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Originally published as:

Lang, C., Flieger, A.
Characterisation of Legionella pneumophila phospholipases and their impact on host cells (2011) European Journal of Cell Biology, 90 (11), pp. 903-912.

DOI: 10.1016/j.ejcb.2010.12.003

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Characterisation of *Legionella pneumophila* phospholipases and their impact on host cells

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Running title: *Legionella* phospholipases

Number of words in abstract: 199

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Abstract

Phospholipases are a diverse class of enzymes produced both by eukaryotic hosts as well as their pathogens. Major insights into action pathways of bacterial phospholipases have been provided during the last years which on the one hand act as potent membrane destructors and on the other hand manipulate and initiate host signalling paths, such as chemokine expression or the inflammatory cascade. Reaction products of bacterial phospholipases may potentially influence many more host cell processes, such as cell respreading, lamellopodia formation, cell migration and membrane traffic. A pathogenic bacterium where phospholipases play a dominant role is the lung pathogen *Legionella pneumophila*. Here so far 15 different phospholipase A enzymes are encoded in the genome dividing into three major groups, the GDSL, the patatin-like and the plaB-like enzymes. The first two lipase families are also found in higher plants (such as flowering plants) and the second family shows similarities to eukaryotic cytosolic phospholipases A, therefore when those enzymes are injected or secreted by the bacterium into the host cell they may mimic eukaryotic phospholipases. The current knowledge on the *Legionella pneumophila* phospholipases is summarised here with emphasis on their activity, mode of secretion, localisation, expression and importance for host cell infections.

Keywords: Bacteria, *Legionella*, phospholipase, free fatty acids, lysophosphatidylcholine, infection, signalling

Introduction on bacterial phospholipases and their manifold impacts on host cells

Phospholipases are ubiquitous and diverse enzymes that mediate various cellular functions, including membrane synthesis, alteration in membrane compositions, cellular turnover, the generation of second messengers and inflammatory responses (Vadas et al., 1993; Granata et al., 2003). They are classified into four major groups (A, B, C, D) based on the position at which they cleave within a phospholipid. On the one hand, phospholipases A (PLA) hydrolyse the carboxylester bonds at sn-1 or sn-2 position and thereby release fatty acids together with a lysophospholipid and phospholipases B act on both fatty acid residues. If only one fatty acid is targeted, a lysophospholipid is generated and may be further cleaved by a lysophospholipase A (LPLA), thus liberating the remaining fatty acid from the glycerol backbone. On the other hand, phospholipases C (PLC) and phospholipases D (PLD) hydrolyse either the glycerol-oriented or the alcohol-oriented phosphodiester bond, respectively. Substrates of phospholipases have an amphipathic nature and are assembled into bilayers or monolayers, of which the most common in mammalian cells are phosphatidylethanolalamine (PE), phosphatidylcholine (PC), phosphatidylserine and phosphatidylinositol (PI) (Schmiel and Miller, 1999). The resulting cleavage products of these substrates participate in multiple signaling pathways and can act as precursors of potent mediators in the host cell (see Fig. 1).

There are many ways by which bacterial phospholipases contribute to the development of disease (Songer, 1997; Schmiel and Miller, 1999; Sitkiewicz et al., 2006; Hyrley and McCormick, 2008). Direct effects of phospholipases resulting from the hydrolysis of phospholipids are the depletion of integral structural lipids or the generation of lytic reaction products both leading to loss of membrane integrity and cytotoxicity. For example, the food-borne bacterial pathogen *Listeria monocytogenes* secretes a phosphatidylinositol-specific PLC (PI-PLC) and a broad-range phosphatidylcholine-specific PLC (PC-PLC) (Camilli et al., 1991; Mengaud et al., 1991; Goldfine and Knob, 1992; Smith at al., 1995). Both PI-PLC and listeriolysin O, a pore-former, are required for bacterial escape from the pathogen-containing vacuole into the cytosol, whereas PC-PLC contributes to disruption of the double-membrane vacuole during cell to cell spread (Goldfine et al., 1995; Smith et al., 1995; Poussin and Goldfine, 2005; Wei et al., 2005; Alberti-Segui et al., 2007). Further, the lung pathogen *Pseudomonas aeruginosa* injects the effector protein ExoU with PLA_2 activity directly into host cells by means of a type III secretion system (Finck-Barbancon et al., 1997; Hauser et al.,

1998; Sato et al., 2003; Phillips et al., 2003; Sato and Frank, 2004; Sato et al., 2005). By lysing cell membranes, it contributes to the ability of *Pseudomonas* to disseminate rapidly from lung tissue to the bloodstream. Also, the generation of lysophosphatidylcholine (LPC) contributes to increasing the permeability of cell membranes (Weltzien, 1979).

LPC is derived from PC as a result of PLA_2 enzymatic action and possesses a variety of functions within the eukaryotic cell. For example, extracellular application of LPC activates RhoA in a protein kinase C α (PKCα)-sensitive manner and impairs endothelial barrier function (Huang et al., 2005). Further, LPC induces oxidant production through activation of the NADH / NADPH oxidase system (Takeshita et al., 2000) and increases the expression of chemokines such as monocyte chemoattractant protein-1 and IL-8 (Murugesan et al., 2003). Obviously, phospholipase reaction products can also act as second messengers and aid the pathogen in manipulation of host signaling events. As an example, arachidonic acid or 1,2-diacylglycerol (1,2-DG) freed from a phospholipid molecule by PLA_2 or PLC, respectively, are involved in cellular signaling. This is the case for ExoU from *P. aeruginosa*, which triggers an arachidonic acid-dependent inflammatory cascade *in vivo* and activates several transcription factors that control proliferation responses and proinflammatory cytokine production (Sitkiewitz et al., 2007; Saliba et al., 2005). 1,2-DG, for example is generated by *Clostridium perfingens* α-toxin, one of the most toxic PLCs characterised to date (Flores-Diaz and Alape-Giron, 2003). Incubation of neutrophils with alpha-toxin leads to the production of 1,2-DG and in addition to release of phosphatidic acid (PA) (Sakurai et al., 2004). This triggers various signal transduction pathways, leads to uncontrolled generation of several intercellular mediators (Bryant and Stevens, 1996; Titball et al., 1999) and induces processes, like the adhesion of neutrophils to fibrinogen and fibronectin, as well as the production of O_2^- (Ochi et al., 2002). 1,2-DG has further been shown to activate protein kinase C, which is known to modulate a variety of cellular processes and growth including activation of neutrophils and macrophages (Nishizuka, 1992).

An additional product, which can be generated by various phospholipase pathways, like particularly PLD-mediated degradation of LPC or PLA_2-dependend hydrolysis of PA, is lysophosphatidic acid (LPA). LPA is a lipid mediator with many possible biological actions, particularly as an inducer of cell proliferation, migration and survival. LPA binds to specific G-protein-coupled receptors and thereby activates multiple signal transduction pathways, including those initiated by the small GTPases Ras, Rho and Rac (Moolenaar, 1995;

Moolenar et al., 1997; Moolenaar et al., 2004). Exogenous PLD from *Streptomyces chromofuscus* triggers Ca^{2+} mobilization, membrane depolarization, Rho-mediated neurite retraction and is acting on cognate G-protein-coupled LPA receptors via the production of LPA (Van Dijk et al., 1998). LPA also promotes cell respreading (adhesion and extension of eukaryotic cells after debonding from the matrix), lamellopodia formation and cell migration by activating the Rac GTPase through a G_i-mediated pathway (Van Leeuwen et al., 2003). These data indicate that products of phospholipases are also significantly involved in the rearrangement of the cytoskeleton and of cellular movement. But phospholipases not only modify the host cell membranes they can also facilitate the regulation of bacteria-induced membrane extensions.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) has the fascinating ability to form tubular structures known as *Salmonella*-induced filaments (Sifs) in host cells. To regulate the level of Sifs, *Salmonella* Typhimurium injects the effector protein SseJ into the host cell cytoplasm showing PLA and glycerophospholipid:cholesterol acyltransferase (GCAT) activities (Lossi et al., 2008, Nawabi et al., 2008). Upon secretion, SseJ localizes to the *Salmonella*-containing vacuole membrane and to Sifs and antagonises the stimulatory effect of SifA, which is essential for Sif formation (Ruiz-Albert et al., 2002).

Another potential substrate of phospholipases is PI. The phosphorylated variants of PI, called phosphoinositides, are an important class of signaling lipids. Their hydrolysis yields second messengers that transmit downstream signals (Di Paolo and de Camilli, 2006), they play a role in the regulation of receptor-mediated endocytosis and phagocytosis (Yeung et al., 2006) and some of them are involved in the recruitment of cytoskeleton elements (Scott et al., 2005). Pathogens have evolved strategies to subvert phosphoinositide metabolism to affect either the uptake process or phagosomal maturation (Weber et al., 2009, Steinberg and Grinstein, 2008). In this background bacterial phospholipases, in particular PLC, could interfere or influence phosphoinositide signaling by PI hydrolysis followed by emission of different inositolphosphates and 1,2-DG as second messengers.

In the following, the bacterial lung pathogen *L. pneumophila* and its manifold PLA enzymes are introduced and especially their action onto host cells is described.

Legionella and its multitude of phospholipases.

Legionella bacteria are characterised by their biphasic lifestyle. On the one hand, they thrive within different kinds of protozoa present in aqueous habitates and on the other hand, when inhaled with or without protozoa, *Legionella* causes a potentially severe pneumonia in humans, termed Legionnaires' disease. The genus *Legionella* comprises about 50 different species; however only one species, *L. pneumophila*, is responsible for more than 90% of the disease cases (Hilbi et al., 2010; Fields, 1996; Winn and Myerowitz, 1981). Therefore, *L. pneumophila*-specific proteins which are not produced by or which are not equipped with comparable functional properties in non-*pneumophila* species are of special interest.

Since phospholipases are classical virulence factors of pathogenic bacteria, including both extracellular (such as *C. perfringens* or *P. aeruginosa*) and intracellular pathogens (such as *L. monocytogenes*), we aimed to investigate the importance of such enzymes for *L. pneumophila* belonging to the intracellular bacteria (Sitkiewicz et al., 2006; Istivan and Coloe, 2006; Schmiel and Miller, 1999; Titball, 1998). Especially here, phospholipases may fulfil a variety of the above mentioned functions for nutrition, adhesion, invasion, modulation of the host (including its signalling) allowing intracellular establishment and also escape from the phagosome and the host cell to subsequently infect another host cell. Surely, one important function of those enzymes may include to mimic or substitute host cell phospholipases to drive processes beneficial for the invader. This is potentially relevant for enzymes injected into the host cell cytosol or the phagosomal membrane via the *L. pneumophila* type IVB secretion system Dot/Icm, a secretion system which transports a multitude of effector proteins (at least 140) and strongly promotes *L. pneumophila* virulence (Hubber and Roy, 2010; Isberg et al., 2009; Ensminger and Isberg, 2009; Franco et al., 2009; Ninio and Roy, 2007; Vogel et al., 1998; Segal et al., 1998). But also other established and putative secretion systems for transport through (at least) the outer membrane have been described for *L. pneumophila* strains, such as a type I Lss, a type II Lsp, several type IVA systems (for example Lvh, Trb-1, Trb-2), and a potential type V autotransporter protein (Schroeder et al., 2010; Glöckner et al., 2008; Albert-Weissenberger et al., 2007; Brassinga et al., 2003; Jacobi and Heuner, 2003; Rossier and Cianciotto, 2001; Segal et al., 1999). So far only the Lsp system exporting more than 20 proteins and, in the absence of the Dot/Icm system, the Lvh system, have been described as additional virulence determinants (Cianciotto, 2009; Bandyopadhyay et al., 2007; DebRoy et al., 2006; Rossier et al., 2004).

During life within a host cell but also during growth in laboratory media, *L. pneumophila* exhibits two different phases which are on the one hand the replicative phase where the bacteria do not show cytotoxicity but instead the expression program is focused on the usage of present nutrients. On the other hand, when the nutrient supply ceases, the bacteria switch to the cytotoxic phase where the expression program favours production of factors facilitating acquisition of new nutrient sources and bacterial exit from the spent host cell (Byrne et al., 1998; Molofsky and Swanson, 2004; Brüggemann et al., 2006). It seems plausible that some of the *Legionella* phospholipases might support firstly intraphagosomal nutrient acquisition (impact on signalling to hijack host cargo?) and catabolism as well as suppression of host defence in the replicative phase, while others might serve to release bacteria from the host in the cytotoxic phase. Therefore, it makes sense that an intracellular pathogen could engage several kinds of phospholipases at different stages for its benefit.

To assess the impact of *Legionella* phospholipases on bacterial pathogenicity and also characterise their substrate specificity and their mode of secretion, about ten years ago, we started to identify the proteins and genes responsible. At that time, we never thought that our bacterium of interest would hide an unexpected variety of lipolytic enzymes comprising a multifaceted redundancy, which we now know is a very characteristic feature of *L. pneumophila*. This actually might not be surprising for a bacterium which has been shown to infect many different kinds of protozoa (Fields, 1996). Today we know that the genomes of several *L. pneumophila* strains contain at least 15 genes coding for potential PLAs which classify into three different families, the GDSL family, the patatin-like protein (PLP) family, and the PlaB-like family (Fig. 2, Table 1; Banerji et al., 2008). In principal, paralogs of many of those are also found in other *Legionella* species, such as *L. longbeachae* and *L. drancourtii*, however the panel of genes present is not absolutely identical (see Table 1). There are surely more lipolytic enzymes in addition to the PLAs included in the genome, for example also classical lipases and potential phospholipases C (Aragon et al., 2002). In the following paragraphs, the current knowledge on the *L. pneumophila* phospholipases A will be summarised and especially their substrate specificities, mode of secretion, expression and impact on the host cell will be addressed.

Legionella GDSL lipase family

The GDSL enzyme family, a sub-family of the SGNH hydrolases, comprises enzymes with PLA, LPLA, lipase, haemolytic, and GCAT activities mostly of plant or bacterial origin (Molgaard et al., 2000; Upton and Buckley, 1995; Brick et al., 1995). GDSL proteins possess the GDSL signature motif encompassing the putative catalytic nucleophile Ser located in amino acid homology block I and the putative catalytic triad members, Asp and His, in block V (Brick et al., 1995; Upton and Buckley, 1995). The prototype of a bacterial GDSL enzyme is the GCAT SatA of *Aeromonas salmonicida* also showing PLA, LPLA and lipase activities (Buckley et al., 1982). SatA is an important virulence factor and also other GDSL enzymes contribute to the establishment of the pathogen within the host, such as SseJ, a GCAT of *S.* Typhimurium, by fulfilling a function in biogenesis of the *Salmonella*-containing vacuole by cholesterol acylation (Lossi et al., 2008; Nawabi et al., 2008; Ohlson et al., 2005; Ruiz-Albert et al., 2002; Lee and Ellis, 1990).

Enzymes and activities

The species *L. pneumophila* (currently represented by five sequenced strains: Philadelphia-1, Corby, Lens, Paris, and Alcoy) possesses three different GDSL enzymes with distinct activities, PlaA, PlaC, and PlaD, sharing the five typical blocks of amino acid homology (Table 1; D'Auria et al., 2010; Banerji et al., 2008; Glöckner et al., 2008; Cazalet et al., 2004; Chien et al., 2004; Flieger et al., 2002). A screen of the recently sequenced genomes of the non-*pneumophila* species *L. longbeachae* (strains D-4968 and NSW150) and *L. drancourtii* (strain LLAP12) uncovered that those are coding for orthologs of all three proteins (Cazalet et al., 2010; Kozak et al., 2010; Moliner et al., 2009). Interestingly, in *L. drancourtii* PlaA and PlaD seem to have two close relatives each and the *L. longbeachae* strains even contain two to three PlaD paralogs (Table 1).

PlaA was originally identified via biochemical purification and N-terminal sequencing from *L. pneumophila* culture supernatant and was found to be the major secreted LPLA with some additional PLA and lipase activities (Flieger et al., 2002; Flieger et al., 2001). The progression of the *L. pneumophila* genome projects allowed identification of the other homologs and subsequent experimental studies. PlaC exhibited some LPLA but majorly PLA and GCAT activities but PlaA interestingly did not show the latter activity (Banerji et al., 2005; Flieger et al., 2002). The GCAT activity of PlaC is via an unknown mechanism directly or indirectly

dependent on the secreted zinc metalloprotease ProA, and the putative activation process also leads to an increase of *L. pneumophila* secreted PLA compared to LPLA activity (Banerji et al., 2005; Flieger et al., 2002). For other bacterial GDSL enzymes, an activation procedure which obviously prevents the enzyme's premature action is also described. *A. salmonicida* SatA is processed between two cysteine residues and *S.* Typhimurium SseJ even requires binding of eukaroytic RhoA (Christen et al., 2009; Lossi et al., 2008; Hilton et al., 1990). The host lipids PC and also phosphatidylglycerol (PG), the latter especially found in lung surfactant, are efficiently cleaved by the here described *L. pneumophila* PLA activities to the lysophospholipids and fatty acids are released. Subsequently, LPLA frees the remaining fatty acid and thereby degrades and detoxifies the pore-forming agent and signal transducer LPC (Murugesan et al., 2003; Prokazova et al., 1998; Kume et al., 1992; Niewoehner et al., 1987; Weltzien, 1979). The detoxifying function has been shown especially for PlaA supporting bacterial survival under LPC exposure (Flieger et al., 2002). Enzymatic activity of the third *L. pneumophila* GDSL hydrolase PlaD and further properties still remain to be comprehensively determined, but first results suggested presence of PLA and LPLA activities (Rastew, Lang, Flieger unpublished).

Mode of secretion, localization, and expression

Both PlaA and PlaC are secreted proteins because they are found in *L. pneumophila* culture supernatant. *plaA* knock out mutants accordingly possess severely reduced secreted LPLA and to some extent diminished secreted PLA activity. Further, *plaC* knock out mutants show a complete loss of secreted GCAT and a marked reduction in PLA and LPLA activities (DebRoy et al., 2006; Banerji et al., 2005; Flieger et al., 2002). PlaC is not only found free in the *L. pneumophila* culture supernatant, but also is associated with outer membrane vesicles shed from the bacteria (Galka et al., 2008). PlaA and PlaC contain a predicted N-terminal signal sequence and secretion of both depends on the functional type II secretion system Lsp (DebRoy et al., 2006; Banerji et al., 2005; Flieger et al., 2002; Flieger et al., 2001; Rossier et al. and Cianciotto, 2001). Type II secreted proteins, such as the zinc metalloprotease ProA, are potentially secreted into the phagosome, and therefore, the presence of PlaA and PlaC within the lumen of the phagosome also is very likely (Rechnitzer et al., 1992). Since the type II secretion mutant still exhibits residual secreted PLA and also LPLA activities, it is expected that further lipolytic enzymes are secreted into the culture supernatant by another secretion mechanism (Flieger et al., 2001; Rossier and Cianciotto, 2001). Secreted lipolytic activity is most prominent in the late logarithmic growth phase (Flieger et al., 2000). Interestingly, *L.*

pneumophila GCAT activity was not found in the culture supernatant of mutants in the two component regulatory system LetA/S or RpoS, which are important for the switch from the replicative into the transmissive phase. Moreover, *plaC* mRNA was severely reduced in the mutants, and therefore, we conclude that those regulators directly or indirectly impact *plaC* expression (Broich et al., 2006). Further, secreted PLA activity was dramatically reduced and secreted LPLA activity was increased in the mutants, showing a further induction of a so far not known secreted PLA and further repression of a major LPLA, such as PlaA, respectively, by LetA/S and RpoS.

GDSL enzyme gene expression profiles comparing the replicative with the transmissive growth phase during an amoeba infection by means of microarray analyses pointed out that expression of *plaA* and *plaC* was not significantly changed whereas *plaD* expression increased twice in the transmissive growth phase (Brüggemann et al., 2006). Those data suggested some importance of PlaD within the transmissive rather than the replicative growth phase, whereas the other two enzymes might be expressed at similar levels in the two phases.

Importance for host cell infections

Single knock out mutants in *plaA* and in *plaC* were tested for host cell infection in human macrophages and amoebae and the *plaA* mutant was also checked in a mouse model. However, the mutants were as virulent as the wild type bacteria (DebRoy et al., 2006; Banerji et al. 2005; Flieger et al., 2002). This suggests that the three enzymes may balance the loss of a specific lipase. Although they exhibit different major activities, they are mostly overlapping in their LPLA activities, which might be the most important activity under infection conditions. In general, the fine-tuning of GDSL protein enzymatic PLA and LPLA activities might not only support bacterial survival by detoxification of LPC, but at the same time also guarantee host cell survival and integrity until the end of the intracellular infection. Further, the release of fatty acids, their binding onto acceptors such as cholesterol and specific amounts of LPC might influence host cell signal transduction. Interestingly, PlaC is yet the only *L. pneumophila* enzyme contributing to cholesterol acylation, however currently it can not be excluded that under *in vivo* conditions also PlaA and PlaD may develop GCAT activity (e.g. by means of an eukaryotic factor). Such an activity may modify important cholesterol-rich regions within mammalian cells and influence for example receptor presentation and membrane organisation. In the future, analysis of *L. pneumophila* double and triple GDSL protein mutants will shed light on the issue of GDSL protein redundancy.

Legionella patatin-like protein family

Patatins are a family of plant proteins with acyl hydrolase activity fulfilling important functions in signal transduction processes and plant parasite defence. Some of the plant patatins are upregulated during colonization by bacteria or fungi (Holk et al., 2002; Strickland et al., 1995). More interestingly, the knock out of certain up regulated patatins renders the plant more resistant towards pathogen attacks, implying that the invader may just hijack the lipolytic activity for its own nutritional purposes (La Camara et al., 2005). Patatin-like proteins (PLP) have been found as well in bacteria and they are considered to be eukaryotic-like phospholipases because they are more similar to the eukaryotic patatins than to any other family of bacterial lipases (Banerji and Flieger, 2004). Both bacterial PLPs and eukaryotic patatins are characterized by the following: 1) four blocks of amino acids homologous to the active site residues serine (GxSxG motif) and aspartate (DGx motif) in blocks II and IV, respectively, representing the catalytic dyade (instead of a more common serine-aspartate-histidine triade) and 2) a stretch of glycines (mostly three) close to the N-terminus in block I (Banerji and Flieger, 2004; Rydel et al., 2003; Hirschberg et al., 2001; Schrag and Cygler, 1997). Differences between the eukaryotic and bacterial proteins are also found, namely in block III, where a conserved proline is preceded by the distinct motifs, AAP and ASxxxP, respectively. Successive to block IV, an additional region involving a conserved serine is found only in eukaryotic patatins and the related eukaryotic cytosolic phospholipase A_2 (Banerji and Flieger, 2004; Hirschberg et al., 2001). At present about 4400 potential proteins containing the typical patatin domains are coded in the sequenced bacterial genomes (http://www.ebi.ac.uk/interpro) and currently only a minority of bacterial PLP (*Pseudomonas aeruginosa* ExoU and PlpD, *B. subtilis* YvdO, *Rickettsia typhi* RT0522, *L. pneumophila* VipD/PatA, VpdA/PatC, VpdB/PatG, VpdC/PatF, and PatD) have been characterised to some extent, which shows that there is a research need to understand the function of the bacterial PLP especially with respect to pathogenesis (Kato et al., 2010; Rahman et al., 2010; Salacha et al., 2010; Aurass et al., 2009; VanRheenen et al., 2006; Shohdy et al., 2005; Philipps et al., 2003; Sato et al., 2003).

Enzymes and activities

Most of the yet sequenced *L. pneumophila* strains encode 11 PLP and therefore *L. pneumophila* currently is the bacterium with the highest number of PLP and the highest genomic density of 3.7 PLP per 1000 genes, followed by *Mycobacterium tuberculosis* with 8

PLP and a density of 2.0 (Fig. 2, Table 1; Banerji et al., 2008). Since the mentioned two bacterial species and many others coding for PLP are important bacterial pathogens or symbionts, those *in silico* analyses suggest that PLP may influence their specific host cell-associated life style.

Enzymatic activity of *L. pneumophila* PLPs has so far just been published for PatD which is, comparable to PlaB, a cell-associated PLA and LPLA contributing to about 20% to the total cell-associated activity (Aurass et al., 2009). *patD* is organised in an operon together with *bdhA* which codes for a protein with significant full-length homology to *Sinorhizobium* sp. 3-hydroxybutyrate (3-HB) dehydrogenase BdhA (Aurass et al., 2009; Aneja and Charles, 2005). 3-HB dehydrogenases oxidise depolymerized polyhydroxybutyrate (PHB), an important storage lipid of bacteria, to acetoacetate, allowing metabolisation of the energy reserve (Aneja and Charles, 2005; Jendrossek and Handrick, 2002; Anderson and Drawes, 1990; Dawes and Senior, 1973). A *L. pneumophila bdhA/patD* mutant accordingly accumulates higher amounts of PHB granules compared to the wildtype, indeed suggesting a function of the operon in PHB usage. Currently, it is however not clear whether PatD directly contributes to PHB cleavage, for example as a PHB depolymerase, hydrolysing PHB into 3-HB monomers. A PHB depolymerase is not obviously encoded in the *L. pneumophila* genome sequence (Aurass et al., 2009).

VanRheenen et al. were not able to detect enzymatic activity for VipD and its expression as a whole protein was relatively well tolerated in yeast contrasting the immediate cytotoxicity of *P. aeruginosa* ExoU (VanRheenen et al., 2006; Hauser et al., 1998; Finck-Barbancon et al., 1997). Only upon overproduction in yeast, VipD slowed growth (VanRheenen et al., 2006) and interestingly, it was further found that VipD lacking the patatin domain perturbed the late secretory pathway more severe than the full length protein (Shohdy et al., 2005) whereas the main toxic effect of ExoU depended on its phospholipase domain. Those experiments show that there might be two different functional domains contained within the VipD protein, however their mode of cooperation and their protein or lipid target in the host cell still remains elusive.

Mode of secretion, localization, and expression

Some data are available on *L. pneumophila* PLP localisation and mode of secretion. Since PatD remains associated with the bacterial cell, it therefore seems important for processes

within the pathogen (Aurass et al., 2009). With respect to its likely involvement in storage lipid metabolism, PatD is expected to be present in the cytoplasm, within the inner bacterial membrane or rather at lipid inclusion membranes but it is certainly not directed towards the outside of the bacterium. VipD, VpdA and VpdB conversely are injected via the Dot/Icm type IVB secretion machinery directly into the host cell, but their target location within the host cell is currently not known (VanRheenen et al., 2006; Shohdy et al., 2005).

Gene expression analysis during the infection of the environmental host amoeba *Acanthamoeba castellanii* has also shed light on the differential expression of multi member protein families in *L. pneumophila*, such as the 11 PLPs. Seven *L. pneumophila* PLP genes (*vipD*, *patB*, *vpdA*, *patD*, *patE*, *patI* and *patK*) are upregulated at least two-fold from the replicative towards the transmissive growth phase. Especially *vipD*, *patD*, *patE*, and *patI* expression increased 8-11 times showing that the respective proteins might be of major importance for survival (i.e. additional acquisition of nutrients at a phase when those become rare) or exit of the bacteria at late stages of intracellular growth, or their expression is required for the subsequent infection cycle in a new host cell (Brüggemann et al., 2006).

Importance for host cell infections

L. pneumophila seems to implement a huge variety of proteins to modulate host cell functions for its benefit. Already the number of Dot/Icm system-dependent injected effector proteins is exceeding 140 and there are other secretion systems, such as the Lsp machinery, which deliver proteins at least to the phagosomal space (Hubber and Roy, 2010; Cianciotto, 2009; Ensminger and Isberg, 2009; Franco et al., 2009). It is known that *L. pneumophila* produces protein families with a multitude of members, for example the PLPs, therefore work with single effector knock out mutants will very seldomly identify single genes with an essential importance in infection models. This is exactly the case for single knock out mutants in *vipD*, *vpdA*, *vpdB*, and *vpdC*, but also a knock out mutation in all four genes did not show any defect in a macrophage infection model or even resulted in a growth advantage in a *Dictyostelium discoideum* which again highlights the redundancy issue for *Legionella*. But this does not mean that the PLP might not at all serve the bacterium in host modification. Many ways of bacterial PLP interactions are imaginable starting from mimicking host cell PLAs which are very important to drive membrane traffic through the secretory pathway and continuing with very dominant activities which might be rather important for bacterial release from the phagosome (Schmidt et al., 2010; Brown et al., 2003; Choukroun et al., 2000; de Figueiredo et al., 2000; Drecktrah and Brown, 1999).

Although redundancy might mask an essential role of at least four *L. pneumophila* PLPs for host cell infection, the *bdhA*/*patD* knock out mutant nevertheless exhibits a very severe infection defect both in a macrophage and an amoeba infection model (Aurass et al., 2009; VanRheenen et al., 2006). The infection defect is very comparable with a Dot/Icm secretion system mutant, suggesting that PHB metabolism is essential for the life style of *L. pneumophila* and intracellular propagation. One can now speculate that provision of energy by PHB cleavage is an essential determinant to efficiently energise the Dot/Icm secretion system or another essential component; however currently it is not known whether the mutant is defective in the export of effector proteins.

Legionella PlaB

A novel lipolytic enzyme was discovered by screening a *L. pneumophila* gene library expressed in *E. coli* for haemolytic clones. Since the clone also showed PLA and LPLA activities, the gene was designated *plaB* (Fig. 2, Table 1). PlaB did not exhibit significant protein homology to any characterised protein, but instead to a large number of uncharacterised and therefore under the category hypothetical annotated proteins of many (sea) water-associated bacteria, such as *Psychromonas ingrahamii*, *Shewanella paleana*, *Marinobacter algicola*, *P. aeruginosa*, and *Persephonella marina*. Very minor and actually not significant protein homology was found to the characterised secreted lipases LipB and weakly also LipA for which the cleavage of phospholipids substrates so far has not been shown (Flieger et al., 2004, Aragon et al., 2002).

Enzymes and activities

To further characterize the enzymatic profile of PlaB and its contribution to *L. pneumophila* total activity, a *L. pneumophila* knock out mutant was constructed and tested for changes in lipid hydrolysis. Here, no differences in the secreted PLA/LPLA/GCAT activities were found but instead a very dramatic loss of cell-associated PLA and LPLA activities. Those experiments showed that PlaB is the most prominent cell-associated PLA/LPLA, especially cleaving PG and PC as well as the respective lysophospholipids. Importantly, the detected PlaB-associated PLA activity exceeded ~100-times the lipolytic activities present in the culture supernatant of *L. pneumophila* (Flieger et al., 2004). Since PlaB seemed to be the first representative of this novel enzyme family, we wanted to define the residues essential for catalytic activity. In many lipase families a catalytic diade or triade, mostly combinations of a

serine together with an aspartate and a histidine, has been found to be essential. Also in PlaB, serine 85, aspartate 203 and histidine 251, embedded into the uncommon motifs TH**S**TG, GS**D**GVV, and S**H**S, respectively, were determined as the catalytic triade (Bender et al., 2009). Interestingly, other changes such as serine 129 and histidine 270 did to ~90% reduce cleavage of PC but not PG and resulting mutant proteins were not haemolytic, revealing that the specificity for PC hydrolysis is absolutely essential for PlaB haemolytic activity. Therefore, the fact whether an organism or a protein exhibits PLA activity is not sufficient to conclude on the potential activity towards eukaryotic cells. But rather an activity targeting typical major eukaryotic lipids, such as PC or PE, might be decisive host cell disintegration. This is also underlined by the investigation of PlaB from *L. spiritensis*, a *Legionella* species so far not involved in human disease cases (Muder and Yu, 2002; Fang et al., 1989). Compared to *L. pneumophila* PlaB, *L. spiritensis* PlaB showed a less prominent relative PC-specific activity (~50%) when normalised to PG-hydrolysis which correlated to a lower potential (~50%) to lyse human blood cells (Bender et al., 2009). Therefore, the presence of an enzyme gene in a specific strain might not give sufficient information to explain virulence or avirulence. Instead, it is important to additionally include considerations on the time of gene expression and quantity as well as enzyme specificity, which is not easy to analyze, because single amino acid changes or alterations in the reaction habitat, such as temperature, pH, ion concentration, and others might severely influence enzyme activity and specificity. This is also illustrated by *L. spiritensis* PlaB showing all so far determined residues for catalytic activity (serine, aspartate, histidine triade and surroundings) or substrate specificity (additional critical serine and histidine) exactly as *L. pneumophila* PlaB. In the future, it will be very interesting to analyze additional protein examples of *Legionella* species other than *L. pneumophila* or further related enzymes in the context of substrate specificity and virulence potential.

Mode of secretion, localization, and expression

Differential centrifugation and separation of bacterial membranes from the cytosol showed presence of PlaB in the outer membrane of *L. pneumophila*. Also, PlaB-associated PLA activity was cleaved from the bacterial cell by an external proteinase K treatment and was then found in the culture supernatant (Schunder et al., 2010). Those data strongly suggest that the enzyme is embedded in the bacterial outer membrane and is exposed to the external environment, a precondition allowing a direct interaction of PlaB with host cell lipid targets. However, how PlaB localizes to the outer membrane remains to be determined; known

secretion systems (Dot/Icm, Lvh, Lss, Tat) did not play a role and even a signal peptide is not predicted for the enzyme (Schunder et al., 2010). *plaB* expression in *L. pneumophila* is most prominent during early logarithmic growth and in the following decreases, showing that *plaB* is expressed at a rather early bacterial life stage. Accordingly, PlaB-dependent lipolytic activity develops most prominently during *L. pneumophila* late exponential growth in broth medium (Schunder et al., 2010). Microarray analyses did not reveal significant changes of *plaB* expression comparing the replicative and transmissive phase during an amoeba infection, suggesting that mRNA production might be induced at the beginning of the replication cycle within the cells and might be kept at that level during the intracellular stage (Brüggemann et al., 2006). The determination of bacteria-specific PLA/LPLA activities during an amoeba or human cell infection indicates that PlaB is indeed produced and is the most prominent *L. pneumophila* PLA/LPLA during an infection (Bender et al., 2009). Since the bacteria-associated PLA/LPLA activity partially decreased in mutants of the regulators LetA/S and RpoS impacting the switch from the replicative into the trasmissive phase, PlaB activity directly or indirectly depends on these regulators (Broich et al., 2006).

Importance for host cell infections

Although PlaB activity was high during an *in vitro* host cell infection of human macrophages and amoebae, there was no difference in CFU isolated from wildtype *L. pneumophila* and a *plaB* knock out mutant in those infection models (Bender et al., 2009; Flieger et al., 2004). We then speculated on a possible function of PlaB within the in vivo setting of the lung and performed Guinea pig infection experiments. Here, the *plaB* mutant compared to the wildtype was indeed severely attenuated, illustrated by an about 400-fold increase of CFU in the lung after two days post infection for the wild type, compared with only 20-fold increase in CFU of the *plaB* mutant. Further, dissemination of the mutant from the lung to the spleen was severely inhibited. Prominent destruction of lung tissue, such as accumulated endothelial debris within the alveoli, and signs of inflammation were only observed for *L. pneumophila* wildtype infections but almost not apparent with the *plaB* mutant (Schunder et al., 2010). This shows that *plaB* is an important virulence factor of *L. pneumophila* and its role became apparent only after an *in vivo* infection.

Conclusion

Many phospholipases A are found in *L. pneumophila* bearing an enormous redundancy with respect to lipid acquisition and membrane modulation, highlighting their importance for the bacterium. These enzymes promote cell-destructing enzymatic activity of *L. pneumophila* and may also be important for modification of eukaryotic signalling by the pathogen. So far two (PatD and PlaB) of the 15 enzymes, all belonging to the class of serine esterases, have been shown to be essential for host cell and/or animal infection. Future studies are intended to identify the precise action mechanisms and cellular targets of the single enzymes. Important topics for future research are: 1) Further characterisation of PlaC which not only catalyses fatty acid release from phospholipids but also transfers the fatty acid to an important eukaryotic lipid, cholesterol. Where exactly in the host cell is cholesterol acylated by PlaC and what does that mean in terms of a host cell infection? May also PlaA and PlaD develop GCAT activity? 2) Is PatD essential for energising major virulence determinants, such as the Dot/Icm-dependent protein secretion? 3) How does PlaB support host infection, rather via signal transduction interference or “mere” tissue damage? 4) Importantly, for all mentioned enzymes no crystal structures are yet available. Those analysis might help to confirm genetically acquired data on catalytic residues and contribute to an understanding of the additional protein domains attached to the lipase domain of some here presented lipases, such as PlaD (last 170aa), VipD/PatA (last 120aa), VpdA/PatC (last 150aa), VpdC/PatF (first 240 and last 300aa), VpdB/PatG (last 250aa) and PlaB (last 170aa).

Acknowledgements

The authors acknowledge the German Research Foundation (DFG) for providing grant DFG FL 359/5-1 within the priority program “Signalling to the cytoskeleton and bacterial pathogenicity” as well as grants DFG FL 359/3-1/3-3 and DFG FL 359/4-1. We thank Dorothea Eitze for support on Fig. 2 and Erika Kleindienst for skilled help on manuscript preparation.

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Table 1. Potential and established phospholipase A enzymes coded in the sequenced genomes of *Legionella pneumophila, L. longbeachae and L. drancourtii.* Only protein homologs with an expect value < 0.01 (output of a BLAST protein homology comparison, Altschul et al., 1997) and presence of the potential catalytic domains were considered. In gray: additional homologs with lower similarity to the specific proteins. The locus tag numbers of the proteins are given with respect to the different *L. pneumophila* strains, i.e. Lpg numbers refer to strain Philadelphia-1 (Phil-1) (Chien et al., 2004), Lpc to strain Corby (Glöckner et al., 2008), Lpl to strain Lens, Lpp to strain Paris (Cazalet et al., 2004), Lpa to Alcoy (DÁuria et al., 2010), LLB to *L. longbeachae* clinical isolate D-4968 (Kozak et al., 2010), LLO to *L. longbeachae* strain NSW150 (Cazalet et al., 2010) and LDG to *L. drancourtii* or *Legionella*-like amoebal pathogen (LLAP12) (gene bank ACUL00000000).

Strain / Family	Protein	Activity	*L. pneumophila*					*L. longbeachae*		*L. drancourtii*	References
			Phil-1 Lpg	Corby Lpc	Lens Lpl	Paris Lpp	Alcoy Lpa	D-4968 LLB	NSW150 LLO	LLAP12 LDG	
GDSL	PlaA	LPLA, PLA	2343	1811	2264	2291	03353	2504	2819	2220 2854	Flieger et al., 2001 Flieger et al., 2002
	PlaC	PLA, LPLA, GCAT	2837	3121	2749	2894	04118	1661	0210	0760	Banerji et al., 2005
	PlaD	unpublished	2587	0558	2510	2640	03783	2716 0726	2624 0995	1000 0625	Banerji et al., 2008
PlaB	PlaB	PLA, LPLA	1603	1029	1422	1568	02320	1067	0672	2542	Flieger et al., 2004; Bender et al., 2009; Schunder et al., 2010
Patatin-like	PatA/VipD	unpublished	2831	--------	------	2888	------	------	------	------	Van-Rheenen et al., 2006; Shohdy et al., 2005; Aurass et al., 2009
	PatB	unpublished	2807	3093	2722	2853	04079	1602	0263	0829	
	PatC/VpdA	unpublished	2410	2065	2334	2479	03513	2148	3207	------	
	PatD	PLA, LPLA	2317	1784	2237	2265	03323	3073	2301	2345	
	PatE	unpublished	1944	1417	1914	1925	02828	3073	2301	------ 3102	
	PatF/VpdC	unpublished	1426	0842	1377	1381	02090	3606	1791	2407	
	PatG/ VpdB	unpublished	1227	0696 1707	1235	1235	01899 03221	------	------	------	
	PatH	unpublished	0952	2335	0981	1014	01436	1154	------	------	
	PatI	unpublished	0670	2623	0706	0726	01050	3651	1748	3100 1414	
	PatJ	unpublished	0290	0369	0343	0368	00502	2097 0980	3249 0751	------ 2200 1948	
	PatK	unpublished	0014	0015	0014	0014	00018	2972	2395	0012	

Figure legends.

Figure 1: The variety of possible impacts of phospholipases on host cells (Abbreviations: LPC = lysophosphatidylcholine; PKCα = protein kinase Cα).

Figure 2: Overview of *L. pneumophila* phospholipases A modified from Banerji et al., 2008. Enzymes highlighted in grey have not been characterised yet.

Figure 1
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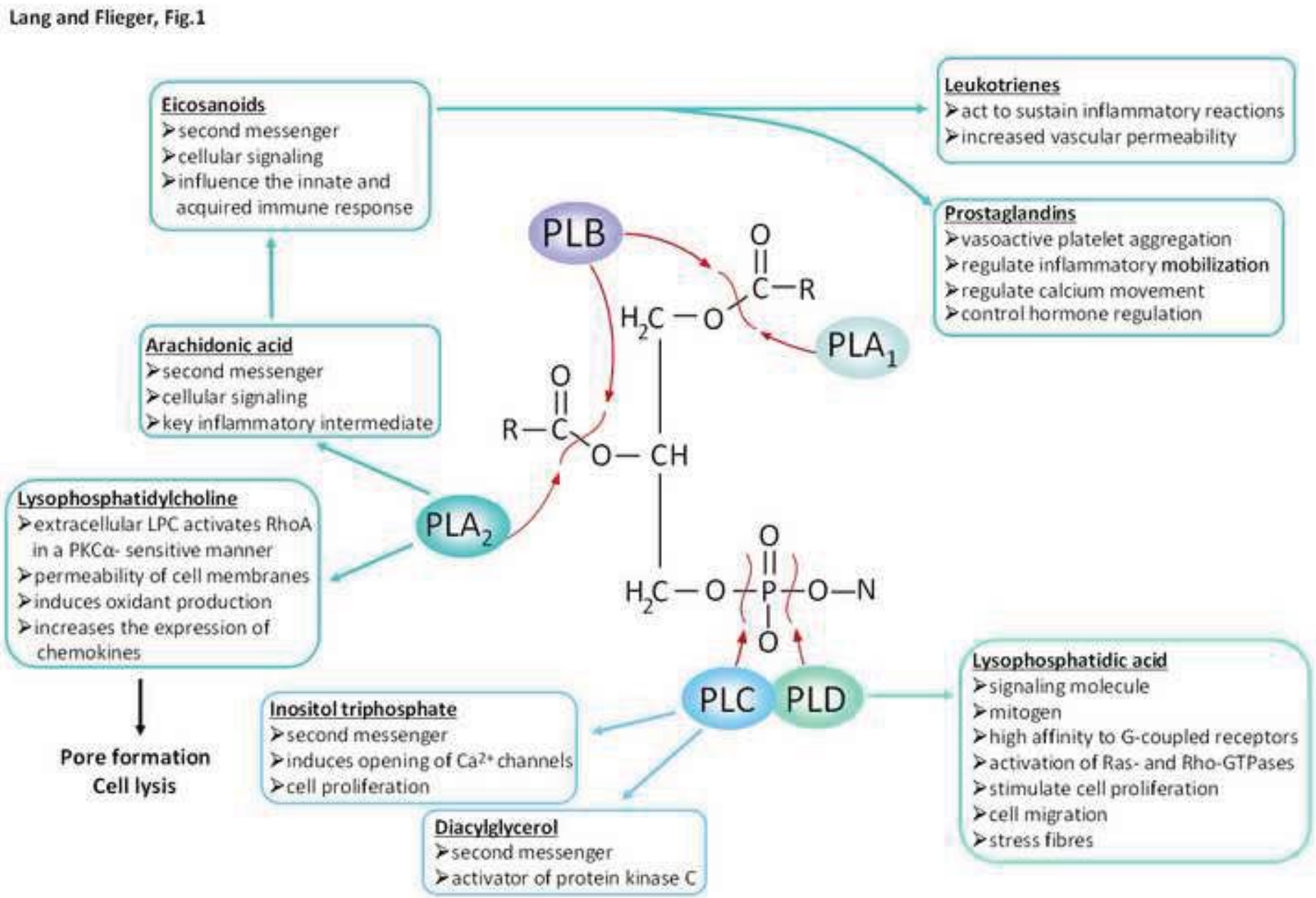

Figure 2

Lang and Flieger, Fig. 2