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REVIEW ARTICLE

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Tools and mechanisms of vacuolar escape leading to host egress in *Legionella pneumophila* infection: Emphasis on bacterial phospholipases

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Abstract

The phenomenon of host cell escape exhibited by intracellular pathogens is a remarkably versatile occurrence, capable of unfolding through lytic or non-lytic pathways. Among these pathogens, the bacterium *Legionella pneumophila* stands out, having adopted a diverse spectrum of strategies to disengage from their host cells. A pivotal juncture that predates most of these host cell escape modalities is the initial escape from the intracellular compartment. This critical step is increasingly supported by evidence suggesting the involvement of several secreted pathogen effectors, including lytic proteins. In this intricate landscape, *L. pneumophila* emerges as a focal point for research, particularly concerning secreted phospholipases. While nestled within its replicative vacuole, the bacterium deftly employs both its type II (Lsp) and type IVB (Dot/Icm) secretion systems to convey phospholipases into either the phagosomal lumen or the host cell cytoplasm. Its repertoire encompasses numerous phospholipases A (PLA), including three enzymes—PlaA, PlaC, and PlaD—bearing the GDSL motif. Additionally, there are 11 patatin-like phospholipases A as well as PlaB. Furthermore, the bacterium harbors three extracellular phospholipases C (PLCs) and one phospholipase D. Within this comprehensive review, we undertake an exploration of the pivotal role played by phospholipases in the broader context of phagosomal and host cell egress. Moreover, we embark on a detailed journey to unravel the established and potential functions of the secreted phospholipases of *L. pneumophila* in orchestrating this indispensable process.

KEYWORDS
host cell exit mechanisms, *Legionella pneumophila*, lytic escape, secreted phospholipases, vacuolar egress

Abbreviations: DAG, Diacylglycerol; ER, endoplasmic reticulum; GCAT, glycerophospholipid-cholesterol-acyltransferase; LCV, *Legionella*-containing vacuole; LPLA, lysophospholipase A; OM, outer membrane; PC, phosphatidylcholine; PCD, programmed cell death; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PLA, phospholipase A; PLA_2, phospholipase A2; PLB, phospholipase B; PLC, phospholipase C; PLD, phospholipase D; PLPA, patatin-like phospholipase A; PtdIns4P, phosphatidylinositol 4-phosphate; PV, parasitophorous vacuole; RBC, red blood cell; T2SS, type II secretion system; T4BSS, type IVB secretion system.

1 | INTRODUCTION

Intracellular pathogens constitute a heterogeneous group of microorganisms, encompassing bacteria, protozoans, and fungi. Upon infection, the majority of these pathogens establish residence within intracellular compartments. Notable examples of bacteria adept at manipulating their vacuoles to elude lysosomal fusion and foster intraphagosomal bacterial replication include *Legionella pneumophila* (Horwitz, 1983; Isberg et al., 2009), *Mycobacterium tuberculosis* (Russell, 2001), and *Salmonella enterica* (Steele-Mortimer, 2008). Conversely, not all intracellular microbes require enclosure in vacuoles for their lifestyle. Some bacteria, including *Listeria monocytogenes* (Joseph & Goebel, 2007), *Shigella flexneri* (Sansonetti et al., 1986), and Rickettsiae, such as *Rickettsia conorii* (Teysseire et al., 1995), actively lyse their endosomal compartment soon after infection and replicate in the cytoplasm.

Despite their evolutionary diversity and distinct intracellular survival strategies, all the aforementioned microbes share a common challenge: breaching cellular membrane barriers to progress in their infection cycle. Intracellular pathogens have evolved numerous analogous exit strategies that can be divided into three categories. (1) Membrane-dependent non-lytic escape, (2) induction of programmed cell death (PCD) of the host cell, and (3) membrane-disruptive lytic escape (Flieger et al., 2018; Friedrich et al., 2012). The latter is defined by the intertwined activity of microbial pore-forming proteins, proteases, and phospholipases that mediate the direct breaching of membranes (Friedrich et al., 2012). It's worth noting that aside from apoptosis, which exhibits non-lytic characteristics (Jorgensen et al., 2017), other forms of PCD also involve the lytic disintegration of the host cell.

For the intracellular replicating bacterium *L. pneumophila*, all the mentioned pathways play a role in egress. *L. pneumophila* is a gram-negative bacterium that is naturally parasitic to environmental amoebae (Richards et al., 2013). However, through the inhalation of droplets derived from contaminated water systems, *L. pneumophila* can be transmitted to humans and cause a severe pneumonic infection, called Legionnaire's disease (Cunha et al., 2016). Within the human host, the bacterium efficiently infects alveolar macrophages and neutrophils (Copenhaver et al., 2014). Subsequent to the internalization by phagocytotic cells, *L. pneumophila* replicates in an intracellular compartment known as the *Legionella*-containing vacuole (LCV). A pivotal element of intracellular survival is the bacterium's ability to modify its compartment to mimic the endoplasmic reticulum (ER) and prevent lysosomal degradation (Isberg et al., 2009; Newton et al., 2010). *L. pneumophila* achieves this by secreting various effectors through both its type IVB secretion system (T4BSS) Dot/Icm which translocates proteins into the host cell cytoplasm, and its type II secretion system (T2SS) Lsp which releases proteins into the LCV lumen. Over 330 secreted Dot/Icm effectors (Ensminger, 2016; Qiu & Luo, 2017) and more than 25 Lsp-secreted proteins (Cianciotto, 2013; Truchan et al., 2017) are currently known. Beyond their role in intracellular replication, secreted effectors are further considered to orchestrate vacuolar escape once the replicative phase is completed. Numerous phospholipases are among *Legionella's* secreted proteins and will be the primary focus of this review (Aurass et al., 2016; Hiller et al., 2018).

This article places its emphasis on *L. pneumophila* and its secreted phospholipases as tools for achieving vacuolar escape. In the ensuing sections, we will provide a brief overview of egress-relevant microbial phospholipases in general, followed by a more in-depth discussion of the characteristics and potential roles of *L. pneumophila's* secreted phospholipases.

2 | SECRETED PHOSPHOLIPASES AND THEIR INVOLVEMENT IN PATHOGEN EGRESS

2.1 | Vacuolar egress of *L. pneumophila* is linked to host cell lysis

As mentioned earlier, *L. pneumophila* employs various mechanisms to exit host cells. The bacterial egress phase commences once *L. pneumophila* has completed its intracellular replication cycle. At this juncture, the bacterium transitions into its motile and transmissive state (Molofsky & Swanson, 2004). In cases where lytic egress is the chosen pathway, *L. pneumophila* ruptures the LCV before ultimately exiting the disintegrating host cell (Striednig et al., 2021). Consequently, phagosomal egress emerges as a pivotal intermediary step in the process of exiting the host cell. Conversely, *L. pneumophila* can also employ a non-lytic exit strategy, which appears to be influenced by the host species it inhabits during replication. For instance, *L. pneumophila* was found to exit the amoebae *Acanthamoeba castellanii* and *Dictyostelium discoideum* in a non-lytic fashion by exploiting the exocytosis pathway. Two Dot/Icm effectors, LepA and LepB, assist in this mild form of release. They share minor structural commonalities with the membrane fusion proteins EEA1, USO1, and SNAREs, and, accordingly, bacterial egress is impaired when the effectors are eliminated (Chen et al., 2004). The mechanisms underlying *L. pneumophila*'s escape from amoebae appear to be more versatile compared to its egress from mammalian cell lines such as macrophages, where all exit routes tend to culminate in the destructive demise of the infected cell. This divergence might be attributed to the bacterium's long history of co-evolution with its native protozoan hosts (Gomez-Valero & Buchrieser, 2019).

Pathogen release via the lytic pathway appears closely connected to the action of host-driven PCD. Intracellular pathogens might have developed strategies to exploit their host's PCD pathways to escape from a cell and spread (Demarco et al., 2020; Lamkanfi & Dixit, 2010). One of these pathways is pyroptosis, a subtype of regulated necrosis which is marked by the lytic disintegration of the cell and is characterized by the activity of gasdermins (GSDMs), caspase-1, and caspase-4/5/11 in a canonical or non-canonical mode, respectively (Brennan & Cookson, 2000; Yu

et al., 2021). Several microorganisms were shown to trigger pyroptosis, among them *Shigella flexneri*, *S. eneterica*, *L. monocytogenes*, and *L. pneumophila* (Xia et al., 2019). Whether *L. pneumophila* purposely stimulates pyroptosis to escape after replication is finished or whether bacterial release simply coincides with an ongoing anti-microbial cell response that the bacterium thwarts until the end of its intracellular life cycle, is not known. Past studies have demonstrated that pyroptotic events in *Legionella*-infected cells are set off by the NAIP5/NRCL4 inflammasome in a caspase-1-dependent manner and that it requires both bacterial flagellin and the Dot/Icm type IVB secretion system (Case et al., 2009; Lightfield et al., 2008; Molofsky et al., 2006; Zamboni et al., 2006). Silveira and Zamboni (2010) have later shown that flagellin is sufficient to prompt pore formation through the NRCL4 inflammasome prior to pyroptosis onset (Silveira & Zamboni, 2010). Given that *Legionella* mutant strains deficient in promoting pore formation in the host were severely delayed in cellular egress (Alli et al., 2000), it suggests that the entire cascade from NRCL4/NAIP5 activation by bacterial flagellin to pore formation and pyroptosis could constitute a process which *Legionella* exploits for exit. This might be paradigmatic for how other intracellular bacteria utilize pyroptosis to spread but further research is required in this matter. Intriguing discoveries were made which demonstrated that *Salmonella* rapidly associates with guanylate-binding protein 1 (GBP1) after vacuolar escape which recruits further GBPs to form a protective coat that in turn activates caspase-4 and primes pyroptosis via gasdermin D (GSDMD) (Santos et al., 2020). In light of the circumstance that flagellin activates caspase-1 (Silveira & Zamboni, 2010), it remains elusive if vacuolar egress is a prerequisite for PCD-exploiting escape during *Legionella* infections. Further factors have to be considered to that end, such as the T2SS-secreted protease ProA which readily cleaves exported flagellin (Scheithauer et al., 2022) and can translocate through the LCV membrane into the host's cytoplasm (Truchan et al., 2017). Nonetheless, it appears that the cytosolic egress of *L. pneumophila* precedes and potentially triggers pyroptotic cell death.

Ultimately, the question arises of how *L. pneumophila* orchestrates vacuolar egress and which bacterial effectors partake in phagosomal membrane disruption. An array of protein groups, including proteases, pore-forming proteins, and lipases like phospholipases (Figure 1), may contribute to phagosomal escape

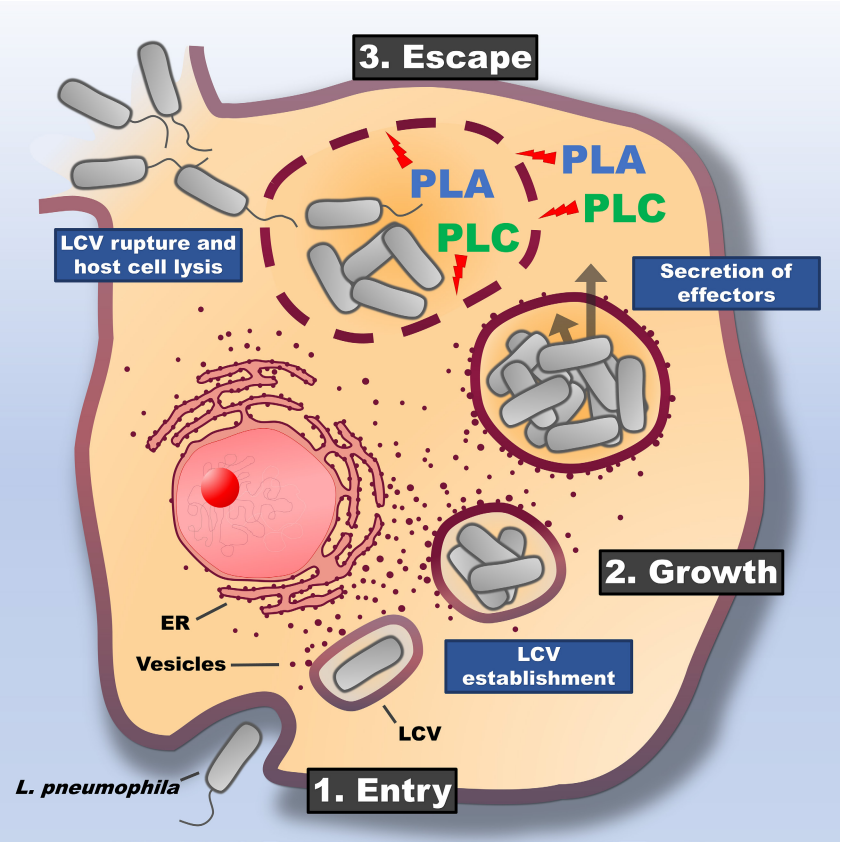

FIGURE 1 Scheme of how secreted exit-associated phospholipases are engaged by *Legionella pneumophila* during lytic escape. 1. *L. pneumophila* enters the host cell in an endocytic manner. 2. The bacterium actively establishes an intracellular niche (*Legionella*-containing vacuole, LCV) which is required for replication. The bacterium modulates its phagosome by interception of the secretory pathway and fusion of ER-derived vesicles with the vacuolar membrane. 3. During growth, *L. pneumophila* secretes various proteins into both the lumen of the vacuole and into the host cell cytoplasm, including phospholipases A and C (PLA, PLC). Secreted phospholipases potentially rupture the vacuolar membrane at the final stage of intracellular replication (indicated by red bolts), resulting in cytoplasmic translocation of the bacteria. This event is a complex interplay of further host and bacterial proteins, culminating in the onset of programmed cell death pathways which cause the lytic death of the host cell and escape of the bacteria.

(Flieger et al., 2018). However, the extent to which these effectors also participate in lytic host cell escape for *L. pneumophila* remains uncertain. It is unclear whether this process is linked to the observed PCD in macrophages or stands as an isolated phenomenon. Illustrating this complexity, *Plasmodium falciparum*'s PfPATPL1 enzyme plays a crucial role in the entire exit cascade by facilitating translocation through both the parasitophorous vacuole (PV) membrane and the red blood cell (RBC) plasma membrane (Singh et al., 2019). These intricate interplays illuminate the multifaceted nature of pathogen exit strategies and prompt the need for further exploration and understanding.

2.2 | Introduction into phospholipases

Phospholipases can be divided into four classes depending on their catalytic activities (Aloulou et al., 2012). They hydrolyze phospholipids which are subcategorized into glycero-phospholipids, sphingosine-phospholipids, and alkyl-phospholipids (Lordan et al., 2017). Phospholipase A (PLA) hydrolyzes glycerol-phospholipids at the sn-1 (PLA_1 activity) or sn-2 (PLA_2 activity) position of the glycerol backbone of phospholipids, respectively, generating one free fatty acid (FFA) and a lysophospholipid. Phospholipase B (PLB) cleaves at both positions and thereby releases two FFA (Ramrakhiani & Chand, 2011). Phospholipases that remove the remaining fatty acid from lysophospholipids are referred to as lysophospholipase A (LPLA). Furthermore, fatty acids may not be released but directly transferred to an acceptor molecule, such as cholesterol, by means of a glycero-phospholipid acyltransferase (GCAT). Phospholipase C (PLC) and phospholipase D (PLD) hydrolyze phospholipids at the phosphodiester bond which in the case of PLC leads to the formation of a phosphoryl ester and diacylglycerol (DAG) (Haas & Stanley, 2007). An overview of phospholipase activities is shown in Figure 2.

Lipids play a fundamental role in cellular functions, not only in membrane formation due to their amphiphilic properties but also in signal transduction pathways (Dowhan, 1997). Moreover, lipids can define the character of a cellular compartment. Many intracellular pathogens hence exploit this feature and alter the lipid composition of their phagosomal membrane after internalization. For instance, *L. pneumophila* was shown to intercept vesicles of the secretory pathway to quickly accumulate PtdIns4P on the LCV membrane to resemble the ER (Weber et al., 2006, 2014, 2018).

2.3 | Secreted phospholipases of *L. pneumophila* contribute to vacuolar egress

In recent years, it has become increasingly evident, that secreted phospholipases could play a role in bacterial escape from their vacuole. Creasey and Isberg (2012) have illustrated the balancing effects of *L. pneumophila* PlaA and SdhA which impressively revealed the potential of secreted phospholipases to execute phagosomal escape. Knockout of the T4BSS-secreted LCV guard SdhA resulted in increased LCV destabilization, whereas this effect was largely compensated in infections with Δ*sdhA* Δ*plaA* double mutants (Creasey & Isberg, 2012). Striednig et al. found further compelling arguments that favor the role of secreted phospholipases in escape and the need to fine-tune this process. Both an *L. pneumophila plaACD* and a *plcABC* triple knockout strain displayed impaired egress from the LCV at the late stage of infection (Striednig et al., 2021). Detailed insights into this crucial topic will be elaborated upon in subsequent chapters, where we will delve into the extensive repertoire of secreted phospholipases employed by *L. pneumophila*. An illustrative schematic depicting how these secreted phospholipases could contribute to lytic escape is presented in Figure 1.

2.4 | Extracellular phospholipases are prevalent escape tools among various intracellular pathogens

Apart from *L. pneumophila*, other intracellular pathogens are likewise thought to use secreted phospholipases to orchestrate vacuolar escape, albeit the full magnitude of ongoing events during exit remains not completely understood. Hence, many extracellular effectors, including studied phospholipases, might perform yet unknown roles. A selection of secreted and surface-exposed phospholipases of different obligate and facultative intracellular pathogens of bacterial, protozoan, and fungal origin is shown in Figure 3. In the realm of phagosomal exit studies, *L. monocytogenes*' LmPlcA and LmPlcB have emerged as pioneer phospholipases. Together with listeriolysin O, these two PLCs play a pivotal role in rupturing the phagosomal membrane shortly after internalization, a critical step in *Listeria*'s intracellular lifecycle (Camilli et al., 1993; Quereda et al., 2018; Smith et al., 1995). Another well-studied enzyme is SseJ of *Salmonella*. This type III-secreted PLA and GCAT antagonizes bacterial SifA and

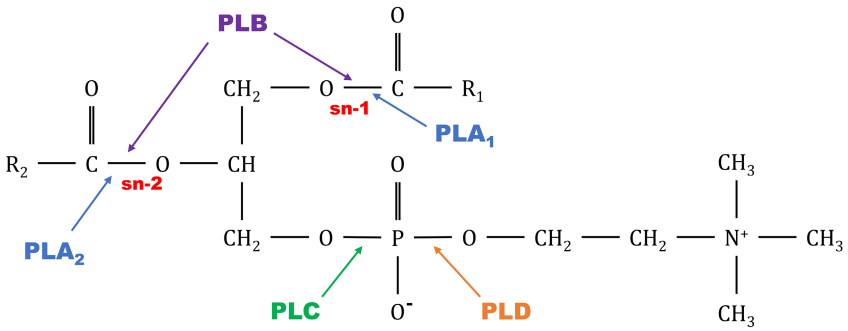

FIGURE 2 PLA, PLB, PLC, and PLD hydrolyze glycerophospholipids (here depicted as phosphatidylcholine) at different sites.

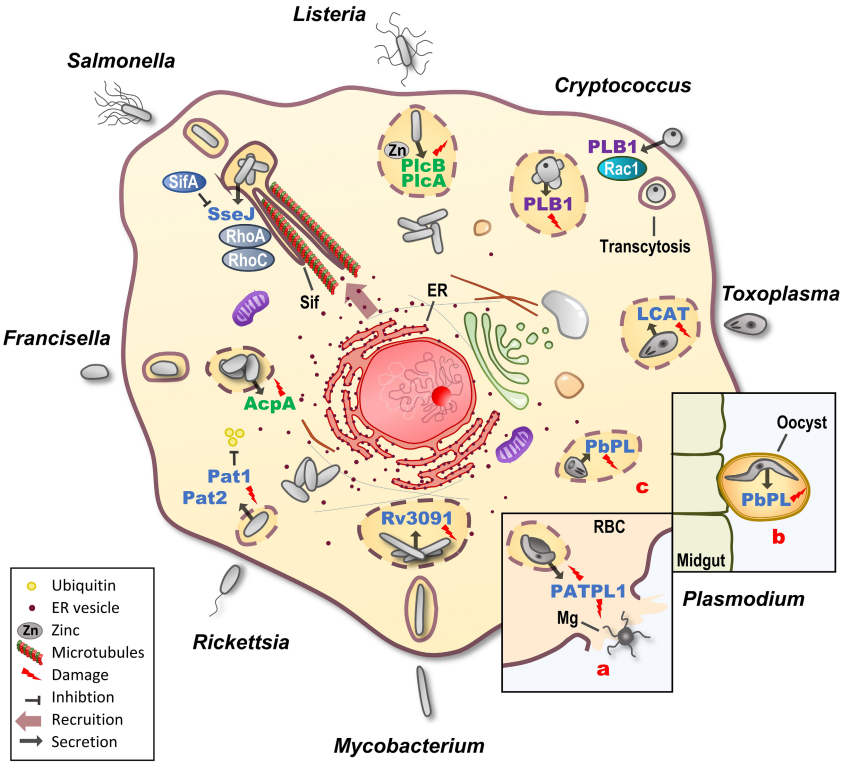

FIGURE 3 Secreted phospholipases of obligate and facultative intracellular pathogens. Phospholipases of interest are indicated and categorized by color: PLA (blue), PLB (purple), PLC (green), PLD (orange). (a) PfPATPL1 disrupts both the PV membrane and RBC plasma membrane for gamete egress. PbPL mediates egress of (b) sporozoites from oocysts in the midgut, and (c) merozoites from the PV during liver stage. ER, endoplasmic reticulum; Mg, (exflagellated) male gamete; RBC, red blood cell; Sif, *Salmonella*-induced filaments. See manuscript text for further information.

contributes to virulence by destabilizing the *Salmonella*-containing vacuole (SCV) (Ohlson et al., 2005; Ruiz-Albert et al., 2002). The relationship between SseJ and SifA shows similarities to *L. pneumophila's* PlaA and SdhA (Creasey & Isberg, 2012), although SseJ appears to exercise a more complex role than simply disrupting the SCV as it also binds Rho GTPases and alters microtubule dynamics (Raines et al., 2017), ultimately aiding in *Salmonella*-induced filament (Sif) formation (Gao et al., 2018). AcpA of the bacterium *Francisella novicida* is predominantly a phosphatase but additionally exhibits PLC activity which is considered to be a crucial feature for disrupting the vacuole during egress (Dai et al., 2012). Further examples include the patatin-like PLA Pat1 and Pat2 found in all *Rickettsia* species, with the exception of *R. parkeri*, which lacks Pat2 (Gillespie et al., 2015; Rahman et al., 2013). In particular, Pat1 plays a superior role in exit and virulence by facilitating phagosomal egress, host cell escape, and cell-to-cell spread, and promotes cytosolic survival by circumventing ubiquitination (Borgo et al., 2022). The extensively studied bacterium *M. tuberculosis* owns a noteworthy range of extracellular phospholipases. However, despite the substantial number of ascertained phospholipases, comparably little is known about their potential contribution to vacuolar damage and escape. Nonetheless, one potential candidate is Rv3091 which was found upregulated during infection and might promote phagosomal egression (Cui et al., 2020).

Not only bacteria but also intracellular pathogens of protozoan and fungal origin employ phospholipases during various stages of their lifecycle. PfPATPL1 of *P. falciparum* regulates gamete rounding, the exflagellation of male gametes, and the sequential translocation of the parasites from both the PV and the RBC following the final stages of gametogenesis (Singh et al., 2019). *P. berghei* PbPL further mediates both the egress of sporozoites from oocysts in the mosquito midgut, as well as merozoite escape from the PV during the liver stage (Burda et al., 2015). Another member of the Apicomplexa, *Toxoplasma gondii*, deploys an orthologue of PbPL, the lecithin-cholesterol acyltransferase TgLCAT which facilitates tachyzoite exit from epithelial monolayers (Schultz & Carruthers, 2018). The yeast *C. neoformans* releases the PLB1 which is essential for the escape from capsules during macrophage infection (De Leon-Rodriguez et al., 2018). The phospholipase was further illustrated to interact with Rac1 to conduct fungal transcytosis through the blood–brain barrier (Maruvada et al., 2012). Overall, PLB1 is considered a crucial virulence factor for central nervous system colonization (Hamed et al., 2023).

It appears increasingly evident that secreted phospholipases pose a common tool for intracellular pathogens that they, despite their fundamental cellular differences, have convergently put into play to facilitate escape.

3 | SECRETED PHOSPHOLIPASES OF *LEGIONELLA PNEUMOPHILA*

Bacterial pathogens harbor a range of secretion systems that they deploy to modulate the surrounding environment (Costa et al., 2015). As mentioned earlier, *L. pneumophila* makes use of two main secretion systems to release proteins, the T4BSS Dot/Icm and the T2SS Lsp. Among its plethora of secreted proteins is a large set of phospholipases. In total, 15 PLA, three PLC, and one PLD are found in *L. pneumophila* and many of those are known to be exported by those means (Hiller et al., 2018; Figure 4). The predominant group within

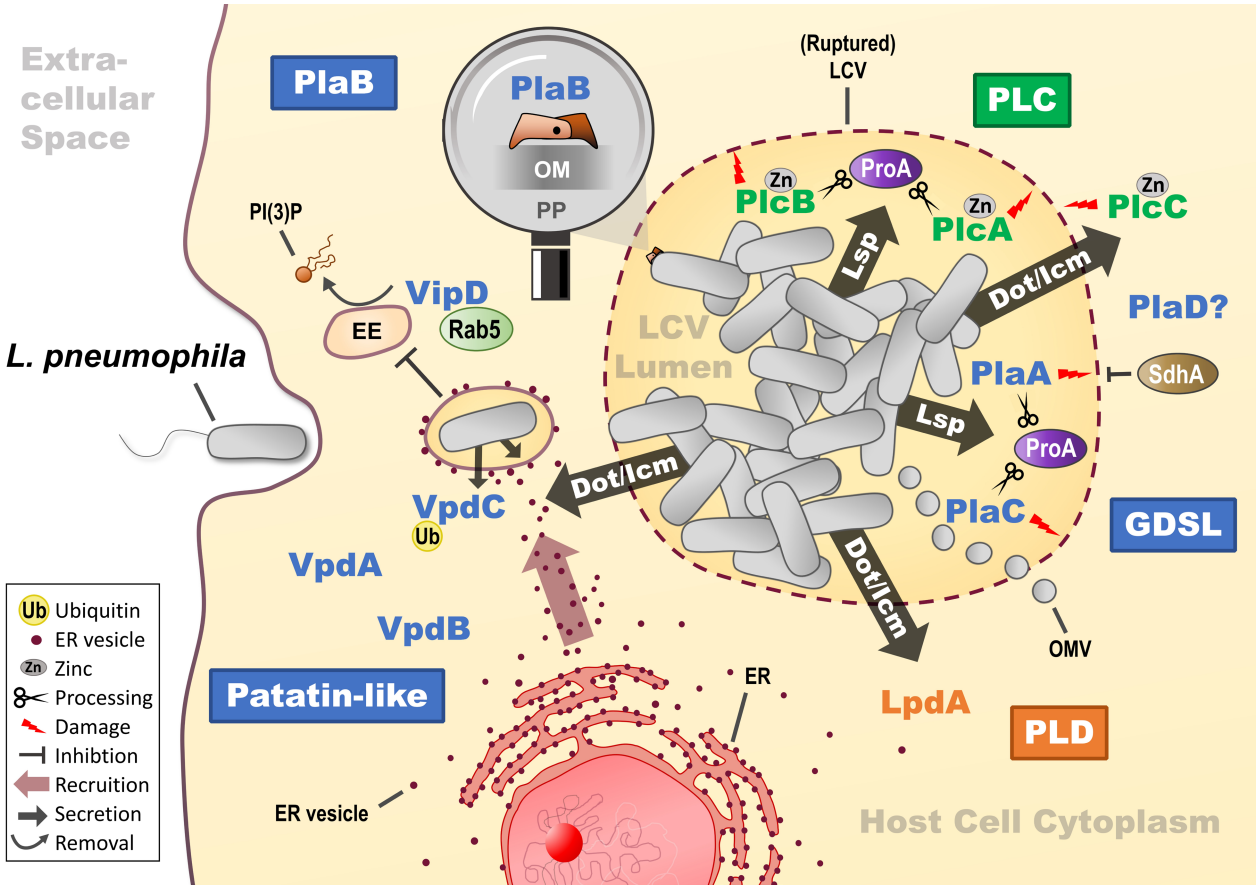

FIGURE 4 Secreted and surface-exposed phospholipases of *Legionella pneumophila*. The bacterium is shown during three stages (from left to right): as an extracellular bacterium before entry, during LCV establishment/endosome modifications after entry, during LCV lysis following intracellular replication. Color codes depict the different phospholipase types: PLA (blue), PLC (green), PLD (orange). Phospholipase groups are shown in color-filled frames. PlaB on the bacterial envelope is shown through a magnifying glass. EE, early endosomes; OM, outer membrane; PP, periplasm.

the PLA category includes three enzymes featuring a GDSL lipase motif (Akoh et al., 2004)—PlaA, PlaC, and PlaD. Additionally, there are 11 Patatin-like phospholipases A (PLPA) (Hirschberg et al., 2001), including VpdA, VpdB, VpdC, and VipD, as well as PlaB which forms its own subset of PlaB-like phospholipases.

4 | GDSL PHOSPHOLIPASES

4.1 | Introduction to the GDSL enzyme family

GDSL hydrolases were initially characterized as a novel family of lipolytic enzymes by Upton and Buckley in 1995 (Upton & Buckley, 1995) These enzymes exhibit a diverse range of activities, including protease, arylesterase, PLA, LPLA, GCAT, and hemolysins. The remarkable breadth of their substrate specificity can be attributed to a dynamic active site, which undergoes conformational changes upon interaction with various substrates (Akoh et al., 2004; Upton & Buckley, 1995). GDSL-enzymes contain five conserved amino acid sequence blocks that are distributed over the protein sequence. The catalytic triad Ser, His, and Asp/Glu are found in block I, whereby the nucleophile Ser is present in the Gly-Asp-Ser-Leu (GDSL) motif that is located close to the N-terminus of the protein. The two other catalytic triad members, His and Asp, are located in block V (Akoh et al., 2004; Upton & Buckley, 1995).

The GDSL enzyme family is abundant in bacteria and higher plants and in the latter is involved in the regulation of plant growth and development, specifically in the synthesis of phytohormones, in seed germination, flowering, and resistance signaling (Shen et al., 2022). A variety of characterized bacterial GDSL enzymes display hydrolytic activity towards different lipids, including phospholipids and other substrates such as acyl-CoA, esters, and amino acid derivates (Shaw et al., 1994; Talker-Huiber et al., 2003; Vujaklija et al., 2002; Yang et al., 2019; Yoshida et al., 2010; Yu et al., 2010). In addition, GDSL enzymes may not only release fatty acids from lipids, like glycerophospholipids but may also transfer fatty acids to an acceptor molecule, such as cholesterol or ergosterol in mammalian or amoebal cells, respectively, and therefore exhibit GCAT activity (Akoh et al., 2004; Lang et al., 2012, 2017).

GDSL enzymes are virulence factors of bacterial pathogens. An illustrative example is SseJ from *S. enterica*, which exhibits both PLA and GCAT activities. Activation of SseJ relies on the eukaryotic

RhoA GTPase and plays a pivotal role in modifying the membrane of *Salmonella*-containing vacuoles. In the absence of the stabilizing factor SifA, SseJ promotes the destabilization of these vacuoles (Christen et al., 2009; Freeman et al., 2003; Lawley et al., 2006; Ohlson et al., 2005, 2008; Ruiz-Albert et al., 2002). Importantly, three of the at least 15 PLAs encoded by *L. pneumophila* belong to the GDSL lipase family, namely PlaA, PlaC, and PlaD.

PlaA was originally discovered as the protein responsible for the majority of secreted *L. pneumophila* LPLA activity after fractionation of culture supernatants by means of anion exchange chromatography (Flieger et al., 2001, 2002). BLAST analyses revealed two homologs of PlaA within *L. pneumophila*, designated as PlaC and PlaD (Akoh et al., 2004; Banerji et al., 2005, 2008). Sequence analyses of the three proteins showed that they contain the five conserved sequence blocks of the GDSL hydrolases (Lang et al., 2012). PlaA (34.4 kDa) and PlaC (49.7 kDa) comprise a predicted N-terminal signal peptide for Sec-dependent export but not PlaD (59.6 kDa).

4.2 | PlaA is a type II secreted LPLA activated by the *L. pneumophila* zinc metalloprotease ProA

To assess the contribution of PlaA to the lipolytic activities of *L. pneumophila*, analysis was conducted using a *plaA*-deficient mutant which was incubated with various lipid substrates. The *plaA*-mutant is strongly reduced in secreted LPLA but no change was observed for the PLA activity (Flieger et al., 2002; Lang et al., 2017). Western blot analysis revealed that secreted PlaA is found as a 25 kDa protein instead of the predicted molecular weight of 34.4 kDa but absent in the *lspDE*-mutant which confirms the secretion of PlaA via the T2SS subsequent to sec-dependent transport. Due to the shift in the molecular weight of secreted PlaA in *L. pneumophila* wildtype versus a zinc metalloproteinase ProA knockout mutant, proteolytic processing of PlaA by ProA was assumed (Flieger et al., 2001, 2002; Lang et al., 2017).

For in-depth investigation, PlaA was recombinant expressed in *E. coli* and subsequently purified. The purified recombinant PlaA exhibited prominent LPLA, lipase, and GCAT activities. Notably, GCAT activity was only observed when lysophospholipids were utilized as fatty acid donors. Incubation with ProA increased LPLA and diminished GCAT activity showing that LPLA activity requires proteolytic activation (Lang et al., 2017). Processing of PlaA by ProA occurred within a predicted disulfide loop structure between glutamate 266 and leucine 267 (Lang et al., 2017). Crystallization and 3D structure analysis of PlaA demonstrated that the disulfide loop functions as a lid that covers the catalytic triad explaining the increased LPLA activity upon proteolytic activation (Hiller et al., 2023). Accordingly, the deletion of 20 amino acids in the disulfide loop results in activated PlaA without the addition of ProA (Lang et al., 2017). PlaA further showed substrate preference for lysophospholipids harboring ~16 carbon atom fatty acid chains and this preference is explained by the presence of a hydrophobic channel in close proximity to the catalytic triad in PlaA which matches linear fatty acids of approximately 16 carbon atoms (Hiller et al., 2023).

A particular feature of PlaA links it to bacterial egress. PlaA was found to destabilize the phagosomal membrane in the absence of the type IVB-secreted protective factor SdhA. Knockout of *sdhA* resulted in early egress of the bacteria from the phagosome and subsequent activation of host cell death, an effect which was relieved by knockout of *plaA* (Creasey & Isberg, 2012). Therefore, the destabilizing effect of PlaA on the LCV might be due to the direct targeting of vacuole membrane lipids. As mentioned above a similar interplay is described for SseJ and SifA of *S. enterica* (Kolodziejek & Miller, 2015; Raines et al., 2017; Ruiz-Albert et al., 2002). Molecular relationships between disruptive elements and phagosome protective factors have been reported for a small number of pathogens so far and this topic is comprehensively covered and discussed in a recent review by Anand and colleagues (Anand et al., 2020). Taken together PlaA is a type II secreted LPLA targeting phagosomal lipids which requires proteolytic activation by ProA.

4.3 | PlaC is a type II secreted PLA and GCAT activated by ProA

The second GDSL enzyme of *L. pneumophila* PlaC, when derived from *L. pneumophila* culture supernatants, shows PLA, LPLA, and GCAT activities. In contrast to PlaA, PlaC reveals GCAT activity towards diacyl phospholipids as donor substrates (Lang et al., 2012). PlaC like PlaA is a type II-secreted protein and absent in the supernatants of a *L. pneumophila lspDE*-mutant. Further, secreted PlaC of wildtype *L. pneumophila* was ~10 kDa smaller than the predicted molecular weight of 49.7 kDa, which was found in *proA* mutant culture supernatants. A similar shift in molecular size was seen for PlaA and therefore this suggested alike processing and activation of PlaC by ProA (Lang et al., 2012, 2017).

PlaC expressed in *E. coli* showed PLA, LPLA, and lipase activities but without ProA-dependent processing only minor GCAT activity (Banerji et al., 2005; Lang et al., 2012). The addition of ProA induced GCAT activity and increased PLA activity. Like in PlaA, the deletion of the stretch of amino acids within the disulfide loop resulted in increased PLA and GCAT activities. As no specific single cleavage site for ProA but rather several possible sites were identified in the disulfide loop sequence, ProA might rather recognize the loop structure of PlaC in general instead of a specific amino acid sequence (Lang et al., 2012). The 3D structure of PlaC was predicted by using the AlphaFold algorithm. Similar to PlaA, a lid domain including an α-helix that was delimited by a disulfide bridge was found. This structure is slightly shifted away from the active site, resulting in a larger entrance funnel for PlaC corresponding to the preferential hydrolysis of diacyl phospholipids instead of lysophospholipids as observed for PlaA. The length of PlaC's hydrophobic substrate channel is identical to PlaA's, indicating that in both cases linear fatty acids with ~16 carbon atoms are the preferred substrates (Hiller et al., 2023). In conclusion, both PlaA and PlaC share a similar enzyme activation mechanism via proteolytic disulfide loop cleavage after sec and type

II-dependent protein secretion. This activation mechanism might prevent self-damage of *L. pneumophila* via the phospholipolytic activity before protein export.

Exit from the LCV is particularly crucial during the later stages of infection when the bacteria have replicated within the phagosome. The *plaC* gene together with the *lapA* and *lapB* genes, coding for aminopeptidases, belong to the genes most highly upregulated among the type II- secreted substrates during the late infection phase of *L. pneumophila* in its host *Acanthmoeba castellanii*. Double *L. pneumophila* mutants of *plaC- lapA-* showed a severe replication defect during infection of *A. castellanii*. Deletion of *plaC* resulted in increased expression levels of *lapA* and *lapB* and vice versa suggesting that the enzymatic activities of the associated proteins might act in concert and therefore counterbalance each other (White et al., 2018). PlaC is found in outer membrane vesicles (OMV), which are used by gram-negative bacteria to deliver proteins to the extracellular environment and into host cells. It has been shown that OMVs fuse to the LCV which might impact LCV composition and based on the fact that lipolytic enzymes fuse, also LCV stability (Galka et al., 2008). In summary, PlaC is a type II secreted PLA and GCAT requiring ProA-mediated processing. It shares many similarities with PlaA and may likewise act on the LCV.

4.4 | PlaD is a GDSL enzyme with many yet unknown features

PlaD, identified as a homolog of PlaA, has not undergone extensive characterization to date. Among the three proteins, it is the largest, featuring a C-terminal appendage of approximately 170 amino acids after block V (Banerji et al., 2008). The protein is not found in the culture supernatant of *L. pneumophila* and accordingly, the *plaD*-mutant shows no change in secreted lipolytic activities (Lang et al., 2017). PlaD expressed in *E. coli* revealed low PLA activity but major LPLA activity and on the contrary, both activities are reduced by ProA addition. Minor sterol acylation with short-chain fatty acids was observed in the absence of ProA with diacyl phospholipids as donor substrates (Lang et al., 2012). Whether PlaD is secreted after host-cell contact remains an open question.

Utilizing the AlphaFold modeling, the 3D structure of PlaD was predicted. PlaD consists of an α/β hydrolase domain in its N-terminal section and is predicted to possess an additional α-helical domain of unknown function at its C-terminus. In contrast to PlaA and PlaC, PlaD lacks a disulfide loop covering the catalytic triad. However, the length of the hydrophobic substrate channel in PlaD aligns with that of PlaA and PlaC, suggesting a preference for linear fatty acids with approximately 16 carbon atoms (Hiller et al., 2023). As no lid structure is present in the PlaD structure model and the addition of ProA does not lead to increased activities like for PlaA and PlaC another activation mechanism could be assumed. The mode of PlaD activation mechanism and secretion are subjects of current studies. Importantly, the expression of PlaD is upregulated in the post-exponential growth phase making it another possible factor for exit contribution (Brüggemann et al., 2006; Faucher et al., 2011).

4.5 | Possible contribution of PlaA and PlaC to bacterial LCV exit

In addition to the involvement of PlaA in LCV membrane degradation, experiments of *D. discoideum* amoebae infected with an *L. pneumophila plaACD-* mutant versus wildtype bacteria further indicate that the GDSL-phospholipases promote LCV rupture and bacterial escape (Striednig et al., 2021). How exactly these phospholipases contribute to the LCV escape in this infection model and how the mutant behaves in mammalian cells needs to be analyzed in future studies. As previously mentioned, PlaA has been implicated in promoting LCV membrane destabilization in mouse bone marrow-derived macrophages, particularly in the absence of the protective type IVB-secreted Dot/Icm effector SdhA (Creasey & Isberg, 2012). This is not caused by the direct interaction of both proteins as SdhA binds OculoCerobroRenal syndrome of LOWE protein (OCRL) thereby preventing early endosomal compartments of the host cell from docking to the replication vacuole and destabilizing it (Choi et al., 2021). PlaA is secreted into the lumen of the phagosome and after ProA-mediated processing, PlaA activity shifts to a potent LPLA (Lang et al., 2017). Recent data show that the amount of lysophospholipids is critical for vacuole expansion as increased lysophospholipid contents block membrane fusion (Li et al., 2022) but they might also be important in LCV exit. For this process, it is possible that PlaA and PlaC act in concert. PlaC mainly is a PLA and can act directly on diacyl phospholipids, whereas PlaA may target the lysophospholipid reaction product of PlaC. Both activities are even increased after processing by ProA at later time points and for PlaC, in addition, GCAT is activated (Lang et al., 2012). The acylation of sterols due to GCAT activity might influence the membrane fluidity, lead to destabilization of membranes, and alter the activity of membrane-bound enzymes (Volkman, 2003). Therefore, the combined action of PlaA and PlaC could contribute to bacterial egress by LCV lysis.

5 | PLAB-LIKE PHOSPHOLIPASES

5.1 | Outer membrane-located PlaB shows the highest PLA activity of *L. pneumophila* and is an important virulence factor

Another example of the PLAs present in *L. pneumophila* is the 55 kDa protein PlaB. PlaB was originally found when a genomic library of *L. pneumophila* expressed in *E. coli* was tested for hemolysis (Flieger et al., 2004). The clone containing the *plaB* gene showed increased hemolysis toward human erythrocytes. Homology of the coded protein sequence to lipases suggested lipolytic activity for the respective protein. Indeed, pronounced PLA and LPLA activities towards a variety of phospholipids, including phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine

(PE), different phosphatidylinositol (PI) species, lysophospholipids, and lipase activity towards mono-, di- and triacylglycerols were found for PlaB when the gene was expressed in *E. coli* or for *L. pneumophila* wildtype compared to a *plaB* knockout mutant (Bender et al., 2009; Diwo et al., 2021; Flieger et al., 2004).

Analysis of *L. pneumophila plaB* knockout mutants further showed that compared to the wildtype strain no change in activities was noted in the culture supernatants, however interestingly for the cell lysates. Specifically, the mutant almost completely lost the PLA/LPLA/lipase activities which in the wildtype is about 100-fold higher than the respective activities found in the culture supernatants (Flieger et al., 2004). This suggests that PlaB is an exceedingly potent phospholipase, and unlike the GDSL hydrolases PlaA or PlaC, it is not secreted into the culture supernatant but remains associated with the bacterial cell. Since PlaB seemed to convey *L. pneumophila* hemolytic activity, we favored a model where PlaB is presented as a surface protein that associates with the bacterial outer membrane (OM). However, no transmembrane domains or other protein regions indicative of membrane association, and neither sequence hints about the mode of protein secretion were found (Bender et al., 2009; Flieger et al., 2004; Kuhle et al., 2014). Further experiments using immune detection of PlaB and proteinase K digestions of intact cells or cell fractionations confirmed surface and OM localization of PlaB (Diwo et al., 2021; Schunder et al., 2010). Further, *L. pneumophila* mutant analysis in established *L. pneumophila* secretion systems, such as the type II Lsp, type IVB Dot/Icm, type IVA Lvh, and regulators of flagellin-export related systems revealed no block of PlaB surface localization (Diwo et al., 2021). Very interestingly however, the protein was able to surface localize when expressed in *E. coli* suggesting that a common mechanism of transport may exist in *Legionella* and *E. coli* or may be coded in PlaB itself (Diwo et al., 2021).

The presence of potent cell-destructive activities on a pathogen's surface can function as a crucial virulence factor. Indeed, multiple infection models have provided evidence that PlaB serves as one such virulence factor. First, in a guinea pig infection model, the *plaB* knockout mutant replicated less in the lungs, produced less lung destruction, and further disseminated less prominently to the spleens (Schunder et al., 2010). In addition, in RAW macrophage infections, a replication defect of the *plaB* mutant and the contribution of PlaB enzymatic activity to the full level of intracellular replication were observed. Here, both catalytic inactive mutants and C-terminally truncated protein versions expressed in the *L. pneumophila plaB* mutant and which lost activity were analyzed and all mutants failed to reestablish Wt replication efficiency (Kuhle et al., 2014). These studies indicate the importance of PlaB and its catalytic activity as a virulence factor of *L. pneumophila*.

5.2 | PlaB consists of a N-terminal lipase domain and is activated by depolymerization

Analysis of the protein sequence of PlaB revealed that it consists of two distinct protein regions where the N-terminal half showed homology to lipolytic proteins and harbored the typical catalytic triad Ser-Asp-His of lipases but the embedding of these amino acids was unusual. However, the C-terminal protein half did not show relatedness to known proteins in its primary sequence (Bender et al., 2009; Flieger et al., 2004). PlaB shortages from the C-terminus sequentially reduced enzymatic activity which indicated the significance of this region for activity although the specific involvement remained unclear (Kuhle et al., 2014).

A striking observation was the unique activity pattern of PlaB. Surprisingly, the protein showed higher specific activity at lower concentrations (i.e. in the nM range) but lower activity at higher concentrations (i.e. in the μM range) (Kuhle et al., 2014). Such a behavior has been rarely reported and the opposite is known for *E. coli* OM PldA where specific activity increases with increasing protein amounts. This nicely fits the activation mode of PldA which is based on dimerization when the bacterial OM is damaged (Dekker, 2000; Dekker & Parker, 1997). Consequently, we hypothesized that de-oligomerization, rather than oligomerization, might play a role in PlaB activation. Indeed, analytical ultracentrifugation revealed the presence of tetrameric PlaB at higher concentrations in the μM range, while dimers were prevalent at lower nM concentrations (Kuhle et al., 2014). We posited that the tetrameric state represents the inactive version of PlaB, whereas the dimeric or monomeric state represents the active form.

To elucidate the structure–function relationship of PlaB in regard to its activation and to get an insight into protein regions for possible OM association, we analyzed its 3D structure. We crystalized PlaB and solved the structure in its tetrameric inactive state best described as a dimer of dimers. The active dimers are formed by head-to-tail interaction between the N-terminal phospholipase domain of the first monomer and a protruding hook-like extension at the C-terminus of the second monomer. The position of sheet β9/β10 in the tetramer suggests that it may be involved in dimer/dimer interactions. As already indicated by the primary sequence, each monomer in the tetramer consists of an N-terminal and a C-terminal half. The N-terminal domain reveals a typical α/β hydrolase fold as commonly present in lipases (Akoh et al., 2004; Diwo et al., 2021; Mindrebo et al., 2016; Rauwerdink & Kazlauskas, 2015). However, the C-terminal region showed a novel mosaic bilobed β-sandwich structure which was somewhat related to immunoglobulin-like domains (Diwo et al., 2021). The catalytic center is readily discernible by the catalytic triad S85/D203/H251 and is shielded from the solvent by a closed lid as is frequently observed in ABHs in the absence of substrates (Khan et al., 2017).

5.3 | NAD(H) binding is critical for PlaB export and regulates its transformation from an inactive tetramer to an active dimer

An intriguing feature within the PlaB structure was the presence of an electron density at the center of the tetramer. The shape of this density indicated the presence of NAD(H), and indeed, the use

of thio-NAD improved crystallization efforts. NAD(H) serves as a central cofactor in energy homeostasis (Pollak et al., 2007), typically expected to be confined to the intracellular milieu. It appears to be an especially attractive compound for regulating PlaB activity.

In summary, PlaB stands out as a crucial and highly potent surface-associated virulence factor of *L. pneumophila*. It remains inactive within the μM NAD(H) concentration range, which is typically found in the cytosol of bacteria and host cells. This prevents activation of PlaB and intrinsic damage of the bacteria by the lytic factor. When the protein however travels in an unknown manner towards the bacterial surface, NAD(H) concentration decreases, and the protein switches from its tetrameric into a dimeric state where it becomes active and also competent for membrane interaction and surface presentation by β9/β10. Therefore, PlaB provides an example of spatial and temporal activity regulation and for the coupling of a virulence factor to a bacterial low-energy state.

Why might PlaB be an excellent factor for LCV and host cell exit for *L. pneumophila*? PlaB's broad substrate spectrum, including abundant host lipids, such as PC and PE, and its high activity make it a potent tool for compartment egress after intracellular replication. Especially when the LCV is tightly filled with the bacteria this surface-associated PlaB can make contact with the LCV membrane and exert its activity. Furthermore, the onset of activity and also surface localization is linked to NAD(H) reduction which might occur when nutrients are depleted after cessation of intracellular replication and intrabacterial NAD(H) levels. Therefore, PlaB has the potential to couple LCV exit after finalized replication to the exhaustion of nutrients to induce an exit program.

6 | PATATIN-LIKE PHOSPHOLIPASES

6.1 | Patatin-like phospholipases of *L. pneumophila* promote intracellular replication

PLPAs exhibit sequence homology with the potato tuber patatin family, which serves as storage glycoproteins and also show acyl-hydrolyzing phospholipase activity, such as PLA/LPLA activities (Hirschberg et al., 2001). Various observations indicate an implication of plant patatin in fat mobilization, defense against pathogens, stress, and signal transduction during germination (Scherer et al., 2010; Shewry, 2003). Unlike canonical lipases, patatin displays an α/β protein fold but lacks a flexible lid and does not display interfacial activation (Rydel et al., 2003). Outside plants, the catalytic core (i.e. patatin domain) can be found in a wide range of proteins encoded in bacterial genomes, particularly in genomes of pathogenic bacteria (Banerji et al., 2008). PLPA characteristically contains a Ser-Asp catalytic dyad (i.e. PLPA domain, IPR002641) instead of the, among lipolytic enzymes, more common Ser-His-Asp (or Glu) catalytic triad (Rydel et al., 2003). Remarkably, some bacterial PLPAs significantly contribute to virulence and some well-characterized examples can be found in *Pseudomonas aeruginosa* and *L. pneumophila*.

P. aeruginosa carries the first described bacterial PLPA-virulence factor which is its secreted enzyme ExoU. This major toxin is translocated by a T3SS and degrades the PM leading to lysis. ExoU can turn highly active and therefore underlies tight activity control. It unfolds full PLA activity only after experiencing posttranslational modification by the eukaryotic ubiquitination machinery (Foulkes et al., 2019; Stirling et al., 2006).

Indeed, the relevance of bacterial PLPAs for virulence exceeds indiscriminate pore formation and cell lysis as illustrated by two well-established secreted PLPA effectors of *L. pneumophila*. VipD and its paralog VpdC are both translocated into the host cells by the Dot/Icm T4BSS and play crucial roles in LCV biogenesis. VipD phospholipase activity requires allosteric activation through binding of endosomal GTP-bound Rab5. Subsequently, it cleaves phosphatidylinositol-3-phosphate on endosomal membranes, hindering endosome-LCV fusion by suppressing downstream effector binding. This results in membrane fusion events that facilitate escape from lysosomal degradation (Gaspar & Machner, 2014; Ku et al., 2012; VanRheenen et al., 2006).

Recent evidence shed light on the biological role of VpdC which localizes to the LCV's surface and requires allosteric activation by binding host ubiquitin to exert full PLA_2 activity (Li et al., 2022). Evidence from that article suggests that not only establishment but also coordinated expansion of the LCV is critical for full intravacuolar replication of *L. pneumophila*. VpdC-derived lysophospholipids likely contribute to that process by adjusting the compartment's lysophospholipid content to a physiological dose supportive to membrane fusions of the LCV with surrounding host organelles resulting in coordinated expansion of the LCV (Li et al., 2022).

Notably, *L. pneumophila* strains generally contain an unusually high number of 10 to 11 PLPA encoding genes, further indicating the significance of this enzyme group for the lifestyle of that pathogen (Banerji et al., 2008). The high conservation of the catalytic core among the *L. pneumophila* PLPAs suggests that each one of them possesses at least low-level phospholipase activity and consequently holds the capacity to contribute to pathogen-triggered vacuole homeostasis. This is further supported by the circumstance that a subset of these PLPAs is secreted by the Dot/Icm T4BSS. Indeed, four genes of the PLPA gene group, *vipD*, *lpg2317/patD*, *lpg1944/patE*, *lpg0670/patI* were among the top-100 upregulated genes at the late stage of intra-amoebal growth (14 hpi) relative to an earlier infection stage (8 hpi) (Brüggemann et al., 2006). Those data suggest an implication of PLPAs in the very late stage of intracellular growth including the processes culminating in phagosomal exit. However, transposon insertional mutagenesis and Tn-Seq of pools before and after 24 h of *A. castellanii* challenge did not suggest significant intracellular growth defects when either of the singular *Legionella* PLPA genes is inactivated (Park et al., 2020).

7 | PHOSPHOLIPASES C AND D

Phospholipases C and phospholipases D share a common molecular target—phospholipids. Phospholipids play integral roles in the

membranes of eukaryotes, as well as in prokaryotic and eukaryotic cell envelopes. Consequently, they become a frequent focus of phospholipase-mediated interactions between pathogens and host cells, presenting a significant challenge for intracellular bacterial pathogens during invasion and exit. While PLAs cleave lipid-acyl-ester bonds to liberate fatty acids from glycerophosphate backbones, PLCs and PLDs cleave phosphodiester bonds either proximal or distal to the glycerol moiety, respectively. In addition to the inherent structural consequences of phospholipase action, such as pore formation and membrane rupture, its principal products, phosphatidic acid, arachidonic acid, and DAG, can impact signal transduction and differentiation and as such have a multitude of effects on downstream cellular pathways (Flores-Diaz et al., 2016).

7.1 | Phospholipases C support bacterial escape

Phospholipases C constitutes a group of enzymes that predominantly act at cellular membranes by cleaving phospholipids at the phosphodiester bond proximal to the phosphate atom and attached glycerol. PI–PLCs are well-known key enzymes in mammalian cellular signal transduction since important second messengers, such as DAG and inositol 1,4,5-trisphosphate are liberated by their action on phosphatidylinositol 4,5-bisphosphate (PIP2). Several secreted PLCs of important bacterial pathogens, such as *L. monocytogenes*, *Bacillus cereus*, *Clostridium perfringens*, and *P. aeruginosa* have been described and covered in detail in the review by Flores-Diaz et al. (2016). These secreted PLCs employed by pathogenic bacteria induce various effects, such as cytotoxicity, proinflammatory cytokine production, activation of the arachidonic acid cascade, autophagy suppression (Tattoli et al., 2013), facilitation of bacterial release from the phagosome, and cell-to-cell spread (Wei et al., 2005).

L. pneumophila encodes three putative phospholipases C, PlcC, PlcA, and PlcB, of which PlcC activity has been comprehensively analyzed (Aragon et al., 2002; Aurass et al., 2013; McCoy-Simandle et al., 2011). *L. pneumophila* PlcC (synonymously CegC1) expressed in *Escherichia coli* hydrolyzed a broad phospholipid spectrum, including PC, PG, and PI. The enzyme is activatable by Zn2+ ions and can be inhibited by ion chelation. While PlcC is a Dot/Icm T4BSS effector, the export of PlcA and PlcB depends on the Lsp T2SS (Aurass et al., 2013). The PLC family in *L. pneumophila* is required for full virulence in the *Galleria mellonella* model, however, no clear evidence for its mode of action in host cells has been uncovered to this day.

In *L. pneumophila*, a recent study proposed type II secreted effectors, namely the zinc-metalloprotease ProA and chitinase ChiA, which both bind to the cytoplasmic face of the LCV after accessing the cytosol via a semipermeable LCV membrane and then eventually aid vacuolar escape of the pathogen (Truchan et al., 2017). Explicit indication of PLCs PlcC/CegC1, PlcA, and PlcB promoting vacuolar rupture and exit at late stages of infection was found in a recent publication (Striednig et al., 2021). When *D. discoideum* amoebae were infected with a *L. pneumophila plcABC* triple-gene-deletion mutant, significantly more cells infected with that mutant harbored intact LCVs at late timepoints of infection relative to wildtype–infected cells. Reciprocally, significantly more wildtype bacteria were found cytosolically when compared to *ΔplcABC* infected amoebae consistent with full capability to escape from the vacuole being dependent on PLCs.

7.2 | *L. pneumophila* phospholipase D supports bacterial fitness

PLD-encoding genes are prevalent in several intracellular and extracellular pathogens, including *Neisseria gonorrhoeae*, *Acinetobacter baumannii*, *Rickettsia prowazekii*, *Yersinia* spp., *Chlamydophila* spp., *H. pylori*, *P. aeruginosa*, and *Klebsiella pneumoniae* (Selvy et al., 2011). *L. pneumophila* encodes a high number of phospholipid-converting enzymes including PLD LpdA. This translocated effector was initially identified in a yeast screen for *L. pneumophila* effectors with toxicity to yeast (Viner et al., 2012) and later was shown to associate with membranes after experiencing post-translational S-palmitoylation. Ectopically expressed LpdA triggered massive cellular phosphatidic acid redistribution as well as Golgi apparatus fragmentation in HeLa cells. Indeed, in vitro, LpdA possessed PLD activity towards lipids including phosphatidylinositol phosphate. PLD activity has been previously linked to the vacuolar escape of a pathogen (Whitworth et al., 2005), however, the work of Viner et al. and Schroeder et al. provides no indication of LpdA-mediated LCV destabilization as a result of PLD-catalyzed lipid turnover (Schroeder et al., 2015; Viner et al., 2012). However, *ΔlpdA* mutants showed moderately reduced fitness in mice indicating its relevance as a virulence factor in vivo (Schroeder et al., 2015). In contrast, *lpdA* deletion mutants exhibited no growth defects when challenged with amoeba or THP-1 macrophages, suggesting that disruption of LpdA function alone unlikely disrupts cell egress and spread of the pathogen in the most widely used in vitro models of intracellular growth.

8 | OUTLOOK ON EXIT: THE OVERLOOKED KEY EVENT

To date, the majority of studies on microbial intracellular pathogens have primarily focused on unraveling the mechanisms of host cell entry and intracellular replication. However, there is a significant knowledge gap when it comes to understanding the pathways of phagosome and host cell escape. These pathways could potentially serve as critical targets for the development of novel antimicrobial therapies. The intracellular lifestyle of pathogens premises a sophisticatedly balanced series of events that initially mediate survival by innate immune evasion (Finlay & McFadden, 2006; Ta & Vanaja, 2021) and replication niche establishment (Eisenreich et al., 2019) and eventually set off an egress cascade that ultimately renders the pathogen vulnerable to immune detection. In this sense, comprehending exit is largely paralleled by understanding the

strategies pathogens drive to thrive until it is time to escape. An insight into such molecular "yin and yang" was provided by Creasey and Isberg (2012) who illustrated the balancing effects of SdhA and PlaA on LCV integrity (Creasey & Isberg, 2012), as mentioned earlier. *Legionella* strongly relies on the T4BSS Dot/Icm to establish its replicative niche (Hubber et al., 2014). Considering the sheer number of >300 secreted effectors (Ensminger, 2016; Qiu & Luo, 2017), it appears we have only caught a glimpse into the scope of how *Legionella* but also other pathogens orchestrate intracellular events.

Interfering with infection by targeting vacuole maintenance while simultaneously stimulating the clearance of infected cells by, for instance, inducing apoptosis could be one conceivable way for the development of antimicrobial therapies. In the case of *L. pneumophila*, this would potentially involve the type IVB-secreted phospholipases we have discussed in this review. Both VpdC with its effects on LCV expansion (Li et al., 2022), as well as VipD with its role in preventing lysosomal fusion (Gaspar & Machner, 2014; Ku et al., 2012) present putative druggable targets during the early stages of intracellular infection before massive bacterial replication occurs.

Oppositely, given the powerful lytic potential of phospholipases such as PlaA, PlaB, and PlaC (Flieger et al., 2002; Lang et al., 2012, 2017), direct inhibition of phospholipases and other egress factors might constitute another considerable approach to interfere with infection at its final stage by blocking escape. Preventing exit could ameliorate disease as otherwise released infectious agents and remnants of dying cells trigger inflammatory responses in the host that, when dysregulated, can cause permanent tissue damage or manifest in a chronic form (Chen et al., 2018). Exemplary, the inhibition of the *L. monocytogenes* kinase Taok2 by the chemical compound SW172006 was demonstrated to halt endosomal escape into the host cytosol, thereby preventing bacterial replication and reducing the overall bacterial count (Quereda et al., 2022). Inhibitors of phospholipases exist and predominantly find their application in treatments of neurological disorders (Ong et al., 2015) and snake bites (Marcussi et al., 2007). However, specific phospholipase inhibitors for *Legionella* are not known. Yet, intriguing developments in this context have been made for the ExoU of *P. aeruginosa* (Sato et al., 2003). The inhibitor pseudolipasin A (PSA) was shown to specifically inhibit ExoU activity (Lee et al., 2007), albeit (Foulkes et al., 2021) showed that its sole application in HCE-T cell cultures did not prevent the lysis of infected cells due to the general cytotoxic burden of replicating bacteria. Nonetheless, in the latter study they further demonstrated that once combined with antimicrobial moxifloxacin, cell lysis was strongly mitigated. The big hurdle that remains is to implement these approaches in the in vivo environment.

Taken together, phospholipases are intriguing enzymes that play diverse roles in various physiological processes. Further research is needed to fully elucidate how intracellular pathogens utilize phospholipases throughout their infectious cycle, from entry to host cell exit. This review has provided an overview of the current state of research on *L. pneumophila*'s secreted phospholipases. As one of the most extensively studied organisms in this field, the insights discussed here suggest that secreted phospholipases play central roles in both establishing and disrupting replication niches, making them compelling targets for antimicrobial therapy.

AUTHOR CONTRIBUTIONS

Antje Flieger: Conceptualization; supervision; writing – original draft; writing – review and editing; funding acquisition; methodology. **Jonathan Neuber:** Visualization; writing – review and editing; writing – original draft; conceptualization; methodology. **Christina Lang:** Writing – original draft; writing – review and editing; conceptualization; methodology. **Philipp Aurass:** Writing – original draft; writing – review and editing; conceptualization; methodology.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ETHICS STATEMENT

No human or animal subjects or materials were used in this review.

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