to support intracellular bacterial infection without undergoing apoptosis (Nogueira et al., 2009), indicating that infection by *L. pneumophila* activates the mitochondrial pathway of apoptosis by inducing an imbalance between the pro-apoptotic and pro-survival members of Bcl-2 protein family. The activation of the mitochondrial apoptotic pathway also occurs in macrophages but at a much slower pace (Abu-Zant et al., 2005; Nogueira et al., 2009). The drastic differences between macrophages and dendritic cells in response to cell death stimuli such as those caused by *L. pneumophila* infection may be due to higher level or more active of the putative receptors or sensor proteins responsible for engaging the cell death signals from the bacterium in the latter cell type. It has been proposed that the sensitivity to pathogens exhibited by dendritic cells serves a protective role to the host by preventing infectious agents from using these cells as vehicles to reach deep tissues of the organism (Nogueira et al., 2009).

The induction of host cell death by bacterial toxins has been well documented. For example, Shiga toxins trigger apoptosis in many cell types, probably by inducing stress in the ER through inhibition of protein synthesis (Lee et al., 2008). Although a number of Dot/Icm substrates toxic to both yeast and mammalian cells have been identified, none of them has been shown to specifically induce host cell death. For example, at least five *L. pneumophila* proteins capable of inhibiting host protein synthesis have been reported. When overexpressed, these proteins are highly toxic to host cells (Belyi et al., 2006, 2008; Shen et al., 2009; Fontana et al., 2011). However, whether these proteins play roles in the induction of host cell death under infection conditions is unknown. It is worth noting that a mutant lacking all five genes failed to induce prolonged NF-κB activation, suggesting that these toxic proteins contribute to protect host cell death during infection (Fontana et al., 2011). Such outcomes clearly are opposite to the phenotypes observed by over-expressing these proteins in host cells. Nevertheless, since the Dot/Icm transporter but not bacterial replication is required for the activation of the apoptotic pathway (Abu-Zant et al., 2005; Nogueira et al., 2009), substrates of this transporter must be involved in the activation process. Either the activities of one or more Dot/Icm substrates or the "stress" caused by the collective impact of multiple effectors, or a combination of both can be the mechanism used by the bacterium for such activation. Interestingly, macrophages lacking Bak and Bax or overexpressing Bcl-2 still undergo extensive apoptosis upon being challenged by the *sdhA* mutant (Nogueira et al., 2009), suggesting that *L. pneumophila* is able to activate a cell death pathway independent of several critical components of the mitochondrial apoptotic pathway.

Besides the mitochondrial apoptotic pathway, *L. pneumophila* also induces pyroptosis, a form of inflammatory cell death in macrophages from mice harboring a functional *Naip5* allele (Fortier et al., 2005). Naip5 is a member of the Nod-like receptor (NLR) family, and an important component of the cytosolic protein complexes called inflammasomes, which induce autoactivation of caspase-1 (**Figure 1**). The NLRs are considered functionally equivalent to the TLRs localized on the surface or within endosomes of immune cell (Davis et al., 2011), which recognize components of the pathogen called PAMPs such as bacterial flagellin, peptidoglycan, and nucleic acid variants (West et al., 2006). Caspase-1 induced pyroptosis, which is accompanied by the release of mature IL-1β and IL-18 and other cytokines, and is inherently proinflammatory (Fink and Cookson, 2006). The observation that flagellin deficient *L. pneumophila* mutants gained the ability to grow productively in macrophages expressing functional Naip5 without activating caspase-1-dependent cell death indicates that flagellin activates Naip5 in the cytoplasm (Molofsky et al., 2006; Ren et al., 2006). A subsequent study using a retroviral transduction to express flagellin directly in macrophages clearly showed that flagellin is necessary and sufficient for the pyroptosis induction via a pathway controlled by the Ipaf inflammasome (Lightfield et al., 2008). Consistently, macrophages from Naip5-deficient mice completely failed to activate caspase-1 and were able to support robust growth of wild type *L. pneumophila*, further indicating that flagellin-mediated pyroptosis induction absolutely requires a functional Naip5 (Lightfield et al., 2008). Thus, the induction of pyroptosis in immune cells from restrictive mice by *L. pneumophila* flagellin is "accidental," but has provided an excellent model to dissect the host immune surveillance mechanisms (Vance, 2010).

POTENTIAL BENEFITS OF *L. PNEUMOPHILA*-INDUCED APOPTOSIS

Clearly, in permissive mammalian cells, infection by *L. pneumophila* activates the classic mitochondrial apoptotic pathway and possibly other yet unrecognized cell death processes, leading to activation of several caspases, including caspases 3 and 7. Concurrently, the bacterium employs a combination of mechanisms to inhibit infected cells from fully executing the apoptotic cascade to allow productive bacterial replication. Whereas the benefit of inhibiting apoptosis by *L. pneumophila* is obvious, the benefit for activation this pathway is less clear. Activated caspases may participate in cellular processes in not directly related to cell death but important for the biogenesis of the LCV. For example, active caspase-3 appears to cleave Rabaptin-5, an Rab5 effector, and cell treated with caspase-3 inhibitor (DEVD-fmk) or the pan inhibitor of caspases (Z-VAD-fmk) abolished intracellular bacterial growth (Molmeret et al.,

2004). However, in contrast to this observation, macrophages from permissive mice (A/J) lacking caspase-3 (*Casp3*$^{-/-}$) still support robust intracellular bacterial growth (Zamboni et al., 2006; Nogueira et al., 2009). These discrepancies may result from the non-specificity of the caspase inhibitors or the different host cells used in these studies. Recently, Srikanth et al. (2010) showed that cleavage of bacterial effectors by caspase-3 is important for pathogenicity of *Salmonella enterica* serovar Typhimurium. It is possible that active caspases also participate in cellular processes that are not directly related to cell death but are important in the biogenesis of the LCV.

CONCLUDING REMARKS AND PROSPECTIVES

A number of important questions remain outstanding in our understanding of the modulation of host cell death pathways by *L. pneumophila*. First, it is believed that amebae but not metazoan play a role in the evolution of *L. pneumophila* pathogenicity. However, NF-κB signaling and many components of apoptosis have now been documented to be targeted by proteins of this bacterium are not present in amebae cells. It is possible that *L. pneumophila* co-evolved with some as yet unrecognized lower metazoan. In support of this notion, two recent studies found that *L. pneumophila* can infect *Caenorhabditis elegans* (Brassinga et al., 2010; Komura et al., 2010), an organism with well-defined cell death pathways (Conradt, 2009). Alternatively, *L. pneumophila* can acquire genes specific for metazoan pathways from other more adapted mammalian pathogens which also are parasites of amebae (Moliner et al., 2010). Second, the nature of the bacterial signals that triggers the mitochondrial cell death pathway remains elusive. Particularly, the *sdhA* mutant appears to elicit a cell death pathway independent of the canonical mitochondrial pathway controlled by the Bcl-2 protein family (Nogueira et al., 2009). The identification of these proteins will undoubtedly facilitate the search of the host "receptors" that directly sense the bacterial signals. Research in this field aiming at characterizing both pro-survival and pro-death *L. pneumophila* proteins and their host targets will surely generate more exciting discoveries in years to come.

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Innate immunity to *Legionella pneumophila*

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Innate immune cells, such as macrophages, are highly adapted to rapidly recognize infections by distinct pathogens, including viruses, bacteria, fungi, and protozoa. This recognition is mediated by pattern recognition receptors (PRRs), which are found in host cell surface membranes and the host cell cytoplasm. PRRs include protein families such as the toll-like receptors, nod-like receptors, RIG-I-like receptors, and sensors of cytosolic DNA. The activation of these PRRs by pathogen-associated molecular patterns leads to transcriptional responses and specific forms of cell death. These processes effectively contribute to host resistance to infection either via cell-autonomous processes that lead to the intracellular restriction of microbial replication and/or by activating pathogen-specific adaptive immune responses. *Legionella pneumophila*, the causative agent of Legionnaires' disease, is a Gram-negative bacterium that triggers responses by multiple PRRs. Here, we review a set of studies that have contributed to our specific understanding of the molecular mechanisms by which innate immune cells recognize and respond to *L. pneumophila* and the importance of these processes to the outcome of infection.

Keywords: *Legionella pneumophila*, innate immunity, pattern recognition receptors, nod-like receptors

INTRODUCTION

Activation of innate immune cells is critical for the initiation of adaptive immune responses. This process relies mostly on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Among the canonical PAMPs are molecules such as lipopolysaccharide, peptidoglycan, bacterial lipoproteins, flagellin, and nucleic acids derived from viruses, bacteria, fungi, and protozoa (Janeway and Medzhitov, 2002; Akira et al., 2006; Gazzinelli and Denkers, 2006). Upon direct or indirect ligand recognition, toll-like receptors (TLRs) dimerize and trigger a signaling cascade leading to the activation of proinflammatory responses (Uematsu and Akira, 2006). TLRs are transmembrane proteins containing an extracellular leucine-rich repeat (LRR) domain that facilitates PAMP recognition and an intracellular domain that mediates intracellular signaling via four different adaptor proteins: TRAM, MAL/TIRAP, MyD88, and TRIF (O'Neill, 2008). Depending on the nature of their specific ligands, TLRs are embedded in either the extracellular membrane (TLR-1, -2, -4, -5, -6, -10, -11) or in the membranes of endocytic vacuoles (TLR-3, -7, -8, -9). In addition to TLRs, other PRR families have already been described; these include DNA/RNA-sensing proteins such as the RIG-I-like receptor (RLR) family and sensors of membrane damage and intracellular PAMPs such as the nod-like receptors (NLRs; Creagh and O'Neill, 2006).

The RNA helicase domain-containing proteins retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) comprise a group of cytoplasmic receptors important for the recognition of viral nucleic acids. PAMP recognition by RIG-I and MDA5 triggers the activation of IRF3 via MAVS (IPS-1), culminating in the production of type I interferons (IFN). Recent studies have demonstrated that these receptors also induce type I IFN production upon recognition of nucleic acids from intracellular bacteria (Cao, 2009). In addition, some studies have shown that the recognition of DNA by RIG-I/MDA5 is dependent on cytosolic RNA polymerase III (Pol III; Ablasser et al., 2009; Chiu et al., 2009).

The NLRs comprise a PRR family that can be classified into three sub-groups. The first sub-group is composed of receptors that trigger intracellular signaling pathways leading to the activation of transcriptional factors mediating the expression of inflammatory response genes. Both NOD1 and NOD2 are members of this first group, and they signal via RIP2, a kinase that ubiquitinates NEMO to induce the activation of NF-κB and MAPK (Shaw et al., 2008). The second sub-group comprises NLRs that do not require ASC to trigger caspase-1 activation. Among these proteins are NAIP5 (BIRC1e) and NLRC4 (IPAF), which have been suggested to assembly a unique inflammasome (hereafter referred to as the NLRC4 inflammasome). Activation of this inflammasome triggers a specific form of host cell death called pyroptosis (Lightfield et al., 2008; Case et al., 2009; Broz et al., 2010; Silveira and Zamboni, 2010; Whitfield et al., 2010). The third sub-group of NLRs comprises those that trigger caspase-1 activation via the adaptor protein ASC. These proteins assemble into a multimeric molecular platform known as the "classical" inflammasome. Among the NLRs that trigger the ASC-dependent inflammasome is NALP3, which has been extensively characterized and shown to be important for the recognition of danger-associated molecular patterns (DAMPs) reviewed by Schroder and Tschopp (2010).

In addition to TLRs, NLRs, and RLRs, previous studies have described a class of proteins that recognize cytoplasmic DNA (Ishii and Akira, 2006; Stetson and Medzhitov, 2006). These multiple protein families include DNA-dependent activators of IFN-regulatory factors (DAI; Takaoka et al., 2007), RNA polymerase III, which induces type I IFN production through the RIG-I pathway (Ablasser et al., 2009; Chiu et al., 2009), and the recently described protein absent in melanoma (AIM2), which activates

inflammasomes in an ASC-dependentmanner (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).

Legionella pneumophila, a Gram-negative bacterial pathogen that evolved infecting unicellular protozoa in freshwater reservoirs, may not have encountered strong selective pressure to avoid recognition by mammalian PRRs. Consequently, *L. pneumophila* triggers multiple PRR and has been a useful model for understanding the biology of PRRs and the induction of appropriate adaptive immune responses against intracellular pathogens. The successful use of *L. pneumophila* as a tool for studying immunology has been reviewed elsewhere (Vance, 2010). Here, we will review the salient findings that have contributed to our understanding of the molecular mechanisms underlying innate immune cell recognition and response to *L. pneumophila* infection (**Figure 1**). Furthermore, we will sumarize studies that have elucidated the importance of these processes to the outcome of *L. pneumophila* infection.

TOLL-LIKE RECEPTORS

It was originally speculated that TLR4, a general LPS sensor, would recognize *L. pneumophila*. However, work on non-enterobacteriaceae species has indicated that although TLR4/MD2 very efficiently recognizes enterobacterial lipid A, the same is not true for other Gram-negative bacteria. These non-enterobacteriaceae bacterial species often express lipid A containing long fatty acid chains that either fail to trigger TLR4 activation or antagonize the TLR4 receptor (Zamboni et al., 2004). In *L. pneumophila*, studies performed with TLR4-deficient or TLR4 knockout mice have confirmed that this receptor does not effectively participate in the recognition of *L. pneumophila* LPS. Even at high MOIs, there is no difference in *L. pneumophila* infection between wild-type and C3H/HeJ mice, which are defective for TLR4 signaling due to a missense mutation in the *Tlr4* gene resulting in the replacement of a proline with a histidine at position 712 (Poltorak et al., 1998; Lettinga et al., 2002). The initial studies on TLR4 function using C3H/HeJ mice were further corroborated in *tlr4*$^{-/-}$ mouse experiments, which supported the hypothesis that TLR4 deficiency does not influence the outcome of *L. pneumophila* infection (Akamine et al., 2005; Archer and Roy, 2006; Fuse et al., 2007). Studies by Girard et al. (2003) have shown that lipid A of *L. pneumophila* signals via TLR2 to induce the expression of CD14. These findings led to the suggestion that *L. pneumophila* LPS is recognized by TLR2, but the mechanisms underlying the recognition of lipid A by TLR2 have not been completely elucidated; some researchers have speculated that lipid A-mediated TLR2 activation requires either a long chain fatty acid or the presence of a substituent or a branch on the penultimate carbon of a fatty acid chain (Brandenburg et al., 1993). Nonetheless, future studies using a synthetic form of *L. pneumophila* lipid A may be required to unequivocally confirm that *L. pneumophila* LPS is a bona fide agonist of TLR2.

Regardless of the proposed role of TLR2 in LPS recognition, other *L. pneumophila* PAMPs, such as lipopeptides and lipoproteins, are sufficient to activate TLR2. Activation of this receptor is critical to the outcome of *L. pneumophila* infection in mice. This was unequivocally demonstrated by experiments using *tlr2*$^{-/-}$ mice, which show impaired cytokine production and are more susceptible to bacterial multiplication in the lungs (Akamine et al., 2005; Archer and Roy, 2006; Hawn et al., 2006).

In addition to TLR2, other TLRs are also important for the host response to *L. pneumophila*. As a flagellated bacteria, *L. pneumophila* is recognized by TLR5, and a common polymorphism in the ligand-binding domain of TLR5 causes increased susceptibility to Legionnaires' disease in humans (Hawn et al., 2003). These data have been corroborated by studies using *tlr5*$^{-/-}$ mice showing that TLR5 recognition of *L. pneumophila in vivo* contributes to the recruitment of leukocytes to the pulmonary cavity (Hawn et al., 2007). However, TLR5 deficiency by itself does not render mice more susceptible to infection as measured by CFU counts and cytokine production (Hawn et al., 2007; Archer et al., 2009).

Another TLR important in *L. pneumophila* infection is TLR9. Mice lacking this receptor exhibit reduced levels of cytokines when challenged with *L. pneumophila* and are therefore more permissive of *L. pneumophila* replication in the lungs (Newton et al., 2007; Archer et al., 2009). This observation was corroborated by experiments involving the *in vivo* administration of CpG oligodeoxynucleotide, a synthetic agonist of TLR9, which protected mice that were pre-infected with *L. pneumophila* (Bhan et al., 2008).

Importantly, these studies using mice deficient for a single TLR indicate that disruption of a single *tlr* gene does not result in a striking susceptibility to *L. pneumophila*; this is possibly due to redundancy in the signaling pathways triggered by these receptors. In contrast, the deletion of the common adaptor protein MyD88, which is important for the signaling of several TLRs, produces mice that are highly susceptible to infection. Mice deficient for MyD88 show impaired cytokine production in response to pulmonary infection with *L. pneumophila*; they also show high numbers of CFUs in the lungs and succumb to *L. pneumophila* infection even at low multiplicities of infection (Neild et al., 2005; Archer and Roy, 2006; Hawn et al., 2006; Sporri et al., 2006; Archer et al., 2009, 2010). The increased susceptibility of *myd88*$^{-/-}$ mice suggests that the deletion of multiple TLR genes will produce mice as susceptible to *L. pneumophila* infection as those lacking *myd88*$^{-/-}$. To test this hypothesis, Archer et al. (2009) constructed mice deficient for multiple TLRs and showed that mice lacking both TLR5 and TLR9 or deficient for TLR2 and either TLR5 or TLR9 are still able to clear *L. pneumophila* infection. Archer and colleagues elegantly concluded that IL-18 signaling via MyD88 is essential for NK cell production of IFN-γ, a cytokine critical for the restriction of *L. pneumophila* infection *in vivo* (Archer et al., 2009). Interestingly, although the authors showed that NK cells signal via IL-18 to produce IFN-γ, they also demonstrated that mice deficient for the IL-18 receptor are no more susceptible to *L. pneumophila* infection than wild-type animals (Archer et al., 2009). Additional studies will therefore be required to further determine the importance of this pathway *in vivo* and its redundancy with other pathways.

NOD-LIKE RECEPTORS: NOD1 AND NOD2

The first study addressing NOD1 and NOD2 signaling in response to *L. pneumophila* infection was performed by Shin et al. (2008). In this study, the authors evaluated the transcriptional responses of macrophages infected with wild-type and *dotA* mutants of *L. pneumophila* to identify macrophage genes induced in a Dot/Icm-dependent manner. By comparing macrophages deficient for MyD88 and RIP2 kinase, which impairs both NOD1 and NOD2 signaling, or lacking both MyD88 and TRIF, the authors identified

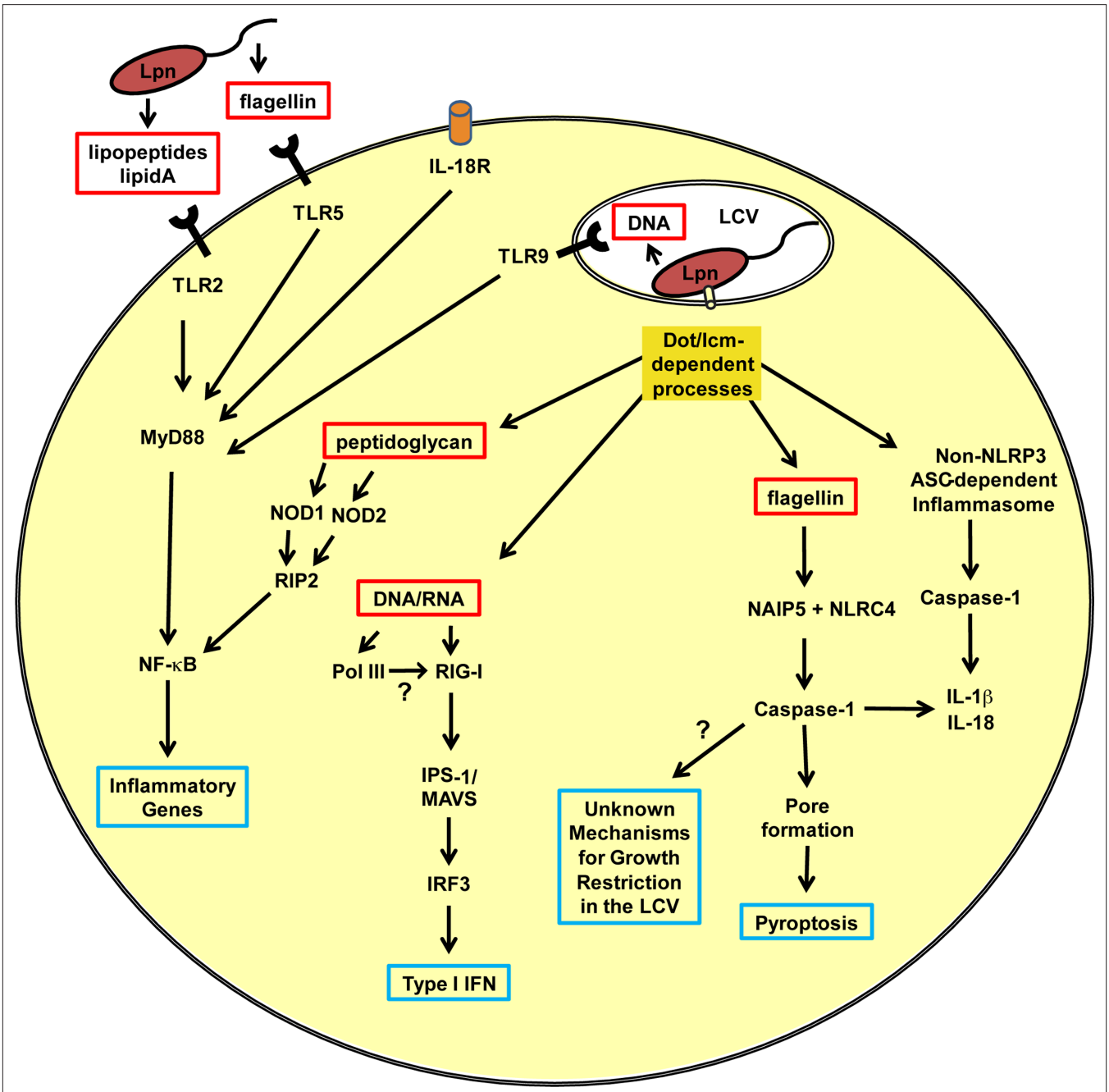

FIGURE 1 | Innate immune responses of a mammalian phagocyte infected with *Legionella pneumophila*. A schematic representation of the pathways activated in a phagocyte after infection with *L. pneumophila*. The red boxes indicate *L. pneumophila*-associated molecular patterns important for the activation of pattern recognition receptors. Blue boxes indicate molecules or processes involved in the cell-autonomous restriction of *L. pneumophila* replication. LCV, *Legionella*-containing vacuole; Lpn, *L. pneumophila*; Dot/Icm, type IVB secretion system; IL, interleukin; IL-18R, IL-18 receptor; TLR, toll-like receptor; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa B; NOD, nucleotide-binding oligomerization domain-containing protein; RIP2, receptor interacting protein 2; Pol III, RNA polymerase III; RIG-I, retinoic-acid-inducible protein I; IPS-I, IFN-β promoter stimulator 1 (also known as MAVS, mitochondrial antiviral signaling); IRF3, interferon regulatory factor 3; NAIP5, neuronal apoptosis inhibitory protein 5; NLRC4, NLR family CARD domain-containing protein 4.

several genes that were regulated by a RIP2-dependent pathway (Shin et al., 2008). Importantly, this study revealed that multiple responses occur after *L. pneumophila* infection of macrophages; some of these were dependent on MyD88, others on RIP2 and some responses that were induced via unknown sensors were independent of both MyD88 and RIP2 (Shin et al., 2008). This study was further corroborated by *in vivo* experiments using mice deficient for both RIP2 and MyD88. The *rip2*$^{-/-}$/myd88$^{-/-}$ mice were significantly more susceptible to *L. pneumophila* than the *myd88*$^{-/-}$ mice, and they succumbed to infection even at low MOIs (Archer et al., 2010).

Importantly, this and another study demonstrated that although a RIP2-dependent response was not critical for restricting *L. pneumophila* infection *in vivo*, a RIP2-dependent response did contribute to the recruitment of phagocytes to the sites of infection (Archer et al., 2010; Frutuoso et al., 2010). Notably, the RIP2-dependent responses that contributed to the recruitment of neutrophils to the lungs of infected mice were at least partially dependent on the NOD1 and NOD2 receptors (Berrington et al., 2010; Frutuoso et al., 2010). These studies confirmed that NOD1 and NOD2 effectively participate in the pulmonary detection of *L. pneumophila* infection, but NOD1 and NOD2 deficiency results only in a minor attenuation of bacterial growth restriction in mouse lungs (Berrington et al., 2010; Frutuoso et al., 2010). Importantly, these studies of *L. pneumophila* infection in mice deficient for TLRs and the NOD/RIP2 pathway demonstrate the substantial redundancy of innate immune receptors in the host response to bacterial infection.

NOD-LIKE RECEPTORS: NLRC4 AND NAIP5

Approximately 30 years ago, it was demonstrated that macrophages from A/J mice fail to restrict the intracellular replication of *L. pneumophila* (Yamamoto et al., 1988). These phenotypic differences between A/J and other mouse strains provided a useful model for investigating the genes responsible for the phenotypic variations. In later years, the genomic region response for *L. pneumophila* resistance was mapped to the autosomal recessive locus *Lgn1* on chromosome 13 (Beckers et al., 1995; Dietrich et al., 1995). In early 2003, it was finally revealed that the susceptibility gene within the *Lgn1* locus was NAIP5 (also known as BIRC1e), a member of the NLR proteins family (Diez et al., 2003; Wright et al., 2003). The mechanisms by which NAIP5 contributes to the host control of infection was unraveled a few years later by the discovery that NAIP5 interfered with caspase-1 activation in response to macrophage infection by *L. pneumophila* (Zamboni et al., 2006). This response is dependent on the Dot/Icm system and effectively contributes to the restriction of bacterial replication in macrophages *in vitro* and *in vivo* (Zamboni et al., 2006). The NAIP5-dependent restriction of *L. pneumophila* growth required another NLR protein called NLRC4 (IPAF), which contributes for caspase-1 activation upon *L. pneumophila* infection (Amer et al., 2006; Molofsky et al., 2006; Zamboni et al., 2006). An elegant screening experiment identified the putative agonist of this NAIP5/NLRC4 inflammasome: *L. pneumophila* deficient for the flagellin gene *flaA* bypassed the NLRC4 inflammasome and replicated in macrophages harboring the restrictive *Lgn1* allele (Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). Although the role of NAIP5 in caspase-1 activation was questioned (Lamkanfi et al., 2007), assays with *naip5*$^{-/-}$ mice unequivocally demonstrated the requirement of NAIP5 for caspase-1 activation in response to *L. pneumophila* flagellin (Lightfield et al., 2008). Furthermore, both NLRC4 and NAIP5 were required for the detection of a carboxy-terminal domain of flagellin, a region not required for TLR5 activation (Lightfield et al., 2008). The same group has recently demonstrated that the N-terminus of *L. pneumophila* flagellin relieves the requirement for NAIP5 during activation of the NLRC4 inflammasome, which suggests that NAIP5 regulates the specificity of the NLRC4 inflammasome for certain species of bacteria (Lightfield et al., 2011). These data explain why for some species, such as *L. pneumophila*, the activation of the NLRC4 inflammasome requires NAIP5, whereas for other species, such as *Salmonella enterica* serovar Typhimurium, NAIP5 is dispensable. Strikingly, activating this NLRC4/NAIP5 inflammasome requires a functional Dot/Icm type IV secretion system. This finding led to the speculation that flagellin may leak from the *Legionella* cell to the macrophage cytoplasm through the Dot/Icm. However, this hypothesis has not yet been experimentally validated.

The mechanisms by which the NLRC4 inflammasome restricts *L. pneumophila* replication remains incompletely understood. Activation of these receptors triggers a caspase-1-dependent pore formation in macrophage membranes and leads to a specific form of cell death called pyroptosis (Derre and Isberg, 2004; Case et al., 2009; Silveira and Zamboni, 2010; Whitfield et al., 2010). However, the activation of NAIP5 and NLRC4 also facilitates a process independent of pyroptosis that culminates with the restriction of *L. pneumophila* multiplication within the replicative vacuole occupied by the bacteria (Swanson and Molofsky, 2005; Amer et al., 2006; Fortier et al., 2007). These processes are possibly dependent on the transcriptional regulation of macrophage genes. This hypothesis has been supported by the demonstration that NAIP5 recognition of *L. pneumophila* triggers IRF1- and IRF8-mediated upregulation of genes important for macrophage resistance to bacterial infection (Fortier et al., 2009). The inducible nitric oxide synthase (NOS2) gene is a possible candidate, as the NAIP5- and caspase-1-dependent induction of NOS2 expression and nitric oxide production has been observed in macrophages transfected with flagellin (Buzzo et al., 2010).

ASC AND THE NLRP3-INDEPENDENT INFLAMMASOME

The adaptor protein called apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC/PYCARD) is a small molecule composed of a PYRIN and a CARD domain. ASC bridges caspase-1 to PYRIN-containing molecules, such as NALP/NLRP family members, via CARD/CARD interactions (Mariathasan et al., 2004). Studies performed with macrophages from ASC-deficient mice have indicated that this molecule is important for the secretion of IL-1β in response to infection but is not required for controlling *L. pneumophila* replication in C57BL/6 macrophages (Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). This finding led to the proposition that ASC may participate in the NLRC4-dependent activation of caspase-1 in response to *L. pneumophila* (Case et al., 2009; Pedra et al., 2009). However, subsequent studies demonstrated that *L. pneumophila* triggers at least two different inflammasomes: one dependent on NLRC4, NAIP5, and flagellin; and another dependent on ASC and independent of NLRP3 (Sutterwala et al., 2006; Case et al., 2009). Although ASC is dispensable for restricting *L. pneumophila* replication in mice and murine macrophages, a recent report demonstrated that ASC does contribute to the control of *L. pneumophila* infection in human monocytes (Abdelaziz et al., 2010). The proteins participating in this ASC-dependent inflammasome and the molecular signals that trigger its activation have not yet been elucidated.

RIG-I-LIKE RECEPTOR AND INDUCTION OF TYPE I INTERFERON

Several studies have reported the production of type I IFN in response to *L. pneumophila* infection (Opitz et al., 2006; Stetson and Medzhitov, 2006; Lippmann et al., 2008; Chiu et al., 2009;

Monroe et al., 2009), and these experiments have contributed to our understanding of the host cell pathways responsible for type I IFN induction. Although RIG-I and MDA5 were initially reported as sensors of viral infection, recent studies indicate that RLRs are also important for the host cell recognition and response to bacterial infection, and both RIG-I and MDA5 have been implicated in type I IFN production by macrophages in response to *L. pneumophila* infection (Opitz et al., 2006; Stetson and Medzhitov, 2006; Ablasser et al., 2009; Monroe et al., 2009). The production of type I IFN in response to *L. pneumophila* infection was shown to be dependent of MAVS and IRF3 (Opitz et al., 2006; Chiu et al., 2009; Monroe et al., 2009). Furthermore, this response accounted to bacterial growth restriction, as the addition of exogenous type I IFN to macrophages lead to the inhibition of *L. pneumophila* replication in non-permissive macrophages (Schiavoni et al., 2004; Plumlee et al., 2009). Several groups have independently demonstrated that this IRF3-dependent innate immune response does not require the flagellin/NAIP5/NLRC4 axis and occurs in both mice and human cells (Opitz et al., 2006; Coers et al., 2007; Lippmann et al., 2008). Activation of the IRF3 pathway was found to be dependent on a functional bacterial Dot/Icm and to require the presence of bacterial DNA in the host cell cytoplasm (Stetson and Medzhitov, 2006). The current hypothesis is that *L. pneumophila* DNA leaks into the host cell cytoplasm via the Dot/Icm. However, a recent report has demonstrated that *L. pneumophila* RNA, but not DNA, is responsible for RIG-I-dependent response to *L. pneumophila* (Monroe et al., 2009). A subsequent study showed that the host protein RNA polymerase III, which converts poly (dA-dT) DNA into 5′-ppp RNA, is important for IFNβ induction through the RIG-I pathway (Chiu et al., 2009). Future studies are required to determine the ligand and receptor responsible for DNA/RNA recognition. Collectively, the investigations of type I IFN in *L. pneumophila* infection indicate that type I IFN signaling effectively restricts *L. pneumophila* replication in phagocytes, thus confirming the importance of this pathway for macrophage resistance to *L. pneumophila*. Conversely, the importance of type I IFN for murine resistance to *L. pneumophila* infection is less pronounced. Work from independent groups has demonstrated that mice deficient for IFNAR, which impairs the activation by both IFN-α and IFN-β, show no increased susceptibility to *L. pneumophila* infection (Monroe et al., 2009; Ang et al., 2010). Nevertheless, further investigation may be required to determine why type I IFN affects macrophage but not mouse resistance to *L. pneumophila*, and future studies are necessary to identify the ligand and receptors involved in the production of type I IFN in response to *L. pneumophila* infection.

CONCLUDING REMARKS

Different families of PRRs, including TLRs, NLRs, and RLRs, effectively recognize *L. pneumophila*. This recognition leads to several events important for the outcome of infection: (1) phagocytes activate cell-autonomous mechanisms to restrict bacterial replication; (2) phagocytes trigger the expression of hundreds of inflammatory genes, including those for cytokines and chemokines; (3) phagocytes then express stimulatory and co-stimulatory molecules important for antigen presentation; (4) the secreted cytokines and chemokines recruit additional cells to the sites of the infection; (5) antigen presentation will proceed and the immune system may generate specific acquired responses that are highly protective against reinfection. The continued use of *L. pneumophila* to dissect these processes will aid us in understanding how the immune system fights to prevent Legionnaire's disease and will continue to provide important insight into the biological functions of the innate immune responses.

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Immune control of *Legionella* infection: an *in vivo* perspective

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Legionella pneumophila is an intracellular pathogen that replicates within alveolar macrophages. Through its ability to activate multiple host innate immune components, *L. pneumophila* has emerged as a useful tool to dissect inflammatory signaling pathways in macrophages. However the resolution of *L. pneumophila* infection in the lung requires multiple cell types and abundant cross talk between immune cells. Few studies have examined the coordination of events that lead to effective immune control of the pathogen. Here we discuss *L. pneumophila* interactions with macrophages and dendritic cell subsets and highlight the paucity of knowledge around how these interactions recruit and activate other immune effector cells in the lung.

Keywords: Legionnaire's disease, inflammation, macrophages, plasmacytoid dendritic cells, cytokines

INTRODUCTION

Members of the genus *Legionella* are Gram-negative, facultative intracellular bacteria of amoebae, including free-living, freshwater, or soil amoebae (Rowbotham, 1980; Tyndall and Domingue, 1982; Fields, 1996). *Legionella pneumophila* was the first species described and is the known causative agent of an acute form of pneumonia termed Legionnaires' disease (Fraser et al., 1977; McDade et al., 1977). Humans become secondarily infected after inhaling or aspirating aerosols containing bacteria. Upon its transmission to the human lung, *L. pneumophila* enters and replicates in alveolar macrophages, leading to inflammation and disease (Horwitz and Silverstein, 1980; Horwitz, 1983a). Replication in macrophages is thus a hallmark of *L. pneumophila* infection. Within macrophages, the bacteria block phagolysosome fusion and intercept vesicles trafficking in the secretory pathway (Horwitz, 1983b; Kagan and Roy, 2002). The resulting *Legionella*-containing vacuole (LCV), ultimately takes on properties of the rough endoplasmic reticulum (Roy and Tilney, 2002; Isberg et al., 2009). The formation of the LCV is dependent on a functional Dot/Icm Type IVB secretion system used by the pathogen to deliver effectors into the host cell cytosol (Segal and Shuman, 1997; Segal et al., 1998; Vogel et al., 1998). At least 275 effectors have been identified (Zhu et al., 2011), that target multiple and overlapping host cell functions including host cell GTPase activity, phosphoinositide metabolism, protein secretion, apoptosis, eukaryotic protein translation, ubiquitination, NF-κB activation and mitochondrial function, reviewed in (Franco et al., 2009; Isberg et al., 2009; Weber et al., 2009; Hubber and Roy, 2010; Newton et al., 2010).

REPLICATION OF *L. PNEUMOPHILA* IN MACROPHAGES

Macrophages and dendritic cells (DC) are important sentinels of the immune system detecting infectious agents by highly conserved microbial motifs, so-called pathogen-associated molecular patterns (PAMPs; Janeway Jr., 1992). Pattern recognition is mediated by a set of invariant pattern-recognition receptors (PRRs) of which four families have been identified: toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I) like receptors (RLRs), C-type lectin receptors (CLRs), and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs; Takeuchi and Akira, 2010). NLRs comprise a large family of cytoplasmic PRRs of which only a few members have been characterized in detail. Some NLRs form multiprotein complexes called inflammasomes (Schroder and Tschopp, 2010) and activation of these complexes leads to the cleavage of the central effector molecule cysteine protease caspase-1, inducing a form of cell death known as pyroptosis which is accompanied by the release of pyrogenic IL-1ß, IL-18, and IL-33 (Davis et al., 2011).

The flagellin sensing Nlrc4 inflammasome plays a central role in the detection of *L. pneumophila*, which is the reason most inbred strains of mice are resistant to *L. pneumophila* infection. The discovery of the Nlrc4 inflammasome began with the observation that macrophages derived from most mouse strains restrict bacterial replication with the notable exception of the A strain (often called A/J, although this terminology refers only to mice derived directly from the Jackson or Janvier laboratories; Yamamoto et al., 1988). Crosses between A mice and non-permissive C57BL/6 mice showed that the susceptibility of the A strain is controlled by a single locus on mouse chromosome 13, designated Lgn1 (Beckers et al., 1995; Dietrich et al., 1995). Genetic studies then identified the new NLR gene, *Naip5*, within this locus as responsible for the increased susceptibility of A mice to infection (Diez et al., 2003; Wright et al., 2003). Subsequent work showed that *Naip5*-dependent restriction of *L. pneumophila* relies on a functional copy of *Naip5* as well as *Nlrc4* and activation of caspase-1 (Zamboni et al., 2006). Restriction results from the presence of

bacterial flagellin in the host cytosol, and recognition of the C-terminus of flagellin is sufficient for activation of the Nlrc4 inflammasome (Molofsky et al., 2006; Ren et al., 2006; Lightfield et al., 2008). Interestingly, the cytosolic localization of flagellin and/or restriction of replication depends on a functional Dot/Icm type 4 secretion system (Amer et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006; Lamkanfi et al., 2007). However, it is not known how the Dot/Icm system contributes to the translocation of flagellin into the host cytosol and whether the detection of flagellin by the inflammasome occurs directly or indirectly with the help of cofactors.

While formation of the inflammasome leads to the activation of caspase-1, as well as maturation and secretion of IL-1ß and IL-18, neither cytokine makes a major contribution to the restriction of *L. pneumophila in vitro* or *in vivo* (Amer et al., 2006; Zamboni et al., 2006; Coers et al., 2007; Akhter et al., 2009; Miao et al., 2010). Nevertheless, caspase-1 knockout macrophages are more permissive for *L. pneumophila* replication and *caspase1*-deficient mice are more susceptible to *L. pneumophila* infection (Amer et al., 2006; Zamboni et al., 2006). Caspase-1 activation upon bacterial infection may also result from an alternative Nlrc4-independent pathway which requires the apoptosis associated speck-like protein (Asc), yet Asc is dispensable for restriction (Zamboni et al., 2006; Case et al., 2009). Although depletion or inhibition of caspase-1 activity leads to decreased targeting of bacteria to lysosomes (Amer et al., 2006; Zamboni et al., 2006), the mechanism of caspase-1-dependent restriction of *L. pneumophila* replication in macrophages and *in vivo* is yet to be fully resolved. Activation of the Nlrc4 inflammasome can lead to macrophage cell death through caspase-1 dependent pore formation, which may account for reduced bacterial numbers through macrophage cell lysis (Case et al., 2009; Silveira and Zamboni, 2010). Downstream molecules such as caspase-7, interferon regulatory factor (IRF) 1 and IRF8 also play a significant role in caspase-1 signaling and in the case of caspase-7, this activation leads to increased macrophage apoptosis (Akhter et al., 2009; Fortier et al., 2009). *caspase7*-deficient mice are also more susceptible to *L. pneumophila* infection (Akhter et al., 2009). However, the ability of *L. pneumophila* to replicate within macrophages *in vitro* does not necessarily equate with virulence in whole animals. For example, type I interferon (IFN-I) receptor-deficient macrophages, support enhanced replication of *L. pneumophila* yet IFN-I receptor-deficient mice are no more susceptible to infection *in vivo* (Monroe et al., 2009; Ang et al., 2010).

In contrast to macrophages derived from restrictive mouse strains, human macrophages or monocytes allow robust replication of *L. pneumophila* despite the presence of *Naip* and *Nlrc4* orthologues. Human Nlrc4 and Naip are functional but only delay *L. pneumophila* replication when overexpressed (Vinzing et al., 2008) suggesting that the level of inflammasome activity may restrict *L. pneumophila* replication in humans cells, similar to mice. A recent report also showed that human Asc is able to restrict bacterial growth in a caspase-1-dependent and independent manner but is downregulated during *L. pneumophila* infection of monocytic THP-1 cells (Abdelaziz et al., 2011). More studies in human cells, ideally in primary macrophages, will provide a useful comparison to the results derived from using mouse infection models.

Immune effector molecules produced by infected macrophages are likely to play an important role in generating a protective immune response and warrant further analysis. In mouse and human macrophages, infection with live *L. pneumophila* induces the production of inflammatory cytokines and chemokines such as tumor necrosis factor α (TNF), interleukin (IL)-10, IL-6, IL-1β, IL-18, CXCL1 and MCP-1 as well as IFN-I (Shin et al., 2008; Case et al., 2009; Monroe et al., 2009; Plumlee et al., 2009; McCoy-Simandle et al., 2011), whereas other cytokines such as IL-12 and IFN-γ appear to be produced at only very low levels, if at all (Matsunaga et al., 2001, 2003). In whole animals, increased susceptibility to pulmonary *L. pneumophila* results from cytokine and/or cytokine receptor deficiencies in IL-12, IFN-γ, and TNF (Brieland et al., 1998; Shinozawa et al., 2002; Fujita et al., 2008). This suggests that cytokine production by cell types other than macrophages is important for controlling infection. At this stage a thorough understanding of the role of distinct cytokines and immune cells in combating *L. pneumophila* lung infection is lacking.

LEGIONELLA PNEUMOPHILA INTERACTIONS WITH DC

Dendritic cells represent a heterogeneous group of cells with specialized functional properties. DC play a critical role in eliciting adaptive immune responses through their role as primary antigen presentation cells (Heath and Carbone, 2009). Several subsets of DC are now recognized in the mouse, which began with the identification of $CD8^-$ and $CD8^+$ DC in the spleen (Heath and Carbone, 2009). Further examination of precursor-product relationships led to the identification of distinct end stage subsets of DC, including $CD4^-CD8^-$ (double-negative) DC, $CD103^+$, and $CD11b^+$ migratory DC and Langerhans cells, as well as plasmacytoid DC (pDC) which are set apart from the other conventional DC by their gene expression profile. The role of pDC in generating adaptive immunity is unclear although evidence for a role in antigen presentation is emerging (Heath and Carbone, 2009).

We recently showed that pDC make an important contribution to the restriction of *L. pneumophila* infection *in vivo* (Ang et al., 2010). pDC are known for their ability to combat viral infection through the production of IFN-I (Colonna et al., 2004; Fitzgerald-Bocarsly et al., 2008). However, a role for pDC in resistance to bacterial infection had not been described before. During *L. pneumophila* infection, pDC are rapidly recruited to the lungs of mice and depletion of pDC significantly increases bacterial burden in the lung (Ang et al., 2010). Currently, the mechanism by which pDC restrict *L. pneumophila* infection is not known. However, it is clear that IFN-I is not necessary as IFN-I-receptor-deficient (IFNAR−/−) mice are not more severely infected by *L. pneumophila* compared to wild type mice (Monroe et al., 2009; Ang et al., 2010). Moreover, depletion of pDC in IFNAR−/− mice results in increased bacterial load in the lung, suggesting that IFN-I signaling is dispensable for the anti-bacterial activity of pDC (Ang et al., 2010). Although *L. pneumophila* can infect pDC (Ang et al., 2010), the number of bacteria per host cell is significantly lower compared to macrophages, suggesting that, similar to conventional DC, bacteria do not replicate intracellularly within pDC (Neild and Roy, 2003). The mechanisms that recruit pDC to the lung are not yet known but as the primary site of *L. pneumophila* replication, macrophages are a likely source of chemoattractant

cytokines. pDC presumably then respond to *L. pneumophila* infection by producing cytokines that activate neutrophils, NK cells, and/or macrophages to kill intracellular bacteria (**Figure 1**). Further investigation is needed to determine the mechanisms by which pDC restrict *L. pneumophila* infection and importantly whether these mechanisms are utilized to combat other bacterial pathogens.

In contrast to macrophages, conventional DC do not allow replication of *L. pneumophila* (Neild and Roy, 2003), even if derived from A strain mice. This is despite the fact that LCV formation in DC appears to be similar to that in macrophages (Neild and Roy, 2003). Restriction of replication by mouse DC is the result of activation of both caspase-1-dependent pyroptosis and classical cell death pathways through Bcl-2-associated X (Bax) and Bcl2 antagonist/killer (Bak) mediated apoptosis (Nogueira et al., 2009). The initiation of the intrinsic (mitochondrial) apoptotic pathway by Bax/Bak leads to early activation of caspase-3 in DC that is delayed in macrophages (Nogueira et al., 2009). *L. pneumophila* is known to induce the intrinsic pathway in macrophages (Hagele et al., 1998; Gao and Abu Kwaik, 1999; Molmeret et al., 2004; Abu-Zant et al., 2005; Furugen et al., 2008; Nogueira et al., 2009) but counteracts the pro-apoptotic stimuli, in part by triggering NF-κB dependent up-regulation of anti-apoptotic genes (Losick and Isberg, 2006; Abu-Zant et al., 2007; Bartfeld et al., 2009) as well as delivering anti-apoptotic Dot/Icm effectors such as SdhA and SidF (Laguna et al., 2006; Banga et al., 2007). In fact, SidF acts directly on pro-apoptotic Bcl2 family members Bcl-rambo and BNIP3 while the anti-apoptotic mechanism of SdhA seems independent of central components of the apoptosis pathway (Laguna et al., 2006; Banga et al., 2007; Nogueira et al., 2009). It is unclear why these effectors are functional in macrophages but do not have the same impact in conventional DC, despite the fact that SdhA appears to be at least partially functional (Nogueira et al., 2009). Nevertheless, rapid apoptosis is key to the difference between *L. pneumophila* replication in macrophages and conventional DC because adding the anti-apoptotic Dot/Icm effector, AnkG, from the evolutionarily related pathogen, *Coxiella burnetii*, inhibits *L. pneumophila* induced apoptosis of DC and reverses the restriction on bacterial replication (Luhrmann et al., 2010). The importance of conventional DC in controlling *L. pneumophila* infection *in vivo* is not known. While DC presumably play a role in antigen presentation and the development of an adaptive response, no direct role for conventional DC in controlling *L. pneumophila* lung infection has been proven. It has been proposed that DC may act as a dead

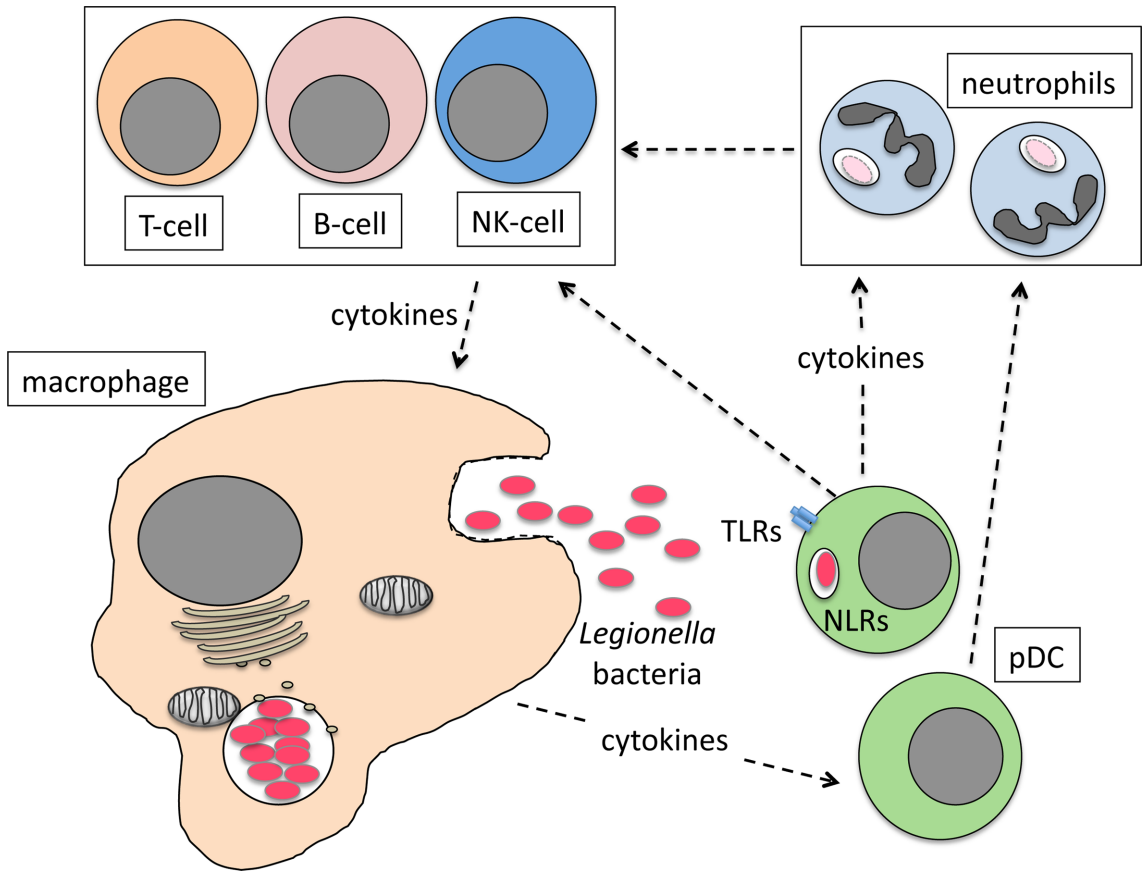

FIGURE 1 | Model for the role of pDC in combating *L. pneumophila* lung infection. Infected macrophages produce cytokines and chemokines that recruit pDC to the lung. Bacteria activate pDC via TLR/NLR interactions or cytokines from infected macrophages stimulate pDC cytokine production that then activates neutrophils, NK cells and macrophages to kill bacteria directly or indirectly.

end for *L. pneumophila* replication thereby restricting bacterial infection but this hypothesis has not been tested directly *in vivo*, for example by depletion of conventional DC (Nogueira et al., 2009).

CONCLUDING REMARKS

Biopsies from patients with Legionnaire's disease show bacteria contained within multi-organism vacuoles in alveolar macrophages (Chandler et al., 1977; Glavin et al., 1979; Hernandez et al., 1980). In guinea pig and mouse lung infection models, alveolar macrophages are the first cells infected by *L. pneumophila* (Winn Jr., 1988; LeibundGut-Landmann et al., 2011). As the initial niche for bacterial replication, macrophages play a pivotal role in initiating the host response to *L. pneumophila*. Indeed recently, IL-1β production by mouse alveolar macrophages was shown to activate cytokine responses in airway epithelial cells (LeibundGut-Landmann et al., 2011). As such, this initial interaction with macrophages is likely to be crucial for the recruitment of immune effector cells including neutrophils and NK cells. In both intravenous and respiratory infection models, IL-18 is required for IFN-γ production by NK cells (Sporri et al., 2008; Archer et al., 2009), however whereas the intravenous model of *L. pneumophila* infection suggested increased susceptibility of IL-18 receptor knockout mice (measured by increased splenic bacterial load; Sporri et al., 2008), this result was not validated in the respiratory infection model (Archer et al., 2009). Therefore it appears that the role of cytokines and immune cells during lung infection differs from interactions during systemic responses. T- and B-cells also ultimately contribute to clear the organism (Susa et al., 1998; Kikuchi et al., 2005; Joller et al., 2007) but their recruitment and mechanism of activation has not been closely examined in the context of *L. pneumophila* infection *in vivo*. Given that the resolution of *L. pneumophila* infection requires multiple cell types and abundant cross talk between immune cells, the role of other cell types such as DC as well as the mechanism of action of protective cytokines should be examined. The coordinated functions of these immune components during *L. pneumophila* infection *in vivo* is likely to yield important new information about immune defense mechanisms in the lung.

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