Chapter

Exploitation of Phosphoinositides by the Intracellular Pathogen, *Legionella pneumophila*

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Abstract

Manipulation of host phosphoinositide lipids has emerged as a key survival strategy utilized by pathogenic bacteria to establish and maintain a replication-permissive compartment within eukaryotic host cells. The human pathogen, *Legionella pneumophila*, infects and proliferates within the lung's innate immune cells causing severe pneumonia termed Legionnaires' disease. This pathogen has evolved strategies to manipulate specific host components to construct its intracellular niche termed the *Legionella*-containing vacuole (LCV). Paramount to LCV biogenesis and maintenance is the spatiotemporal regulation of phosphoinositides, important eukaryotic lipids involved in cell signaling and membrane trafficking. Through a specialized secretion system, *L. pneumophila* translocates multiple proteins that target phosphoinositides in order to escape endolysosomal degradation. By specifically binding phosphoinositides, these proteins can anchor to the cytosolic surface of the LCV or onto specific host membrane compartments, to ultimately stimulate or inhibit encounters with host organelles. Here, we describe the bacterial proteins involved in binding and/or altering host phosphoinositide dynamics to support intracellular survival of *L. pneumophila*.

Keywords: bacteria, infection, effector proteins, pneumonia, *Legionella pneumophila*, phosphoinositides, host-pathogen interactions, membrane traffic

1. Introduction

Bacterial pathogens have evolved diverse and effective strategies to promote their survival in human cells. Some bacteria can circumvent the innate immune response, managing to replicate within macrophages, which are the first line of defense against microbial pathogens and genetically programmed to eradicate foreign particles. Mechanisms that bacteria employ to survive in macrophages include (i) acclimating to the acidic environment within the host lysosome, (ii) escaping the phagosome to persist inside the host cell cytoplasm, and (iii) eluding the endolysosomal pathway by establishing a replication permissive vacuole within the host [1]. The Gram-negative facultative intracellular bacterium, *Legionella pneumophila*, has adopted a survival strategy that relies on the establishment of a protective vacuole that avoids encounters with the endolysosomal pathway. By phagocytosis, macrophages internalize *L. pneumophila* into a membrane-bound compartment termed as phagosome. Upon uptake, *L. pneumophila* directs

membrane remodeling of the phagosomal compartment, employing a sizeable artillery of bacterial proteins that subvert multiple host cellular processes without compromising survival of the host cell throughout infection [2–4]. A specialized secretion system is responsible for translocating these proteins, known as effector proteins, from the bacterial milieu into the host cytosol [5–7]. Effector proteins do not share extensive homology with each other and are often composed of multiple domains that are functionally distinct [8, 9]. An emerging feature among effector proteins is their ability to recognize and bind host phosphoinositides (PIPs) [10], which are a series of phospholipids that play critical roles in coordinating cell signaling and membrane trafficking events in eukaryotic cells [11]. *L. pneumophila* effector proteins exploit the spatiotemporal regulation of host PIPs to facilitate the formation of the *Legionella*-containing vacuole (LCV) and to avoid the endolysosomal pathway. Disruption of the PIP distribution on the LCV membranes leads to bacterial degradation, illustrating that controlling PIP dynamics on and around the LCV is crucial for intracellular survival of *L. pneumophila* [12]. Here we will discuss the *L. pneumophila* effector proteins that contribute to vacuole biogenesis and maintenance through the exploitation of host phosphoinositides.

2. *Legionella pneumophila* replicates in protozoan and innate immune cells

L. pneumophila is ubiquitously found in aquatic environments forming close associations with protozoans and often found as an intracellular parasite of free-living amoeba [13]. In the human lung, *L. pneumophila* infects resident alveolar macrophages leading to severe pneumonia, known as Legionnaires' disease, which can be fatal in immunocompromised individuals [14]. Outbreaks stem from contaminated water systems such as those supplying water towers, cooling systems, and decorative fountains [15]. In 2017, a study by the Centers for Disease Control and Prevention (CDC) found that *L. pneumophila* was the leading bacterial agent responsible for public drinking water-associated outbreaks within the United States [14]. The number of reported Legionnaires' disease cases has been escalating since 2000, presumably due to an increase in urbanization, reliance on industrial water systems, as well as improved diagnostic methods [16]. *Legionella* spp. can exist within biofilms or amoebal hosts in freshwater systems, transitioning between a replicative and a transmissive/virulent phase life cycle [17, 18]. Nutrient deprivation within a biofilm or host triggers the upregulation of genes encoding virulence traits such as motility, osmotic stress resistance, pigmentation production, and multiple virulence factors [17]. This change in gene expression primes the bacterium to be engulfed by a new host cell and tap into their nutrient resources.

Inter-kingdom horizontal gene transfer events and circulating mobile genetic elements over long-term coevolution with multiple hosts have extensively reshaped the plasticity of the *Legionella* spp. genomes [19]. All *Legionella* spp. contain a highly conserved type IV secretion system (T4SS), yet there are differences in the combination of effectors present in each species. An analysis of 38 *Legionella* spp. genomes revealed that DNA exchange between species is rare and only seven core effectors are shared by all sequenced species [8]. *Legionella* effectors share more similarity with eukaryotic proteins than prokaryotic proteins, suggesting *Legionella* spp. have acquired their effector arrays from their hosts [20]. A striking number of effectors across the genus (>18,000) contain eukaryotic-derived domains [9]. This extensive combination of effectors likely stems from intimate coevolution between *Legionella* spp. with diverse protozoan hosts, such as *Acanthamoeba castellanii* [13], *Hartmannella vermiformis* [21], *Dictyostelium discoideum* [22, 23], *Tetrahymena*

pyriformis [24], and *Naegleria fowleri* [25]. Only 20 of the 65 known species have been associated with human disease, suggesting that perhaps *Legionella* species are better adapted for infection within their amoebal hosts [9]. A clear set of effectors that render *Legionella* better suited for human infection is not apparent, although conservation of ankyrin motifs, F-box, or Set18 domains was predominantly found in more virulent strains [9].

The prevailing thought is that the mechanisms that enable *L. pneumophila* to infect and proliferate within protozoa have equipped this bacterium with the ability to survive within innate immune cells. This ability could be due to the high conservation of the pathways involved in uptake and microbial degradation between protozoa and human macrophages. In the lung, resident macrophages and neutrophils engulf *L. pneumophila* by phagocytosis but are often unable to degrade it through phagosome maturation [26–28], a process that entails sequential fusion of the phagosome with endocytic compartments and ultimately the lysosome [29]. *L. pneumophila* is initially encased within a phagosome after macrophage engulfment, but within minutes, the membrane of this phagosome is drastically remodeled into a compartment resembling the endoplasmic reticulum (ER) [2, 4]. Tubular ER and secretory vesicles are rapidly routed toward the phagosome where some eventually fuse with the phagosomal membrane, allowing the phagosome to adopt the identity of the recruited host membrane [30]. While promoting LCV membrane fusion with the ER and Golgi-derived vesicles, *L. pneumophila* prevents fusion with endosomal compartments. Studies have found that *L. pneumophila* effector proteins can target specific host membrane compartments, including early endosomes, recycling endosomes, and autophagosomes. Collectively, these effectors help *L. pneumophila* evade the macrophage's pre-programmed lysosomal degradation pathway [10], although precisely how these events are choreographed is not well understood.

The extensive remodeling of the vacuolar membrane is entirely dependent on a specialized Dot/Icm T4SS that delivers a staggering number of bacterial effector proteins (over 350) [8] into the host cytosol, many of which target membrane transport pathways [31, 32]. Disruption of the T4SS results in lysosomal degradation of the bacterium, indicating that the actions of effector proteins are paramount to bacterial survival [33]. However, it is often a challenge to identify an observable phenotype that can be attributed to a single effector because of functional redundancy among bacterial effectors [34]. Many advances have been made to dissect the molecular contribution of individual effectors toward bacterial infection (reviewed in [35]). A number of these effectors have been reported to hijack host vesicular trafficking pathways. An emerging feature among some of the effectors that target membrane trafficking is the ability to bind key host regulatory lipids, phosphoinositides (PIPs).

3. Phosphoinositides as crucial regulators of vesicular trafficking

Membrane compartments within eukaryotic cells are highly abundant, dynamic, and functionally distinct structures. Their movement must be tightly regulated to ensure that cargo carried by these structures is delivered to the proper destination. The cellular machinery recognizes and distinguishes these compartments based on the unique protein and lipid composition on the cytosolic leaflet of the membrane lipid bilayer [11]. Phosphoinositides are glycerophospholipids that amount to less than 15% of phospholipids within membranes but are essential for coordinating the spatiotemporal regulation of membrane trafficking events [11]. Phosphatidylinositol (PI), the precursor of phosphoinositides, can be reversibly phosphorylated at positions 3, 4, and 5 of its *myo*-inositol ring resulting in the

generation of seven PIP species [11]. Membrane compartments are characterized in part by the presence of distinct PIP species that essentially act as molecular anchors to facilitate protein recruitment and attachment to specific compartments [11]. PI is synthesized in the endoplasmic reticulum and delivered to membrane-bound compartments via vesicular transport or cytosolic PI transfer proteins [11]. The Golgi and plasma membrane are highly enriched with PI(4)P, while lower levels of PI(4)P are also found within membranes of the ER and late endosomes [11, 36, 37]. PI(3)P is mainly found on phagosomes, early endosomes, late endosomes, and multivesicular bodies (MVBs). MVBs and late endosomes also contain PI(3,5)P_2, which is the dominant PIP on lysosomes. Phagocytosis and phagosome maturation are entirely dependent on phosphoinositide dynamics [38]. PI(4,5)P_2 and PI(3,4,5)P_3 are present on the plasma membrane and are critical for recruiting the cellular machinery for initiating phagocytosis. Once phagosomes have been formed, PI(3)P is the predominant PIP on the organelle [29]. PI(3)P then triggers the recruitment of proteins to the phagosome, such as EEA1 and its subsequent effector Rab5, to facilitate docking and fusion with early endosomes and progression down the phagolysosomal maturation pathway [39]. Blocking the formation of these PIP species results in robust inhibition of phagocytosis [40]. Given the crucial importance of PIPs for particle uptake and degradation, it is not surprising that intracellular bacteria have evolved molecular mechanisms to take command of these eukaryotic lipids.

4. Phosphoinositide dynamics on the LCV

The PIP composition on the LCV membrane has profound effects on the fate of the bacteria-bearing vacuole. PI conversion that accompanies LCV maturation was deciphered by tracking the localization of fluorescent PI probes produced in the soil amoeba, *Dictyostelium discoideum*, which serves as a model organism for the study of host-pathogen interactions [41]. As *L. pneumophila* enters *D. discoideum*, the phagocytic cup is coated with PI(3,4,5)P_3. On the membrane of the newly formed phagosome, PI(3,4,5)P_3, PI(3,4)P_2, and PI(4)P persist for less than 60 s on average. By 60 s, the phagosome begins to accumulate PI(3)P. Over the next 2 h, PI(4)P levels increase, the LCV lumen expands, and PI(3)P is slowly lost and excluded from the maturing LCV. The mature LCV maintains a discrete pool of PI(4)P separate from the surrounding ER, in which it acquires 30 to 60 min after uptake. As the bacterium continues to replicate, PI(4)P levels are steadily maintained on the LCV but are present in pools distinct from the surrounding ER network. The conversion from a PI(3)P to a PI(4)P-positive compartment is secretion system-dependent: a mutant strain lacking a functional T4SS accumulates PI(3)P on the LCV, PI(4)P is never acquired, and the LCV is destined for lysosomal degradation [12]. Thus, translocated effectors control the PIP composition of the LCV and potentially other host membranes.

In a recent study, Weber and colleagues [42] pursued the source of the PI(4)P on the LCV membrane. Real-time high-resolution confocal laser scanning microscopy (CLSM) revealed that LCVs of infected *D. discoideum* capture PI(4)P from trans-Golgi-derived vesicles. PI(4)P-enriched vesicles accumulate close to the LCV, even in the absence of the T4SS, but retention of these vesicles relies on the T4SS. This observation indicates that while PI(4)P-positive compartments localize to phagosomes regardless of the internalized cargo, effector proteins are needed to prolong this interaction. The removal of PI(3)P from the phagosome membrane was thought to occur through the actions of PIP-modifying enzymes; however, CSLM imaging of infected *D. discoideum* revealed shedding of PI(3)P-positive vesicles from the LCV. Moreover, the timing of PI(3)P shedding coincided with the

gradual accumulation of PI(4)P-compartments around the LCV [42]. Together, these observations support the notion that *L. pneumophila* adopts a combined strategy to convert the LCV from a PI(3)P- to PI(4)P-enriched compartment, employing both direct modification of PIPs on the LCV membrane and selective association with host vesicles.

5. *L. pneumophila* effector proteins alter the PIP composition of the LCV membrane

To manipulate the PIP composition on the LCV, *L. pneumophila* uses both genetically encoded and host-derived PI kinases and phosphatases (**Figure 1**). Converting the PI(3)P-enriched phagosome to a predominantly PI(4)P-positive compartment requires a concerted effort between enzymes that add and remove

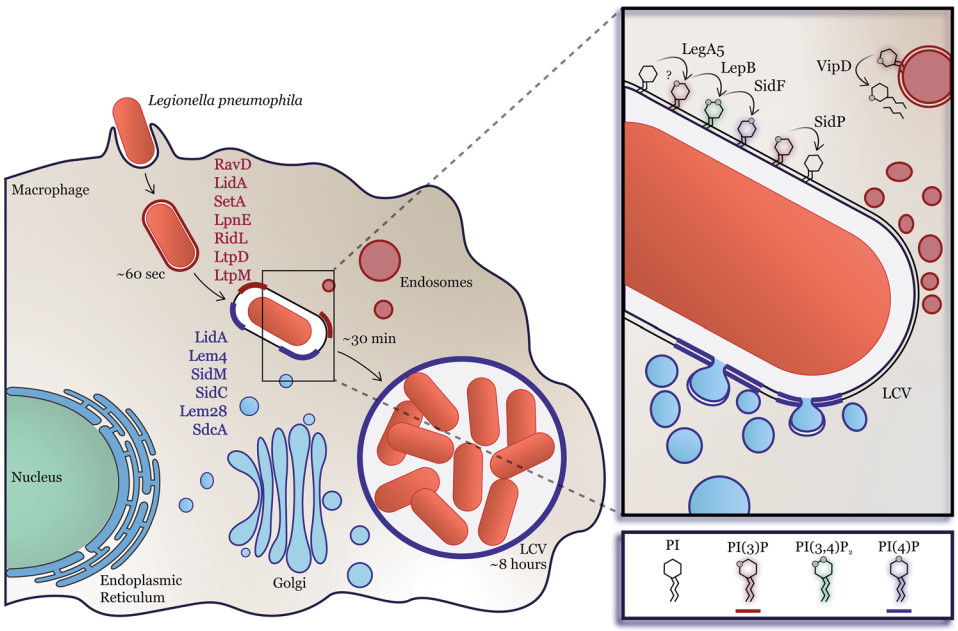

Figure 1.
L. pneumophila converts the phagosome to a PI(4)P-rich vacuole. Within a minute of uptake into the host cell, the LCV acquires the endosomal phosphoinositide, PI(3)P. Within an hour of infection, the LCV starts to accumulate PI(4)P until the bacteria are completely encapsulated in a PI(4)P-rich membrane. To avoid progression down the phagosome maturation pathway, L. pneumophila translocates effectors that alter the phosphoinositide composition on the LCV membrane to a PI(4)P-positive compartment (inset). This process is a result of close association and fusion with host vesicles as well as the direct conversion of existing phosphoinositides by kinases and phosphatases. Golgi-derived PI(4)P-positive vesicles accumulate around the LCV and later fuse with the vacuolar membrane. In contrast, PI(3)P-containing vesicles traffic toward the LCV but do not fuse with it. Additionally, the Legionella effector LepB is a PI kinase that phosphorylates PI(3)P and generates PI(3,4)P2 on the LCV membrane. This PI is a substrate for SidF which dephosphorylates PI(3,4)P2 to PI(4)P. While the origin of PI(3)P that LepB utilizes as a substrate is undetermined, LegA5 is a PI 3-kinase produced by Legionella that phosphorylates PI and could lead to additional PI(3)P on the LCV for conversion to PI(4)P. In combination, LegA5, LepB, and SidF may provide a cascade of enzymatic events for converting the LCV into a PI(4)P-positive compartment. SidP, another direct modifier of phosphoinositides produced by Legionella, may also contribute to the avoidance of the endocytic pathway by removing the phosphate from PI(3)P to hinder vesicle fusion. VipD localizes to endosomes and hydrolyzes a lipid tail from PI(3)P to potentially limit their interaction with the LCV. During this phosphoinositide conversion, Legionella effectors associate with the LCV through phosphoinositide binding domains. Some effectors localize by binding PI(3)P (RavD, LidA, SetA, LpnE, RidL, LtpD, LtpM), and some can associate via PI(4)P-binding (LidA, Lem4, SidM, SidC, Lem28, SdcA). During the later stages of infection, PI(3)P is undetectable and PI(4)P has become enriched on the expanding vacuole.

a phosphate group of the *myo*-inositol head group. The direct PI 4-kinase activity of the effector LepB could potentially initiate the conversion process to a PI(4)P-positive membrane by converting PI(3)P to PI(3,4)P_2. LepB was initially identified as an effector that is involved in bacterial egress [43]. Since then, LepB was found to localize to the LCV, where it contributes to the dynamics of Rab1 by acting as a GTPase-activating protein (GAP) [44, 45]. Found between amino acids 313 and 618, the structure and mechanism of the GAP domain is now well understood [46–48]. The N-terminal domain consisting of the first 311 amino acids garnered interest as this domain alone could disrupt the structure and function of the Golgi. The crystal structure of $LepB_{1–618}$ revealed homology to atypical kinases such as CtkA from *Helicobacter pylori* and actin-fragmin kinase from *Physarum polycephalum*. When mutating residues capable of performing phosphorylation, the yeast toxicity phenotype was found to be suppressed. While this suggested a kinase functionality, the pocket for a substrate was too small to accommodate proteins any larger than Rab GTPases. However, LepB did not phosphorylate any of the tested Rab GTPases. Instead, the LepB substrates were revealed to be phosphoinositides. Studies showed that LepB, but not the catalytically inactive mutant $LepB_{H154A}$, caused the sensor for PI(3,4)P_2 and PI(3,4,5)P_3 to relocate from a cytosolic to punctate distribution, while the signal for PI(3)P diminished dramatically. Ultimately, an in vitro kinase assay validated that LepB is a PI 4-kinase with specificity for PI(3)P and a level of activity comparable to the host kinase PI4KIII. By phosphorylating PI(3)P on the LCV, LepB could be initiating the vacuole's phosphoinositide conversion to PI(4)P by providing the PI(3,4)P_2 intermediate step [49].

As PI(3,4)P_2 is generated on the LCV, it is thought that SidF can dephosphorylate this lipid to PI(4)P. SidF is a membrane protein containing a large N-terminal domain followed by two transmembrane domains triggering localization to the LCV. SidF was the first *L. pneumophila* effector found to directly modify phosphoinositides through a screen for the well-known CX_5R phosphatase motif in the effector repertoire. SidF is a PI 3-phosphatase: this effector hydrolyzes the phosphate group at the third position of PI(3,4)P_2 or PI(3,4,5)P_3 to produce PI(4)P or PI(4,5)P_2, respectively; however, it displays a preference for PI(3,4)P_2. The mutation of the catalytic cysteine at residue 645 to a serine resulted in the abrogation of this phosphatase activity. As described in the following sections, PI(4)P on the LCV membrane can serve as a means for effectors, such as SidC, to anchor onto the LCV. Infection with a mutant lacking *sidF* shows significantly fewer vacuoles positive for SidC, suggesting that SidF contributes to the generation of PI(4)P on the LCV.

Ultimately, the functions of LepB and SidF suggest that PI(3)P can be converted to PI(4)P through the sequential efforts of these enzymes. The deletion of *lepB* and *sidF* individually shows a significant deficiency of SidC on the LCV membrane at similar levels [49, 50]. The deletion of both effectors simultaneously causes a decrease in SidC acquisition on the membrane no greater than the single-mutant strains, suggesting that these effectors are functioning in a linear pathway [49]. However, the complete loss of SidC was not seen in the infection with a *lepB sidF* double-deletion mutant. Additionally, both *lepB* and *sidF* are not always found in other *Legionella* species. Together, this suggests that there are other *Legionella* effectors or host proteins manipulating the LCV PIP landscape.

The screen that identified SidF as a PI phosphatase also yielded SidP as another direct modifier of phosphoinositides. SidP was identified as a candidate due to its CX_5R motif. It was found to have PI 3-phosphatase activity, cleaving PI(3)P and PI(3,5)P_2 in vitro. The *L. longbeachae* orthologue of SidP was only found to hydrolyze PI(3)P, suggesting this lipid may be the true target. SidP was also found to act as a PI 3-phosphatase *in vivo* when it suppressed yeast toxicity in a PI 3-phosphatase-deficient mutant but not a mutant lacking PI 4-phosphatases. This

activity was confirmed when the levels of PI(3)P, but not PI(4)P or PI(4,5)P_2, were decreased in the presence of SidP [51]. Nonetheless, the purpose of SidP's phosphatase activity for successful infection has not yet been determined. We can speculate that SidP may work alongside LepB to quickly eliminate PI(3)P from the vacuole. As LepB converts PI(3)P to PI(3,4)P_2, SidP may be dephosphorylating PI(3)P to PI to completely deplete the membrane of this phospholipid that would otherwise trigger the phagocytic maturation.

As part of an effort to determine the function of a *Francisella* effector, OpiA, LegA5 was found to possess PI 3-kinase activity. LegA5 contains two motifs, DXHXXN and IDH, separated by 14 amino acids that are characteristic of the catalytic and activation loops of PI 3-kinases (and PI 4-kinases) [52]. PI(3)P has been shown to accumulate on the LCV early during infection in a manner independent of effector protein translocation [12]. This lipid is speculated to be the substrate LepB that acts on to initiate the PI(3)P to PI(4)P conversion on the phagosome membrane. However, *Legionella* may encode an effector that also contributes to the PI(3)P pool. These proteins may be delivered to the LCV in a complex so that PI is efficiently converted to PI(4)P. Alternatively, or perhaps in addition, PI(3)P-positive vesicles that accumulate around the nascent *Legionella*-containing phagosome may serve as a source for the initial wave of PI(3)P.

Aside from kinases and phosphatases that change the phosphorylation state of PIPs, *Legionella* also encodes 19 phospholipases. Phospholipases differ from PI phosphatases by cleaving the phospholipid backbone instead of hydrolyzing a phosphate on the *myo*-inositol head group. While these proteins can enter the host through different systems such as the Sec, Tat, T2SS, T4SS, and outer membrane vesicles (OMVs), only phospholipases translocated via T4SS will be discussed here [53]. The best characterized T4SS-secreted phospholipase is VipD. VipD has three paralogs, VpdA, VpdB, and VpdC, which are also T4SS substrates but have yet to be studied in detail [54]. The structure of VipD shows two distinct domains: the N-terminal domain has phospholipase activity A, indicating cleavage of the ester bond releasing a fatty acid chain, and the C-terminal domain causes localization to early endosomes and interacts directly with Rab5 and Rab22 [55]. The phospholipase activity is activated when VipD is bound to Rab5 due to a conformational change that exposes the active site [56, 57]. The activation of VipD causes cleavage of PI(3)P on endosomal membranes that prevents normal localization of membrane trafficking regulators, contributing to endosomal avoidance by *Legionella* [56].

While VipD has phospholipase A activity, *Legionella* also translocates two T4SS effectors with phospholipase C and D activity. A phospholipase C hydrolyzes the phosphorus-oxygen bond, releasing the phosphate of the phospholipid and the attached head group, and a phospholipase D solely cleaves off the attached head group. The phospholipase C effector protein, PlcC, is able to cleave phospholipids such as phosphatidylglycerol, phosphatidylcholine, and phosphatidylinositol [58]. Alone or in combination with two other phospholipase C effectors, PlcA and PlcB, translocated by the T2SS, these effectors were dispensable for growth in amoeba and macrophages. However, a triple mutant of these phospholipases displayed inefficient killing of larvae in the *G. mellonella* infection model compared to the wild type [58]. It is not yet known how this function may contribute to intracellular survival. We speculate that perhaps removing the head group on these phospholipids, specifically PI, would render them incapable of being modified by PI kinases and phosphatase and prevent the vacuole from being quickly converted to an endosome-like membrane. It would be interesting to determine if this phospholipase activity alters the PI composition of the LCV.

Lastly, the phospholipase D effector, LpdA, was first identified due to its homology with known phospholipase D enzymes [59]. LpdA specifically cleaves the head

group from PI, PI(3)P, PI(4)P, and phosphatidylglycerol *in vitro* [60]. While LpdA localizes to the LCV [59], it is not known if or how this effector contributes to phosphoinositide dynamics. Nonetheless, deleting this gene results in the attenuation of growth in a mouse model [60].

LppA is a phytase enzyme that dephosphorylates the compound *myo*-inositol hexakisphosphate, known as phytate. While LppA's phosphatase activity on phytate may play various roles during infection, of interest to this review are its effects on phosphoinositides. The inositol phosphate head group of PIPs is similar in structure to phytate. LppA was shown to dephosphorylate PI(3,4)P_2 and PI(4,5)P_2 as well as, but less efficiently, PI(3,4,5)P_3 to PI(4)P in vitro. However, infection with an *lppA* deletion strain did not impact the presence of PI(4)P on the LCV [61]. It is possible that lack of LppA generates a more subtle phenotype that requires more sensitive detection methods.

In addition to directly manipulating the phosphoinositide composition of the vacuolar membrane, *Legionella* may change the PIP landscape by enlisting host enzymes. For instance, the host PI 5-phosphatase OCRL1 is recruited to the LCV in a T4SS-dependent manner. OCRL1 preferentially removes a phosphate from PI(4,5)P_2 to generate PI(4)P [62]. The homolog of OCRL1 in *Dictyostelium*, Dd5P4, was found to localize to LCVs where it is catalytically active and therefore able to dephosphorylate PIPs [63]. How OCRL1 is recruited to the LCV is not yet clear, but it is thought that *Legionella* protein LpnE may contribute to this process. LpnE is a Sel1-like repeat protein translocated into host cells in a T4SS-independent manner, and it seems to be exported extracellularly through an unknown mechanism [64]. LpnE is important for entry into amoebae and macrophages as well as intracellular replication. *In vitro* LpnE binds PI(3)P and interacts with OCRL1, but it does not seem to be essential for recruitment of OCRL1 to the LCV. It may be that LpnE synergizes with other effectors to stably recruit OCRL1, but this idea remains to be tested [63].

6. *L. pneumophila* effector proteins specifically bind phosphoinositides

Central to the ability of *L. pneumophila* to grow within both mammalian and protozoan cells is the remodeling of the phagosomal membrane through the manipulation of host secretory and endosomal trafficking. The loss of PI(3)P and the acquisition of PI(4)P on the phagosome membrane are achieved through a concerted mechanism carried out by the actions of multiple effector proteins. The acquisition of PI(4)P on the phagosome membrane is imperative for the subsequent recruitment of membranes to promote vacuole expansion [12]. PI(4)P on the LCV can serve as a docking site for effector attachment to ensure effectors are directed to the correct compartment within the cell [65]. Many effectors that bind to PI(4)P on the LCV are involved in the recruitment and fusion of secretory vesicles and ER. In addition to directly producing PI(4)P on the LCV via effector-driven phosphorylation and dephosphorylation of PIPs, it was recently reported that the phagosome also derives PI(4)P from the membrane material of secretory vesicles, demonstrating *L. pneumophila* employs multiple tactics to acquire PI(4)P. A number of PI(3)P-binding effectors have also been identified [66]. The few whose functions have been characterized interfere with phagosomal maturation, retrograde trafficking, and autophagy [67–70]. An overview of *L. pneumophila* effectors that target PIPs is in **Table 1**.

6.1 *L. pneumophila* T4SS effectors that bind PI(4)P

Bacterial effectors translocated early during infection have been shown to facilitate the recruitment and fusion of ER/secretory vesicles with the LCV. SidM

Name	PIP target	PIP-binding domain	Function	Citation
RavB	PI(3)P	LED035	Not Determined	66
CegC2	PI(3)P?	LED006	Not Determined	66
RavD	PI(3)P, PI(4)P		Prevents accumulation of linear ubiquitin chains on the LCV through deubiquitinase activity and prevents endolysosomal maturation of the LCV	67, 87
LtpM	PI(3)P		Glucosyltransferase activity stimulated by PI(3)P-binding	98
AnkX	PI(3)P, PI(4)P		Phosphocholinates Rab1 & Rab35; prevents lysosome-LCV fusion and endocytic recycling	70, 83, 86
LidA	PI(3)P, PI(4)P		RabGTPase interacting protein; contributes to retention of activated Rab1 on LCV and recruitment of secretory vesicles	48, 73, 74, 76, 80
Lem4	PI(4)P	P4M	Phosphotyrosine phosphatase	77, 78
Ceg19	PI(3)P	LED027	Causes secretory trafficking defects in yeast	66
LegK1	PI(3)P	LED006	Activates NF-κB by phosphorylating regulatory proteins	66
Ceg22	PI(3)P	LED006	Not Determined	66
LegC5/Lgt3	PI(3)P	LED006	Glucosylates eEF1A to inhibit translation	66
Lem9	PI(3)P	LED006	Not Determined	66
LegC6	PI(3)P	LED006	Not Determined	66
RavZ	PI(3)P	LED027	Inhibits autophagy through irreversible deconjucation of LC3 from autophagosome membranes	66, 69, 94
Lpg1961	PI(3)P?	LED027	Not Determined	66
SetA	PI(3)P		Glucosyltransferase activity stimulated by PI(3)P-binding	95, 98
LpnE	PI(3)P		Interacts with OCRL1 on the LCV, promotes intracellular uptake	63, 64
Lem21/LotA	PI(3)P	LED035	Prevents accumulation of ubiquitin chains on the LCV through deubiquitinase activity	66, 88
RidL/Ceg28	PI(3)P		Binds the retromer complex to inhibit retrograde trafficking	68, 90
Lpg2327	PI(3)P	LED006	Not Determined	66
MavH	PI(3)P	LED035	Not Determined	66
SidM/DrrA	PI(4)P	P4M	Promotes the recruitment and fusion of secretory vesicles with the LCV, AMPylates Rab1, interacts with exocyst complex	73-75, 85
SidC, SdcA	PI(4)P		Involved in ER recruitment to the LCV and ubiquitination through E3 ligase activity	50, 81, 82
Lem28	PI(4)P	P4M	Not Determined	77

***L. pneumophila* effectors with PIP-modifying activity**

Name	Substrate	Product	Enzymatic activity	Citation
SidP	PI(3)P, PI(3,5)P2	PI, PI(5)P	PI 3-phosphatase	51
LepB	PI(3)P	PI(3,4)P2	Rab1 GAP; PI 4-kinase that generates PI(4)P on the LCV membrane	44, 48, 49, 66
LegA5/AnkK	PI	PI(3)P	PI 3-kinase	52
SidF	PI(3,4)P2, PI(3,4,5)P3	PI(4)P, PI(4,5)P2	PI 3-phosphatase that acts on the LCV	50
VipD	PI(3)P	PI	Rab5-activated phospholipase activity cleaves PI(3)P on endosomal membranes	54-57

Table 1.
Legionella pneumophila *effectors targeting PI(3)P and PI(4)P.*

(DrrA), an effector protein translocated immediately upon infection, localizes to the LCV and plays a crucial role in ER recruitment by exploiting the activity of Rab1, a small GTPase responsible for the transport of vesicles between the ER and Golgi [71–74]. SidM is a modular protein consisting of an N-terminal adenylyl-transferase domain, a C-terminal PI(4)P-binding domain, and a central guanine nucleotide exchange factor (GEF) domain that activates the small GTPase Rab1 by facilitating the exchange of GDP with GTP [73]. SidM's adenylyltransferase activity covalently adds an adenosine monophosphate moiety onto Tyr 77 of Rab1, locking this small GTPase in its active conformation. Activated Rab1 is required for the recruitment of secretory vesicles to the LCV [73, 74]. SidM then promotes the tethering and fusion of these compartments with the phagosome membrane by interacting with an exocyst complex comprised of Sec5 and Sec15 [75]. A

high-resolution crystal structure of SidM revealed a novel fold within the protein structure, termed P4M, that was responsible for binding PI(4)P with an unprecedented high affinity in the nanomolar range [76]. Two additional PI(4)P-binding effectors, Lem4 and Lem28, contain C-terminal domains similar to the P4M domain [77]. While Lem4 and Lem28 localize to the LCV through their PI(4)P-binding domains, they do not act on Rab1. Lem4 was recently demonstrated to be a phosphotyrosine phosphatase [78], although how this enzymatic function contributes to infection has yet to be determined.

Multiple effectors manipulate Rab1 to exploit secretory trafficking [44, 79]. While SidM is required for activating this small GTPase on the LCV, the PI(3)P and PI(4)P binder, LidA, protects Rab1 from being inactivated [73, 74, 80]. LidA also localizes to the early LCV as well as other uncharacterized membrane compartments [73, 74, 80]. Unlike P4M-containing effectors, LidA interacts with PIPs through a central coiled-coil region. LidA interacts with AMPylated Rab1 through the same coiled-coil domain, preventing GAPs from accessing Rab1 to deactivate it. It is unknown whether the PIP interaction contributes to LidA's function.

In addition to SidM, the PI(4)P binders SidC and its paralogue, SdcA, are also required for the recruitment of ER proteins to the LCV. In the absence of *sidC*, only 20% of LCVs acquire the ER marker calnexin, indicating that the interaction of LCVs with the ER is severely impaired upon deletion of this gene [81]. SidC and SdcA interact with PI(4)P using a 20 kDa C-terminal-binding domain (P4C) that does not share similarities with P4M or other eukaryotic PIP-binding motifs. Mutations that abolish P4C-PI(4)P interactions reduced ER recruitment to the LCV, indicating that SidC's PI(4)P-binding activity is critical for remodeling the LCV membrane [82].

6.2 *L. pneumophila* T4SS effectors that bind PI(3)P

Multiple PI(3)P-binding effectors have been identified, and several were shown to be involved in preventing the LCV from entering the phagosomal maturation pathway. AnkX binds both PI(3)P and PI(4)P *in vitro*, and in macrophages infected with a mutant strain lacking AnkX, the lysosomal marker, LAMP1, accumulates around the LCV indicating it is being routed for endolysosomal degradation [70]. AnkX's N-terminal FIC domain harbors phosphocholine transferase activity catalyzing, the covalent attachment of a phosphocholine moiety onto a serine or threonine residue of Rab1 and Rab35 [83, 84]. It is unknown whether AnkX localizes to the LCV, and despite its ability to covalently modify Rab1, it does not enhance retention of Rab1 on the LCV as observed for SidM-catalyzed adenylylation of Rab1. Phosphocholination locks Rab35 in an inactive conformation by preventing interaction with its cognate GEF, connecdenn; however, phosphocholinated Rab1 was still able to interact with SidM, which also acts as a GEF [85]. AnkX disrupts endocytic recycling in infected macrophages in a phosphocholination-dependent manner, suggesting that phosphocholination of Rab35, a key regulator of endocytic recycling, may be responsible for this phenotype [86].

The PI(3)P-binding effector, RavD, also contributes to preventing encounters between lysosomes and the LCV. Transmission electron microscopy and structured-illumination microscopy revealed RavD is present on the LCV membrane and vesicles adjacent to the LCV; however the identity of these vesicles has not yet been revealed. RavD binds PI(3)P via a C-terminal region [67]. A recent study reported that RavD's N-terminal region harbors deubiquitinase activity (DUB) that specifically cleaves linear ubiquitin chains from the LCV using a Cys-His-Ser triad [87]. Deletion of *ravD*

causes the LCV to become decorated with linear ubiquitin and triggers subsequent activation of the NF-κB pathway [87]. Since *Legionella* species have not coevolved with macrophages, it is possible that RavD's DUB activity would be functional in both macrophages and protozoan hosts. It would be interesting to determine RavD's substrates in the context of a macrophage versus amoebae infection. Understanding the functional link between RavD's DUB activity and its contribution to the prevention of LCV-endolysosomal fusion could provide novel insight into why pathogens exploit ubiquitin during infection.

L. pneumophila's cohort of effectors includes multiple deubiquitinases that have evolved to act on different ubiquitin chains. Effector LotA localizes to the LCV through interaction with PI(3)P and harbors dual DUB activity to remove ubiquitin from the LCV [88]. LotA uses a C13 residue that acts against K6 linkages and a C303 residue that acts against K48 and K63 linkages, although C303 has a more considerable contribution to removing ubiquitin from the LCV. A *Legionella* strain lacking LotA and the ubiquitin-associated SidE family of effectors resulted in impaired bacterial growth within murine bone marrow-derived macrophages, indicating LotA has coordinated activity with other *L. pneumophila* ubiquitin-modifying enzymes [88]. While it has not been reported whether the SidE effector family interacts with PIPs, it cannot be ruled out that these ubiquitin-modifying enzymes may also rely on PIPs to correctly direct them to the sites where their enzymatic activity is required.

The effector RidL binds PI(3)P and inhibits retrograde transport through molecular mimicry. Retrograde trafficking serves as a conduit that connects endosomes, the trans-Golgi network, and the ER [89]. Cargo that is cycled from endosomes to the Golgi is recognized and sorted by a retromer complex. Ectopically expressed RidL blocks retrograde trafficking at endosome exit sites through interactions with the retromer complex protein, Vps29 [68]. RidL is present on the LCV membrane and endosomes but does not localize to endosomes through interactions with PI(3)P. Instead, RidL inserts itself into the endosomal retromer complex through interactions with Vps29, displacing Vps29 from binding to the Rab7 GAP, TBC1D5. RidL interacts with Vps29 using a hairpin loop that mimics the same manner in which TBC1D5 interacts with Vps29 [90]. This displacement blocks the movement of retrograde vesicles through an unknown mechanism. In the absence of *ridL*, LCVs accumulate lysosomal markers and retrograde cargo such as CI-MPR, which delivers acidic hydrolases to endocytic compartments [90]. This suggests the LCV may accept cargo or membranes from a subset of endosomal pathways and that RidL could intercept these incoming vesicles.

PI(3)P is also present on autophagosomes [91], and studies found that indeed *L. pneumophila* effectors also interfere with the dynamics of these compartments [69]. Autophagy is a conserved process across eukaryotic species that is triggered by cellular stress and serves as an additional defense mechanism against intracellular pathogens. Autophagy progression relies on a series of membrane reconstruction events, starting with phagophore membrane nucleation, to phagophore elongation and fusion to form the PI(3)P-rich autophagosome and ultimately fusion with lysosomes to degrade the internal cargo [91]. Early phagophore formation events are dependent on the presence of PI(3)P, which stimulates the recruitment of PI(3)P-binding proteins on ER-derived omegasomes [91]. Phagophore closure is completed through conjugation of LC3 to phosphoethanolamine (PE) on the phagophore membrane [92, 93]. Effector RavZ inhibits autophagy by extracting lipidated LC3 from autophagosome membranes and generating a modified LC3 product that lacks the essential C-terminal glycine required for reconjugation back onto autophagosome membranes.

RavZ localizes to autophagosome membranes through a C-terminal domain that recognizes PI(3)P. $RavZ_{1–331}$ contains catalytic activity yet displays reduced LC3-PE extraction, indicating proper localization to phagosomes is needed to inhibit autophagy [94]. This high-affinity PI(3)P-binding domain, termed LED027, contains two conserved tyrosine and lysine residues that are key for PI(3)P binding. LED027 is found in two other effectors, Lpg1121 (Ceg19) and Lpg1961, although Lpg1961 did not display lipid-binding activity when tested *in vitro* [66]. It would be interesting to determine if these LED027-containing effectors also preferentially localize to PI(3)P on autophagosomes, possibly unveiling a novel conserved domain that confers autophagy-related activity in bacterial effectors.

While effectors rely on PIPs for proper localization, binding to PIPs can also induce the enzymatic activity of some effectors. Effector protein SetA possesses an N-terminal region with glucosyltransferase activity and a C-terminal PI(3)P-binding region responsible for LCV localization [95]. Notably, PI(3)P binding enhances SetA's glucosyltransferase activity [96]. *In vitro* SetA has multiple substrates including actin, vimentin, and the chaperonin CCT5 [96], although it is unclear if these substrates are modified during infection.

The cohort of T4SS substrates is not conserved across all *L. pneumophila* strains. Strains harbor variations in their combinations of effectors that have been presumably acquired during the course of coevolution with a variety of protozoan hosts [97]. Despite these variations, PIP binding is emerging as a common feature among effectors of *L. pneumophila* strains. The *L. pneumophila* Paris strain encodes the glucosyltransferase LtpM that resembles the Philadelphia strain effector, SetA, in domain structure and the ability to cause a growth defect in yeast [96, 98]. Unlike SetA which uses a typical DxD motif for catalysis, LtpM harbors a noncanonical DxN motif. The glucosyltransferase activity LtpM is also stimulated by PI(3)P, indicating multiple effectors have evolved to exploit PI(3)P for purposes other than directing proper localization.

7. Eukaryotic and bacterial phosphoinositide-binding domains

In eukaryotes, proteins bind PIPs via domains that are highly conserved. Protein-lipid binding typically occurs through electrostatic interactions between positively charged amino acid residues and the negative phosphate(s) on the *myo*-inositol ring. These protein domains vary in their binding affinity and specificity for the seven PIP species [99]. The well-characterized pleckstrin homology (PH) domain is the eleventh most common domain in humans, found in 275 proteins [100]. Proteins harboring the PH domain are recruited to membranes through interactions with either $PI(3,4)P_2$, $PI(4,5)P_2$, or $PI(3,4,5)P_3$. The FYVE domain confers high specificity for PI(3)P and is present in many proteins that localize to endosomes [101, 102]. The phox domain (PX) is commonly found in sorting nexins and preferentially binds PI(3)P and in some cases $PI(3,4)P_2$ [103]. Intriguingly, bacterial proteins that specifically bind host PIPs do not use eukaryotic-like domains.

Bacteria can acquire protein domains by horizontal gene transfer from the hosts they infect [97]. A number of *L. pneumophila* effectors harbor eukaryotic-like domains such as ankyrin repeats, U-Box, F-box, and Sel1 repeats [9]. Interestingly, prokaryotic PIP-binding domains were not derived from their eukaryotic hosts. Global bioinformatic analysis of 38 *Legionella* genomes revealed a conserved PI(4)P-binding domain found in 36 putative effectors, while a

domain termed LED006 is found in 136 effectors from 30 species [8]. The PI(4)P-binding domain was experimentally validated to be functional in SidM, Lpg1101, and Lpg2603 [73, 77].

A recent study identified three conserved PI(3)P-binding domains present in 14 *Legionella* effectors across 41 *Legionella* species: LED006, LED027, and LED025 [66]. All three domains rely on positively charged or aromatic residues confined to the C-terminus and are accompanied by an adjacent enzymatic or protein-binding domain. LED006 displayed the weakest affinity for PI(3)P yet is the most conserved, found in eight *L. pneumophila* effectors: CegC2, LegK1, Ceg22, LegC5, Lem9, LegC6, LepB, and Lpg2327. Only LegK1, LegC5, and LepB have been studied and shown to possess catalytic activity. While the C-terminal region of these proteins is conserved, the catalytic activity harbored by their N-terminal region varies. LegK1 is a serine/threonine kinase that targets the NF-κB pathway, LegC5 is a glucosyltransferase that modifies eEF1A, and LepB has dual PI 4-kinase activity and a Rab GAP domain. LED027 binds PI(3)P with high affinity and is found in RavZ, Lpg1121 (Ceg19), and Lpg1961, although Lpg1961 did not display lipid-binding activity when tested in vitro. LED035 is present in RavB, Lem21, and MavH, although none have been functionally characterized.

Biochemical analysis of a *Vibrio parahaemolyticus* effector revealed a conserved type III secreted bacterial phosphoinositide-binding domain (BPD) domain that mediates membrane localization in eukaryotic cells. The BPD domain is the first instance of a domain found in both plant and animal pathogens yet shares no homology to eukaryotes suggesting this domain is the result of convergent evolution [104]. Despite the recent discoveries of novel PIP-binding domains, the PI(3)P-binding regions in effectors SetA, RavD, LotA, and AnkX have not been linked to any conserved domains. We could speculate that perhaps this is because phosphoinositide binding is mediated by small, variable motifs or that lipid-binding domains may be quite diverse, as is the case for eukaryotic proteins. A clear perspective on this issue requires further identification, domain mapping, and computational analysis of known and novel phosphoinositide-binding effectors. Therefore, there is much to be learned about the molecular details underlying interactions between bacterial proteins and host phosphoinositides.

8. Conclusions and perspectives

What enables *L. pneumophila* effectors to target multiple membrane trafficking pathways stems in part from their modular structures consisting of various combinations of protein domains. Many of the PIP binding effectors are characterized by the presence of a C-terminal PIP-binding region and an N-terminal region that harbors enzymatic activity or interacts with host proteins.

The presence of PI(3)P on phagosomal membranes serves as a signpost for the recruitment of endocytic proteins that promote fusion with subsequent endocytic compartments and ultimately the lysosome. PI(3)P is therefore an attractive target for intracellular pathogens to eliminate entry into the phagosomal maturation pathway. It is well-established that after phagocytosis, PI(3)P on the nascent phagosome is rapidly depleted in conjunction with PI(4)P acquisition [12, 42]. Multiple studies have supported that this lipid rearrangement is accomplished through the actions of PIP-modifying effectors and effectors that promote the recruitment and fusion of PI(4)P-rich compartments with the LCV (reviewed in [105]). The recent evidence demonstrated that this lipid can also be removed from on or around the LCV in the form of PI(3)P-positive vesicles that are shed from

the LCV. This would indicate that somehow microdomains of PI(3)P within membranes are being recognized, sequestered, and sorted into vesicles for removal or that perhaps PI(3)P-positive vesicles do not stably interact with the LCV. How the LCV can distinguish the simultaneous shedding of PI(3)P-compartments with the fusion of PI(4)P-compartments has yet to be determined. We can speculate that *L. pneumophila* has evolved cohorts of effectors that can independently regulate the acceptance of PI(4)P-rich membrane or the egress of PI(3)P-rich membrane from the LCV.

PI(3)P is completely lost from the LCV membrane after 2 hours; however, it is unclear why there is a strong presence of PI(3)P-binding effectors that are on the LCV membrane after this time point (LpnE, SetA, LotA, RidL, LtpM, LtpD, RavD). At later stages of infection, an accumulation of stagnant PI(3)P-positive vesicles can be seen surrounding the LCV. It is possible that effectors anchored to the LCV could be interacting with these vesicles by recognizing multiple membrane compartments. Most LCV localization studies are assessed using light microscopy, in which the resolution may not be high enough to visualize smaller distinct structures around the LCV. Light microscopy showed RavD is present on the LCV membrane; however higher-resolution imaging techniques like structured illumination and transmission electron microscopy revealed RavD is also present on a subset of unidentified vesicles adjacent to the LCV. It is most likely these vesicles are PI(3)P-rich, as RavD does not localize to PI(4)P-positive compartments. Moreover, RavD does not rely on PI(3)P binding to anchor to the LCV, supporting that effectors may exhibit dual localization patterns and that RavD may interact with the LCV and vesicles through different domains.

L. pneumophila has developed intricate strategies to facilitate intracellular growth by circumventing essential host cellular processes. The arsenal of effectors secreted by the type IV secretion system has evolved to target specific eukaryotic components such as proteins and lipids. Localization to the correct compartments within this host cell is imperative for protein function. A number of *Legionella* effectors rely on phosphoinositides to confer this directionality during infection. Not only are phosphoinositides needed to govern organelle identity, but they also dictate the path the phagosome embarks on once engulfed into the host cell. Thus, some effectors are ingeniously equipped to directly modify the lipid content on the phagosome membrane to avoid being routed toward degradation. Only a small percentage of effectors have been reported to interact with or modify phosphoinositides. Future studies that continue to expand on the repertoire of PIP-binding effectors will undoubtedly enhance our understanding of how intracellular pathogens survive within membrane-bound compartments within eukaryotic hosts.

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