5. Discussion

The present study aimed to further identify the enzymes responsible for the high secreted and cell-associated phospholipase A and lysophospholipase A activities of *L. pneumophila* and to investigate their role in bacterial virulence. Five so far uncharacterized proteins were found to contribute to the bacterial secreted or cell-associated PLA and LPLA activities. Based on their amino acid sequence, these five enzymes could be assigned to four different groups of lipolytic enzymes. PlaC and PlaD belong to the family of GDSL hydrolases, PlaB has a modified lipase motif, PatA is one of 11 *L. pneumophila* Philadelphia-1 patatin-like proteins, and Aas belongs to the group of 2-acylglycerophospholipid acyltransferases.

5.1 Identification of *L. pneumophila* PLA candidate proteins

Mainly two approaches were employed in order to search for PLA candidate proteins. Since the biochemical screen which identified PlaB was conducted by a collaborating group (Klaus Heuner, Universität Würzburg), it will not be further discussed here. The first approach intended to identify putative lipolytic enzymes by screening the *L. pneumophila* genome for proteins containing domains of amino acid homology to known lipolytic families. By this method, two further members of the family of GDSL hydrolases were identified, which were designated PlaC and PlaD, to which the already characterized LPLA PlaA also belongs (75). Furthermore, a new bacterial group of putative lipolytic proteins was identified and designated patatin-like proteins (PLP) and the *L. pneumophila* Philadelphia-1 genome was found to encode eleven members of this family. The screening of the *L. pneumophila* Philadelphia-1 genome (although not completely sequenced at the beginning of this study) and other bacterial genomes was facilitated by the BLAST program provided by the NCBI server (http://www.ncbi.nlm.nih.gov/) and by the genome analysis tools, especially identification of PFAM domains, provided by the pedant web-site (http://pedant.gsf.de/) and PFAM website (http://www.sanger.ac.uk/Software/Pfam/) (38). It was found that the identification of a particular PAFM domain in a protein is a very good indication for its enzymatic function. The three *L. pneumophila* proteins PlaA, PlaC, and PlaD have a PFAM domain of GDSL hydrolases, and all three have been found to possess PLA and LPLA activities (Fig. 4.4, 4.6, 4.8, 4.15, 4.16) (75). *L. pneumophila* PatA and *P. aeruginosa* ExoU share a PFAM domain of patatin-like proteins and both have PLA and LPLA activities (Fig. 4.24, 4,27) (150, 174). However, in absence of a PFAM domain or when the particular group only consists of so far uncharacterized proteins a

BLAST analysis can give important clues as it looks for protein sequence similarities along the whole sequence and often finds relationships of a particular protein to proteins belonging to different enzyme classes. Homologous proteins which possessed relevant domains were then aligned by using the ClustalW method to enable detailed analysis of the homologous regions (figures 4.1, 4.18, Table 4.2).

In order to address secreted lipolytic enzymes which do not contain characteristic domains and as the *L. pneumophila* genome had not been completely sequenced at the beginning of this study a second approach was conducted as well. This approach consisted of a biochemical purification of PLA and LPLA activities from *L. pneumophila* culture supernatant followed by N-terminal protein sequencing. This method had previously led to the identification of the secreted lysophospholipase A, PlaA (73, 75). The method of N-terminal protein sequencing allows the sequencing of proteins from a PVDF membrane that may contain several proteins previously separated by electrophoresis. This is more convenient than the sequencing of a protein in solution as a liquid sample needs to be pure. However, in order to obtain an N-terminal sequence of sufficient length the sample on the PVDF membrane is required to contain a relative high amount of protein ideally 1 µg per lane. In this study, the best results were achieved when the samples from anion exchange chromatography were used for protein sequencing, because the amount of protein present in most of the bands was sufficient to yield N-terminal sequences of 10-18 amino acids length (Table 4.5). When the sample was subjected to a further purification step the amount of protein rapidly decreased (approximately 15-30-fold, see Table 4.4) and the only protein which could be identified from a gel filtration fraction was the zinc metalloprotease ProA which is the most abundant protein secreted by *L. pneumophila* (Fig. 4.36) (48, 59). Increasing the initial culture volume utilized for protein purification resulted in a predominant increase of the amount of ProA, because many of the protein bands then additionally contained the sequence of ProA (data not shown). Three PLA-candidate proteins were sequenced from anion exchange chromatography fractions containing high phospholipase A and lysophospholipase A activities: Unk1, LvrE, and Aas. Only one of these proteins, Aas, may directly contribute to the cell-associated PLA activity of *L. pneumophila* although LvrE indirectly influences the level of secreted PLA and LPLA activities of *L. pneumophila* (Fig. 4.58, 4.59, 4.61, 4.68). Since none of the secreted proteins identified by the genome screening of *L. pneumophila* were also found by N-terminal sequencing from *L. pneumophila* culture supernatant, it appears that in spite of high secreted PLA and LPLA activities the proteins responsible for these activities

occur in comparably low amounts which was indeed confirmed by analytical SDS PAGE (Fig. 4.36, 4.40, 4.49).

5.2 The *L. pneumophila* GDSL enzymes PlaC and PlaD

5.2.1 Regulation of gene expression

Gene expression analysis of *plaC* and *plaD* showed that both paralogs were expressed during extracellular bacterial growth. However, expression of *plaD* in BYE broth was found to be absent in strain 130b and the investigation of PlaD was therefore carried out in strain Corby were a *plaD* expression could be detected (Figures 4.2 & 4.14). An analysis of the upstream region of the *L. pneumophila* 130b *plaD* gene with the web-based program BPROM (www.softberry.com) showed that *plaD* possessed binding sites for four transcription factors, RpoD15, RopD16, Fur, and Crp. The RpoD transcription factor (sigma factor 70) family is known to control the expression of house keeping genes (122). The ferric uptake regulator Fur acts as a Fe^{2+}-dependent regulator of bacterial promoters and in *E. coli* and *Vibrio cholerae* Fur-controlled genes are expressed under low-iron conditions (52, 64, 121). The cyclic AMP receptor protein (CRP), finally, has been found to positively or negatively regulate the expression of many genes, e.g. the carbon regulon in *E. coli*, the hemolysin of *Vibrio vulnificus*, and the cholera toxin of *Vibrio cholerae*, in response to environmental signals like glucose availability which lead to the formation of cAMP by adenlyate cyclase (17, 26, 189, 222). *E. coli* and *Vibrio vulnificus* possess genes which are coordinately regulated by Crp and Fur (39, 222). Compared to strain 130b, the upstream region of the *plaD* gene in strain Corby owned binding sites for the same transcription factors with the exception of the binding site for Crp which was absent (data not shown). This means that the lack of *plaD* expression in strain 130b in rich broth can be attributed to the regulation by Crp. Therefore, it is possible that expression of the *plaD* gene in strain 130b is induced by limitation of the carbon source which in case of *L. pneumophila* is amino acids. As the three other strains Philadelphia-1, Paris, and Lens all have transcription sites for Crp, it appears that the Crp binding site might have been lost in strain Corby leading to a partial deregulation of *plaD* expression. *plaC* on the other hand was found to be expressed in rich broth in strain 130b as well as in strain Corby (Fig. 4.2 and data not shown). A recent study investigating the regulation of several secreted and cell-associated enzymatic activities, including PLA, LPLA, and glycerophospholipid:cholesterol acyltransferase (GCAT) activities, in the *L. pneumophila* strain JR32 showed that all these activities are regulated by the global

regulators RpoS and LetA which are known to induce the transmissive phenotype of *L. pneumophila* (29). The secreted GCAT activity in particular which is directly caused by PlaC was shown to be positively regulated by RopS and LetA in that study.

5.2.2 Mode of secretion of PlaC and PlaD by *L. penumophila*

L. pneumophila PlaC and PlaD are both secreted into the bacterial culture supernatant (Fig. 4.4, 4.15). PlaC is secreted by the *L. pneumophila* type II secretion system; because *L. pneumophila* type II secretion mutants lack GCAT activity and this activity cannot be restored by the addition of *L. pneumophila plaC* mutant culture supernatant containing all other type II secreted factors except PlaC (Fig. 4.7). Thus, PlaC is beside PlaA the second type II secreted GDSL enzyme of *L. pneumophila*. The observed loss of secreted PLA, LPLA, and GCAT activities of *L. pneumophila* type II secretion mutants might therefore be fully attributed to the lack of secreted PlaA and PlaC (9). *L. pneumophila* Corby *plaD* mutants display reduced PLA, LPLA, and lipase activities in their culture supernatant showing that PlaD is also secreted into the bacterial culture supernatant. Due to the absence of an N-terminal signal sequence, however, PlaD is not likely to be a substrate of the *L. pneumophila* type II secretion system. The extended C-terminus of the protein on the other hand renders PlaD to be a likely candidate for type IVB secretion, because this region is considered to be essential for *L. pneumophila* type IVB secretion (Fig. 4.1) (139). This possibility is supported by the finding that several residues are conserved among the C-terminal parts of PlaD and the type IVB secreted enzyme PatA, especially leucine and lysine residues, as seen by alignment of the two C-terminal regions by means of the web-based program MaliP (www.softberry.com) (data not shown) (187).

5.2.3 Enzymatic activities of *L. pneumophila* PlaC and PlaD

PlaC is responsible for the major secreted glycerophospholipid:cholesterol acyltransferase activity of *L. pneumophila* (Fig. 4.5). But it also possesses PLA, LPLA, and lipase activities in addition to its GCAT activity (Fig. 4.4, 4.6). GCAT activity of PlaC was found to be dependent on the presence of the zinc metalloprotease ProA indicating that ProA directly or indirectly activates PlaC (Fig. 4.7). Regarding its enzymatic activities, PlaC is more closely related to its ortholog *Aeromonas salmonicida* SatA than to its two paralogs. Both proteins display GCAT activity as well as PLA and LPLA activities (33). Moreover, both enzymes require activation by a protease. *A. salmonicida* SatA is activated by the AspA serine protease (91, 210). The mechanism of activation is currently not known. The GCAT activity of PlaC requires the presence of ProA which

is not a serine protease but a zinc metalloprotease with a glutamate in its active site (59, 130) (Fig. 4.7). Whether ProA directly activates PlaC and how the proteolytic cleavage leads to enhanced activity of PlaC has still to be determined. Moreover, *A. salmonicida* SatA has been shown to possess a broad substrate specificity accepting a variety of phospholipids as acyl donors but preferring short-chain or unsaturated fatty acids (31). The preferred acyl acceptor of *A. salmonicida* SatA is cholesterol but other steroids with a planar ring system and ß-hydroxyl group at the 3 position are as well suitable, even aliphatic alcohols were found to act as acyl acceptors (32). It was shown that PlaC accepts diacyl and monoacylphospholipids as acyl donors and cholesterol as acy acceptor (Figures 4.5 & 4.40-4.41). It was found, however, that the ergosterol in the membrane of *A. castellanii* is not esterified during *L. pneumophila* infection indicating that ergosterol is not suitable as an acceptor for PlaC (Fig. 4.12). PlaC has only weak phospholipase A activity when expressed in *E. coli*. The difference in substrate specificity between *plaC* expressed in *E. coli* and *L. pneumophila* can be attributed to the absence of *L. pneumophila* ProA and possibly other co-factors present in *Legionella*, but not in *E. coli*.

In contrast to PlaC, PlaD does not have GCAT activity, but only PLA and LPLA activities. Furthermore, a comparison of the enzymatic activities of PlaD and PlaA in strain Corby showed that they have similar activities (Fig 4.15). PlaA and PlaD predominantly possess LPLA activity and some lipase activity although they also contribute to the PLA activities of the wild type. It should be taken into account that the amount of fatty acids released from a diacylphospholipid is in the first step caused by PLA activity but in the second step it also arises from the hydrolysis of the resulting monoacylphospholipid by LPLA activity. It was furthermore observed in strain Corby that PlaA has stronger LPLA activities than PlaD (Fig. 4.14). However, the relative lipolytic activities of the *L. pneumophila* GDSL mutants may not represent the actual situation in the wild type, because it seems very likely that the lack of one of the GDSL hydrolases may be compensated by the upregulation of the others.

5.2.4 Identification of putative active site residues

L. pneumophila PlaA, PlaC, and PlaD belong to the family of GDSL hydrolases comprising plant and prokaryotic lipases, phospholipases, acyltransferases, and hemolysins. The structure of some GDSL-like enzymes have been elucidated such as the *Streptomyces scabies* esterase which forms a Ser-His dyad or the mammalian platelet-activating factor acetylhydrolase PAF-AH(Ib) which froms a Ser-His-Asp triad (93, 215). It was shown for the *Aeromonas hydrophila*

GCAT, which also is a member of the GDSL family, that the active site serine belonging to a catalytical triad is located in block I near the N-terminus and is embedded in the conserved motif G-X-S-X-S (30, 93). The other two members of the active site were proposed to be aspartic acid in block III located in the motif G-X-N-D and histidine in block V set in the motif F-X-D-X-X-H-P (30). However, elucidation of the crystal structure of several GDSL-like enzymes including the above mentioned platelet-activating factor PAF-AH(Ib) and *Aspergillus aculeatus* rhamnogalacturonan acetylesterase revealed that the active site aspartic acid is located in block V in close proximity to the histidine residue rather than in block III (93, 132). An alignment of PlaC and PlaD with *L. pneumophila* PlaA and SatA of *A. salmonicida* showed that the PlaC and PlaD sequences not only contain all five conserved blocks but also possess the three members of the putative catalytic triad in block I and V embedded in the respective conserved motifs (Fig. 4.1). Noticably, PlaC, PlaD, *Salmonella typhimurium* SseJ, and *A. salmonicida* GCAT possess the conserved aspartic acid motif in block III whereas PlaA lacks this motif (Fig. 4.1). In contrast to SatA of *A. salmonicida* and PlaC, which are acyltransferases with additional PLA and LPLA activities, PlaA only possesses LPLA activity and lacks GCAT activity (75). Whether *S. typhimurium* SseJ possesses GCAT activity has not been investigated so far. Yet, in spite of the presence of the conserved aspartic acid motif in its sequence, PlaD does not have GCAT activity (data not shown). The conserved aspartate has been found to stabilize the conformation of the enzyme by participating in several hydrogen bonds (132). Since it is present in most of the diverse GDSL enzymes and is only missing in some, e.g. in the esterase of *Streptomyces scabies*, the phospholipase from *Vibrio vulnificus*, and *L. pneumophila* PlaA (53, 75), it is difficult to predict its effect on the enzymatic activity without data on their acyltransferase activity. Furthermore, it is noticable that the distance between block I and II in the PlaC and PlaD sequence is more than twice as large as in most GDSL enzymes (Fig. 4.1) (30, 53, 75). In case of rhamnogalacturonan acetylesterase of *Aspergillus aculeatus*, the oxyanion of the transition state is stabilized by hydrogen bonds from the main chain NH groups of the active site serine in block I, the conserved glycine residue in block II and the side chain amide of the conserved asparagin in block III (132). Thus, a large distance between block I and II as present in the case of PlaC may allow the entrance of a larger molecule and might indicate that PlaC and PlaD could be able to hydrolyze voluminous substrates characterized by very long or unsaturated fatty acids, e.g. arachidonic acid. Another possibility is that the loop formed between block I and II could be involved in the multimerization of the enzyme as in case of PAF-AH(Ib) where the N-terminal α-helix preceding the first ß-strand is involved in dimer formation of the enzyme

(93). That PlaC might be a multimeric enzyme is suggested by the fact that after gel filtration of *L. pneumophila* culture supernatant, the fraction showing GCAT activity is eluted at a considerably higher molecular mass (248 and 275 kDa) than the approximately 50 kDa of the unprocessed monomer (Fig. 4.45 and 4.46). *A. hydrophila* GCAT is also found in a high molecular weight complex of over 500 kDa as estimated by gel filtration. This complex originates from the association of the enzyme with LPS-containing outer membrane vesicles rather than from a multimeric form (124, 125).

5.2.5 Role of PlaC and PlaD in intracellular infection

PlaC and PlaD were found not to be required for intracellular infection of *A. castellanii* and U937 macrophages in strains 130b and Corby and thereby resemble their paralog PlaA (Figures 4.10 & 4.17) (75). Likewise, it was shown that an *A. salmonicida satA* mutant does not display attenuated virulence. In spite of being a potent toxin, the enzyme is dispensable for the establishment of lethal acute furunculosis in Atlantic salmon (210). There are nevertheless indications, which especially link acyltransferase activity to bacterial virulence. *Staphylococcus aureus* and *Staphylococcus epidermidis* possess an acyltransferase (not belonging to the GDSL family) termed FAME, which neutralizes bactericidal fatty acids by binding them to cholesterol (36). Another putative acyltransferase belonging to the GDSL family is SseJ of *Salmonella typhimurium* a type III translocated effector with an essential role for *S. typhimurium* infection in the mouse model (146). *Salmonella typhimurium* is a pathogen that can invade non-phagocytic cells and replicate in a specialized vacuole (referred to as the *Salmonella*-containing vacuole). It was found that SseJ localizes with the *Salmonella*-containing vacuolar membrane and that it down-regulates the formation of tubular-extensions (*Salmonella*-induced filaments), which are induced by another type III secreted *Salmonella* effector termed SifA (167). Therefore it is assumed that SseJ opposes SifA function by the modification of lipid rafts on the vacuolar membrane by cholesterol esterification and thereby disturbs cell-signalling (146, 167). As the modification of the phagosomal membrane and the recruitment of host proteins are essential hallmarks during *L. pneumophila* intracellular replication, a similar regulatory role mediated by the modification of the phagosomal cholesterol could be assigned to the GCAT activity of PlaC (198). Here it was demonstrated that macrophages infected with *L. pneumophila* indeed accumulate cholesterol ester which is absent in uninfected macrophages (Fig. 4.12). However, this phenomenon was not dependent on the presence of PlaC, because infection with an *L. pneumophila plaC* mutant likewise resulted in the formation of cholesterol ester. A study

investigating the role of cholesterol esterification during the infection of the eukaryotic parasite *Toxoplasma gondii*, an intracellular pathogen of erythrocytes, showed that the formation of cholesterol ester was essential for the establishment of a successful infection and that it was the result of the pathogen's own enzymatic activity as well as an acyl-CoA:cholesterol acyltransferase (ACAT) activity of the host (191). Interestingly, ACAT is located in the ER and the *L. pneumophila* phagosome is known to associate with the host rough ER (166). Therefore, it might be possible that *L. pneumophila* is not solely dependent on its own GCAT activity for cholesterol esterification but might also exploit host enzymes. It should therefore be investigated, e.g. by employing inhibitors, whether the formation of cholesterol ester is essential for *L. pneumophila* infection. PlaC additionally shows a second activity in the presence of phospholipids and cholesterol which leads to the formation a so far unidentified compound, probably another cholesterol derivative (Fig. 4.5, 4.9, 4.45, 4.46). *L. pneumophila* PlaA, the major lysophospholipase A, is also able to form this compound in the presence a lysophospholipid and cholesterol as seen in lysophospholipase A active gel filtration chromatography fractions (Fig. 4.47). This furthermore shows the overlapping enzymatic functions of the GDSL enzymes. Based on the observed lack of *plaD* expression in strain *L. pneumophila* 130b during growth in rich broth and the presence of the binding site for the Crp transcription factor, it can be postulated that *plaD* expression is induced under deprivation of nutrients. Since *L. pneumophila* is likely to encounter such conditions in the host phagosome, *plaD* expression might be induced during intracellular growth. The observation that PlaD is nevertheless not required for intracellular infection suggests that it might predominantly function as a backup enzyme for the loss of PlaA or PlaC. Therefore, another important aspect for further studies would be the investigation of double and triple knockout mutants of the GDSL enzymes in infection models, as it seems likely that due to overlapping enzymatic activities the three *L. pneumophila* GDSL enzymes might replace each other (204).

5.3 The *L. pneumophila* lipolytic enzyme PlaB

L. pneumophila possesses high phospholipase A and lysophospholipase A activities associated with the bacterial cell. Notably, almost all of that *L. pneumophila* PLA/LPLA activity resulted from a single enzyme, because mutants of this protein only retained a very weak MPLPG-hydrolyzing activity in their cell lysate (Fig. 4.18). This enzyme, initially identified by Klaus Heuner (Universität Würzburg) due to its hemolytic activity, was designated PlaB (76).

5.3.1 Classification of *L. pneumophila* PlaB

Although the PlaB sequence did not possess any PFAM domains above the threshold level (expect value = 1) (www.sanger.ac.uk/Software/Pfam/) a secondary-structure prediction (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY) of PlaB showed that the first 138 amino acids of the N-terminal part of the 474 amino acid long PlaB sequence adopt an α/β-hydrolase fold which is then discontinued. The α/β-hydrolase fold is characteristic for a variety of hydrolytic enzymes. This suggests that the active site residues involved in the PLA and LPLA activities of PlaB are located at the N-terminus while the C-terminus might be nessecary for targeting the protein to a specific location. The *L. pneumophila* PlaB protein sequence displays 41 % similarity to the sequence of the *L. pneumophila* lipase LipB. The whole LipB sequence with the exception of the first 56 N-terminal amino acids (probably belonging to the signal sequence) is predicted to adopt the α/β-hydrolase fold. Moreover, LipB possesses two overlapping PFAM domains with similar enzymatic activities: a domain of putative serine esterases (DUF676) and a domain of a PGAP1-like protein (www.sanger.ac.uk/Software/Pfam/). Based on the alignment of PlaB with LipB and human PGAP1, PlaB can be classified as a serine hydrolase consisting of a putative catalytical dyad of serine and aspartate (in block II and IV, respectively) (Fig. 4.18). PGAP1 is an ER membrane protein which deacylates the inositol moiety of glycosylphosphatidylinositol (GPI)-achored proteins, a process found to be necessary for the transport of GPI-anchored proteins from the ER to the Golgi body (202). Thus, the presence of the PGAP1 domain implicates a role for PlaB and LipB in host protein trafficking.

5.3.2. Enzymatic activities of PlaB

PlaB has PLA and LPLA activities, and is able to hydrolyze 1-monopalmitoylglycerol but not the more hydrophobic di-and triacylglycerols indicating that it is not a lipase (Fig. 4.19). Its paralog LipB on the other hand is known as a secreted lipase due to the observation that the culture supernatants of *L. pneumophila lipB* mutants show a reduced ability to hydrolyze tricaprylin, a substitute for triacylglycerol substrates (10). PlaB was further found to influence phospholipid catabolism, because in contrast to the 130b wild type osmotic shockate fractions of *plaB* mutants were defective for the formation of DPPG and DPPE by addition of MPLPG and fatty acids. This defect appears to be distinct from the loss of PLA and LPLA activities in the same mutants which was found in the osmotic lysate fraction instead of the shockate. It might nevertheless be possible that the osmotic shockate fraction contained trace amounts of PlaB

which hydrolyzed MPLPG to fatty acid and glycerophosphorylglycerol. Glycerophosphorylglycerol might have been hydrolyzed to glycerol-3-phosphate by an *L. pneumophila* phospholipase D thereby providing precursors for other enzymes involved in lipid catabolism. Since the membrane lipid composition of *plaB* mutants is similar to that of the 130b wild type, the conditions at which the defect of the *plaB* mutants to generate DPPG and DPPE was observed might not be physiologically relevant (data not shown).

5.3.3 Localization of PlaB

The reduced contact-dependent hemolysis of *L. pneumophila plaB* mutants suggests that PlaB is present in the *L. pneumophila* outer membrane (76). The lack of a signal peptide in the PlaB sequence indicates that PlaB is probably secreted by the type I or type IVB secretion system. However, when *L. pneumophila* cells were subjected to osmotic shock, the cell associated PLA/LPLA activity representing PlaB was not found in the osmotic shockate which is generally considered to contain mainly periplasmic and outer membrane proteins but was found in the remaining fraction assumed to predominantly contain cytoplasmic and inner membrane proteins (Fig. 4.69, 4.70). Since the two osmotic shock fractions were not analyzed for the presence of specific periplasmic and cytoplasmic marker proteins, it is not certain whether the two fractions really consisted of mainly periplasmic and cytoplasmic proteins. Although the PlaB-associated activity was found in the "cytoplasmic" fraction, the "periplasmic" fraction of the *plaB* mutant displayed a defect in the synthesis of DPPG and DPPE in the presence of MPLPE and FFA (4.70). Apart from the possibility that the "periplasmic" fraction contained trace amounts of PlaB one other explanation might be that the protein consists of two subunits with different localizations. These results would then suggest that the subunit with the PLA activity (comprising the N-terminal part of the protein) is located in the bacterial cytoplasm or inner membrane while the second subunit involved in the bacterial phospholipid catabolism is located in the periplasm or outer membrane and that the two subunits might have been separated during the experimental procedure. As mentioned above, an alignment of PlaB with the lipase LipB showed that the homologous region only included the N-terminal 168 amino acids of the PlaB sequence which furthermore suggests the existence of two domains (Fig. 4.17). When the sequences of the two putative PlaB subunits were separately used for the prediction of the cellular localization, the N-terminal part (amino acids 1-170) containing the lipolytic domains was predicted to be located in the cytoplasm while the C-terminal part was predicted to be secreted implying that the C-terminal part contained a secretion signal (www.softberry.com).

5.4 The *L. pneumophila* Patatin-like proteins

In the present investigation, a new group of bacterial lipolytic enzymes was identified and the members of this group were found to possess four blocks of amino acid homology which did not resemble the conserved domains so far found in other bacterial lipolytic proteins (11). Since these conserved domains were similar to those of the potato storage glycoprotein patatin, the proteins were designated patatin-like proteins (PLPs). It was shown that the genome of *L. pneumophila* Philadelphia-1 codes for eleven PLP genes. These PLP genes possess all four domains of bacterial PLPs. The three sequenced genomes of the *L. pneumophila* strains Philadelphia-1, Paris, and Lens possessed eleven, eleven, and ten PLP genes, respectively. Although the genomes of three *L. pneumophila* strains have been completed and analyzed, only two of the *L. pneumophila* Philadelphia-1 PLPs have been annotated as patatin-like proteins (PatB/gi 52843003 and PatC/gi 52842619) and an additional one has been annotated as a putative phosphoesterase (PatH/gi 52841187), showing the limited overall protein homology of this group (http://genome3.cpmc.columbia.edu/~legion/) (Table 4.1) (35, 38).

5.4.1 Correlation of the number of patain-like proteins with bacterial pathogenicity

With eleven PLP genes, the *L. pneumophila* genome encodes the highest number of patatin homologues among the 216 screened bacterial genomes (Table 4.1). An analysis of the number of PLP genes among the bacteria with at least one PLP gene showed that pathogenic and symbiotic bacteria encoded a higher number of such genes compared to non-pathogens especially if the number of PLP genes was put into relationship to the total number of open reading frames (which enables an assessment of the importance of PLPs among the whole enzymatic machinery of a bacterium). This finding suggests that PLPs might be involved in bacterial virulence or host-pathogen interaction processes. Since other pathogenic bacteria, e.g. *Bacillus anthracis* Ames or *Yersinia pestis* possess a comparatively low number of PLPs (0.56/1000 ORF and 0.51/1000 ORF, respectively), PLPs seem to be important for specific lifestyles of bacteria, in particular for some intracellularly replicating bacteria (Table 4.1). For instance, *Mycobacterium tuberculosis* which is like *L. pneumophila* an intracellular pathogen causing pneumonia also possesses a high number of PLPs (2.04/1000 ORF). No studies were found which investigated the phospholipase A activity of *M. tuberculosis* but phospholipase A activity has been reported for other *Mycobacteria* species, in particular *Mycobacterium leprae*

(possesses 1 PLP gene, Table 4.1) (217). Even *Rickettsia prowazekii* which, as an obligate intracellular pathogen, has a reduced genome of only 834 ORFs, possesses 2 PLPs (2.40/1000 ORF) (Table 4.1). *Rickettsia* species cause a number of tropical diseases like typhus and the bacterial PLA activity is considered to promote the invasion of host cells (175, 188). So far, the protein which causes the PLA activity has not been identified therefore the *Rickettsial* PLPs identified here seem likely candidates for being responsible for this activity. *Coxiella burnettii* and *Chlamydia* sp. on the other hand which are also intracellular pathogens do not have any PLPs which might indicate differences in the lifestyles of the bacteria compared to *L. pneumophila*. A recent study provides an explanation for the lack of PLPs in some parasitic bacteria. This study investigated the role of an inducible patatin-like protein, PLP2, in *Arabidopsis* and demonstrated that PLP2 was upregulated during challenge with fungal and bacterial pathogens and that PLP2 activity promoted parasite colonization of the plant (113). Therefore, it seems likely that those pathogens, which do not have their own PLPs, might exploit host phospholipases for their needs.

5.4.2 Are *L. pneumophila* patatin-like proteins candidates for type IVB export?

P. aeruginosa ExoU, the first identified bacterial PLP, is directly injected into the host cell via a type III secretion system where it is activated by a so far unidentified eucaryotic factor and leads to acute lung injury and death due to its PLA/LPLA activity (4, 69, 148, 174, 201). A recent investigation by Rabin and colleagues revealed that a C-terminal part of ExoU comprising amino acids 550-687, i.e. outside of the catalytical domains (Table 4.2), directs ExoU to the plasma membrane of eukaryotic cells (158). An investigation by Shohdy and colleagues screening for substrates of the *L. pneumophila* type IVB secretion system identified among two other *L. pneumophila* proteins a protein named VipD which is identical to the here described PatA as a type IVB secretion substrate (187). In that study, Shohdy and colleagues demonstrated that the C-terminal part of PatA/VipD is essential for translocation by the type IVB secretion system and leads to a defect in vacuolar traffic in yeast (187). This implies that the C-terminal part of PatA/VipD might be likewise important for targeting the protein within the host cell. This is in accordance with another study which showed that a hydrophobic residue at the -3 or -4 position from the C-terminal amino acid is conserved among a number of known substrates of the *L. pneumophila* Dot/Icm secretion system (139). And indeed the type IVB translocated PatA/VipD contains a hydrophobic phenylalanine residue at position -4. In order to find out whether any of the ten paralogs of PatA/VipD might be candidates for type IVB secretion, the proteins were

classified into two groups according to the length of the C-terminal extension after the catalytical domain and additionally analyzed for the presence of a hydrophobic C-terminal residue (Table 4.2). Based on the length of the C-terminus, PatC, PatF, and PatG were classified into the same group as PatA. The C-terminal extensions of this group, however, did not possess protein homology to each other or to any known proteins or catalytic domains. Furthermore, in addition to PatA, only PatB, PatG, PatH, and PatJ possess a hydrophobic residue at the -3 or -4 position. In conclusion, PatG is the only PatA paralog with an extended C-terminus (>200 amino acids) and a hydrophobic C-terminal amino acid and thus fulfils these two criteria for possibly being a substrate of the Dot/Icm type IVB secretion system. The *patA* locus of strains Philadelphia-1 and Lens encodes in addition to PatA two more proteins; the type IVB secreted protein SidH and a eukaryotic-like U-box protein (35, 123). SidH possesses a hydrophobic isoleucine residue at position -4 and the U-box protein in the *patA* locus contains a hydrophobic methionine residue at position -3. Therefore, the U-box protein might be a further candidate for type IVB secretion. In eucaryots, U-box proteins catalyze the ubiquitylation of proteins and thereby mark them for degradation in the lysosome (86).

5.4.3 Comparison of *L. pneumophila* PatA with *P. aeruginosa* ExoU

It was shown that PatA possessed phospholipase A and lysophospholipase A activities (Fig. 4.24, 4.27). The PatA ortholog *P. aeruginosa* ExoU also possesses PLA and LPLA activities as well as the *Solanum tuberosum* patatin isozym PatB2 (92, 201). The lipolytic activities of ExoU are activated by an unknown eukaryotic factor (150, 174). The lipolytic activity of PatA was not activated by a factor present in yeast or monocyte lysates suggesting that unlike *P. aeruginosa* ExoU PatA does not require a eukaryotic factor (4.26, 4.27). Based on the observation that PatA only shows distinct PLA activity when it is expressed in *L. pneumophila* and has predominantly LPLA activity in *E. coli*, a *Legionella* factor might be required for the activity of PatA (Fig. 4.24, 4.27). This factor has not been further investigated in this study and therefore no further information on this factor presently exists. It might be speculated that similar to PlaC, PatA is activated by the zinc metalloprotease ProA which would enable the bacterium to co-ordinately regulate the activity of its type II and type IVB secreted PLAs. The biochemical purification of *L. pneumophila* culture supernatant consistently revealed the presence of a 70 kDa protein in fractions showing PLA and LPLA activities which well matches the molecular mass of unprocessed PatA (Figures 4.32, 4.33, 4.35, 4.45) suggesting that PatA is exported into the bacterial culture supernatant in the absence of a host cell. All isolated *L. pneumophila patA*

mutants were severely defective for intracellular replication in amoeba and macrophages (Fig. 4.28) indicating that similar to *P. aeruginosa* ExoU PatA might be an essential virulence factor. This finding would match the fact that PatA is a substrate of the *L. pneumophila* Dot/Icm secretion system which predominantly translocates effectors that mediate intracellular replication (187). Yet, the growth defect of the *patA* mutants could not be complemented by introducing the *patA* gene in *trans*. Since it is possible that a specific protein level of PatA and/or a specific regulation of its gene expression are crucial for the infection process, the reintroduction of an intact *patA* gene into the genome of an *L. pneumophila patA* mutant might lead to the recovery of virulence. A recent study showed that *P. earuginosa* ExoU releases arachidonic acid from the membrane of endothelial cells and in this way leads to the overproduction of prostacyclin and prostaglandin D in these cells which in turn induce inflammatory processes (171). It should be noted that *L. pneumophila* does not only replicate in macrophage cells but also exploits alveolar epithelial cells for growth. Therefore, it might be possible that PatA could in a similar way mediate inflammation in the lung tissue which would then result in the attraction of macrophages to these sites. The induction of prostaglandin production in the macrophage host on the other hand would lead to a deactivation of macrophage defense mechanisms by the induction of anti-infammatory cytokines and the inhibition of pro-inflammatory cytokine production. This is a strategy used by *Salmonella enterica* Serovar Typhimurium (by the induction of macrophage cycloogycenase 2 expression) to downregulate macrophage activity (207). This means that PatA and the other *L. pneumophila* PLPs might enable the bacterium to differentially modulate the immune response. Since this study did not investigate the capacity of *L. pneumophila* PatA to release arachidonic acid from host cells and the induction of the arachidonic acid cascade, this would be an interesting point for future investigations (Fig. 1.9). These might also examine the capacity of *patA* mutants to infect epithelial cells. Another important issue of future investigations would be to find the *Legionella* factor that is required for PatA activity. The characterization of the remaining 10 *L. pneumophila* PLPs also represents an important future task.

5.5 Proteins identified by N-terminal sequencing of chromatographic fractions containing PLA

Three PLA candidate proteins were identified by biochemical protein purification of secreted PLA activity followed by N-terminal protein sequencing.

5.5.1 *L. pneumophila* Unk1

The first protein, designated Unk1, displayed 37% sequence similarity to a lipase, Lip3, of *M. pneumoniae*. However, the analysis of *L. pneumophila unk1* mutants and expression of *unk1* in *E. coli* only suggests if at all weak esterase and PLA activities (Fig.4.52, 4.53, 4.54). This lack of lipolytic properties is in accordance with the finding that Unk1 lacks the lipase motif which comprises the active site serine (Fig. 4.50). Furthermore, unlike *M. pneumoniae* Lip3 which displays a α/ β-hydrolase fold Unk1 lacks this typical fold of hydrolytic enzymes further suggesting that it may not possess hydrolytic properties. Due to the similarity between the protein sequences of Unk1 and the lipase of *M. pneumoniae*, it could be speculated that Unk1 once indeed possessed lipolytic properties which were weakened by a mutation in the lipase motif (Fig. 4.50). Initial experiments with *unk1* over-expressed in *L. pneumophila* 130b support this hypothesis, because there was a trend of increased phospholipase A activity in these clones (data not shown). This could possibly mean that Unk1 is a weak phospholipase A which requires a *Legionella* factor for its activity. Due to the lack of significant homology to any characterized protein and the lack of any PFAM domain it is not possible to speculate on any other enzymatic function of Unk1. The paralog of Unk1 which is encoded in the *L. pneumophila* genome shares 50% sequence identity with Unk1 but likewise lacks homology to other known proteins. Only three *L. pneumophila unk1* mutants were obtained by the method of homologous recombination and all three mutants showed different restriction patterns as found by Southern blot analysis (see Appendix). This could have resulted from the mutagenesis process which might have lead to alterations in the sites recognized by the employed restriction endonuclease (EcoRV). Southern blot analysis furthermore showed that all three mutant clones had integrated the Km^R gene cassette into their genomes. Only one of the mutants (unk1- cl1) had correctly inserted this cassette into the *unk1* gene. Like the wild type, this mutant showed a single *unk1* fragment but this fragment was approximately 1 kb larger than that of the wild type indicating the insertion of the resistance cassette (see Appendix). RT-PCR analysis furthermore showed the absence of an *unk1* transcript in this clone, confirming that unk1- cl1 was a true *unk1* mutant. Although the two other mutants (unk1- cl2 and cl3) showed an *unk1* fragment that was larger than the wild type fragment they additionally showed a fragment which corresponded or was similar in size to the wild type *unk1* indicating that they might be merodiploids (having both the wild type and the mutant gene). Indeed, a RT-PCR analysis of the *unk1* expression of unk1- cl2 revealed the presence of an *unk1* transcript thereby confirming the results from Southern blot analysis and corroborating that it is a merodiploid. In spite of *unk1* expression in this

merodiploid, it also showed a weak reduction in its phospholipase A and lysophospholipase A activities and both defects were complemented by introduction of the *unk1* gene in *trans* (Fig. 4.52). One explanation might be that the amount of Unk1 protein in the merodiploid was lower than in the wild type. It was also shown that Unk1 contributes to the intracellular replication of *L. pneumophila* 130b (Fig. 4.56). The mutant unk1- cl2 even showed a stronger growth defect than unk1- cl1 and unk1- cl3. However, complementation of the infection defect in any of the three *L. pneumophila* 130b *unk1* mutants was not achieved by the introduction of *unk1* including its native promoter region in *trans* (Fig. 4.56). Yet, the fact that only mutants with an infection defect were isolated strongly suggests that this defect is not due to a second site mutation or favours a specific second site mutation.

5.5.2 *L. pneumophila* LvrE

The second PLA candidate protein, LvrE, is encoded by a gene which is located in the genomic region of the *L. pneumophila* Philadelphia-1 Lvh type IVA secretion system but probably is not functionally associated with this system (172). LvrE was recently identified as a Tat secretion substrate (54). The Tat machinery is distinguished by the fact that it transports folded proteins across the inner membrane. Generally Tat substrates are characterized by two successive arginine residues at their N-terminus. There are, however, exceptions and LvrE is such an exception as it does not have the twin arginine motif in its N-terminal signal sequence (54). Since many Tat substrates require a metal ion cofactor and are transported with this cofactor, it might be possible that LvrE requires a cofactor as well. In this study, the effect of a mutation in the *lvrE* gene was studied in two *L. pneumophila* strains in parallel. The reason for this was based on earlier reports concerning the *lvh* lokus (which includes *lvrE*) one of which not only revealed high variation of this lokus within different *L. pneumophila* strains but also stated the existence of two *lvrE* copies in strain 130b, i.e. the strain from which the LvrE protein had been sequenced (28, 172, 180). Southern blot analysis of 130b wild type and Philadelphia-1 with an *lvrE* probe confirmed the observations made in this earlier report by Samrakandi and collegues, because the 130b wild type showed at least four fragments that bound to the probe while the Philadelphia-1 wild type displayed only one fragment indicating that strain 130b might have a second copy of the *lvrE* gene (see Appendix) (172). Since strain Philadelphia-1 seemed to have only one *lvrE* copy, this strain was additionally used for *lvrE* knockout studies presuming that a possible defect might be more readily detectable in this strain. Four *lvrE* mutants were obtained in strain Philadelphia-1. Southern blot analysis showed that all four had integrated the Km^R

cassette into the chromosomal *lvrE* gene and RT-PCR confirmed the absence of an *LvrE* transcript during early logarithmic growth phase (see Appendix and data not shown). It should still be noted that clone lvrE- 16 and lvrE- 23 showed one and two additional fragments that hybridized with the Km^R cassette probe (Appendix). *L. pneumophila* Philadelphia-1 *lvrE* mutants showed reduced PLA, LPLA, and lipase activities in their culture supernatant during early logarithmic growth phase while during late logarithmic growth phase the lipolytic activites were no more reduced but rather displayed a tendency of increased PLA and LPLA activities (Fig. 4.58). Initially, only two *lvrE* 130b mutants, lvrE- 1.1 and lvrE- 2.1 were obtained and most of the studies presented here were carried out with these two mutants (two further mutants were obtained later by a second mutagenesis experiment). All four 130b *lvrE* mutants displayed identical hybridization patterns with the *lvrE* and Km^R cassette probes as found by Southern blot analysis (see Appendix). All of them furthermore displayed two *lvrE* bands in the Southern blot indicating that they possessed a wild type and a mutant *lvrE* gene. However, RT-PCR analysis of the initial two clones, lvrE- 1.1 and lvrE- 2.1 revealed that the former clone no longer expressed a functional *lvrE* gene while the latter clone as would be expected from the Southern blot analysis still showed an *lvrE* transcript. In spite of an *lvrE* transcript the mutant lvrE- 2.1 showed altered lipolytic activites although the difference was perhaps not as strong as in the mutant lvrE- 1.1. The effect of a mutation in the *lvrE* gene in strain 130b lead to a decrease in PLA activity and to an increae in LPLA activity during early logarithmic growth phase which were not so pronaunced but still detectable during late logarithmic growth phase (Fig. 4.59). Overexpression experiments in *E. coli* revealed that no increased PLA activity could be conferred to *E. coli* clones expressing the *L. pneumophila* Philadelphia-1 *lvrE* gene (Fig. 4.61, 4.62). Even the addition of *L. pneumophila lvrE* mutant's culture supernatant to supply the LvrE protein with possibly lacking cofactors did not lead to enhanced lipolytic activities of *E. coli* clones harbouring the *lvrE* gene indicating that the altered lipolytic properties of the *L. pneumophila lvrE* mutants resulted from indirect effects (data not shown). *E. coli* expressing *lvrE* in *trans* showed reduced cell-associated LPLA activity which correlates with the increased LPLA activity of the *L. pneumophila lvrE* mutants further showing that LvrE inhibits LPLA activity (Fig. 4.61). Since the experiments indicated an indirect and perhaps regulatory effect of LvrE on the secreted PLA and LPLA activites, further experiments addressed the possibility that LvrE might be a protein phosphatase as was suggested by the partial protein sequence homology to a bacterial protein phosphatase. No reduced phosphatase activites were detected in 130b and Philadelphia-1 *lvrE* mutants during mid logarithmic growth phase suggesting that LvrE is not a phosphatase

(Fig. 4.60). This finding was supported by an initial experiment with *E. coli* clones over expressing the Philadelphia-1 *lvrE* gene (data not shown). Thus, it is presently not clear how LvrE influences the secreted PLA and LPLA activity of *L. pneumophila*. As LvrE also possesses partial sequence homology to porins, it might be speculated that LvrE is a secreted porin similar to that found in culture supernatant of *Borrelia borgdorferi* (45). Nothing is known so far about the role of this secreted *Borrelia* porin. However, normally porins enable the transfer of small molecules through the cell wall and are also known to mediate susceptibility to antibiotics (126, 192). Then LvrE (provided that some of the protein is located in the membrane) might mediate the influx of factors which influence *L. pneumophila* PLA and LPLA activities. For *M. tuberculosis*, it was shown that a porin mutant grew better in infection models than the wild type (186). The reason for this is the fact that porins are the only gateway for soluble factors to penetrate the thick *M. tuberculosis* membrane. Indeed, one of the *L. pneumophila* Philadelphia-1 *lvrE* mutants (clone 16) as well as the 130b mutant lvrE 2.1 showed a better infection capability than the wild type (Fig. 4.63 and data not shown). Furthermore, it was observed that over expression of *lvrE* in the *L. pneumophila* 130b *lvrE* mutants resulted in a more rapid consumption by amoebae while Philadelphia-1 *lvrE* mutants were unsusceptible to amoebal consumption (Fig. 4.66). This observation could again be explained by higher or lower membrane permeability due to the presence or absence of porins in the bacterial membrane.

5.5.3 *L. pneumophila* Aas

5.5.3.1 Localization of Aas

The third protein which was identified by N-terminal protein sequencing is *L. pneumophila* Aas, a protein owning a PFAM domain for acyltransferases which transfer fatty acids to lysophospholipids (www.sanger.ac.uk/Software/Pfam/). The Aas ortholog in *E. coli* consists of two subunits, an acyl carrier subunit which binds free fatty acids which are subsequently transferred to the second subunit which binds the lysophospholipids (105). *E. coli* Aas is an inner membrane enzyme (102). When the Aas protein sequence was analyzed for the prediction of transmembrane helices using the web-based tool TMpred (ch.embnet.org), the Aas protein was predicted to have seven strong transmembrane helices between amino acids 1-46 with the N-terminus outside of the membrane and the C-terminus inside. When the Aas subcellular localization was predicted using the program psortb (www.psort.org) the protein was predicted to be localized in the bacterial cytoplasmic membrane. *aas* mutants of *L. pneumophila* show a

reduction in their PLA and LPLA activities which are associated with the bacterial cell suggesting that Aas in *L. pneumophila* is also a cell-bound enzyme (Fig. 4.68). Still, part of this protein was found in the bacterial culture supernatant and was sequenced from anion exchange fractions with PLA activity (Table 4.5). The Aas fragment starting from the sequenced N-terminus (sequenced from position 481) has a predicted isoelectric point of approximately 5 so that the fragment would bind to an anion exchange column at pH 7.5. In contrast, the whole Aas protein is predicted to have an isoelectric point of 8 so that it would not have bound to an anion exchange column at pH 7.5. Since the fragment starting from position 481 only contains part of the AMP binding domain (consisting of amino acids 248-657) but not the catalytic domain, it is unlikely that it retained enzymatic function (Fig. 67). Therefore the fragment might have been accidentally generated during the purification process by proteolysis in spite of the addition of EDTA to inhibit ProA activity. Alternatively, as the experimentally found N-terminal protein fragment which was used for a BLAST search against the *L. pneumophila* Philadelphia-1 genome showed some ambiguity (the ambiguous amino acids are indicated in brackets, see Table 4.5), it is possible that the assignment to the Aas sequence was not correct. Due to the acyltransferase domain present in the *L. pneumophila* Aas sequence its enzymatic activites were further characterized.

5.5.3.2 Function of Aas

L. pneumophila aas mutants had an increased amount of lysophosphatidylcholine in their cell membrane and reduced amounts of phosphatidylcholine and phosphatidylethanolamine which points at the role of Aas in the acylation of lysophospholipids (Fig. 4.71). This finding is in accordance with the results from Hsu and coworkers which showed that *E. coli aas* mutants accumulated lysophosphaditylethanolamine in their membrane (102). Although the physiological function of *E. coli* Aas is supposed to be the generation of DPPE from MLPLE, it was found that the enzyme also accepts the non-physiologic substrate MPLPC and generates DPPC from it (103). Unlike *E. coli*, *L. pneumophila* contains DPPC in its membrane as well as DPPE and DPPG (70). It was found that DPPG was present in much lower amount in the *L. pneumophila* membrane than DPPE and DPPC (Fig. 4.71) (70). Therefore, MPLPG was chosen as a substrate for the assessment of *L. pneumophila* acyltransferase activity supposing that an increase in the amount of DPPG during the reaction would be more distinctly detected by thin layer chromatography due to the low DPPG background. As the Aas protein was predicted to be localized in the cytoplasmic membrane, *L. pneumophila* were lysed by osmitoc shock which is

considered to release periplasmic proteins into the supernatant (osmotic shockate) and thereby separates them from the cytoplasmic proteins which remain in the lysate (156). The acyltransferase activity of Aas was assessed by separately incubating the *L. pneumophila* osmotick lysate and shockate fractions with fatty acids and MPLPG in the presence of ATP and Mg^{2+} and by analyzing them for the (increased) formation of DPPG (Fig. 4.69 and 4.70). However, no phospholipids were detected in the reaction mixture containing lysates from 130b wild type and *aas* mutants after the incubations (Fig. 4.69). The most obvious reason for the loss of membrane lipids was the disturbing activity of the cell-associated PLA/LPLA PlaB which hydrolyzed virtually all phospholipids present in the reaction. Therefore *plaB/aas* double mutants were constructed and the osmotick lysates were analyzed (Fig. 4.69). Still there was no qualitative difference in the membrane phospholipids detectable between *plaB* single and *plaB/aas* double mutants (Fig. 4.69). In both cases, all lipids found in the single mutant were also found in the double mutants and no increase in the amount of DPPG was detected suggesting the absence of 2-acylglycerophospholipid acyltransferase activity under the chosen conditions (Fig. 4.69). The osmotick shockate fraction of the 130b wild type on the other hand showed the formation of DPPG in the presence of MPLPG and FFA indicating acyltransferase activity (Fig. 4.70). Since the Aas mutants also showed the capacity to generate DPPG under the same conditions, the observed acyltransferase activity cannot be attributed to Aas (Fig. 4.70). A Blast search of the *L. pneumophila* genome with the Aas protein sequence yielded four homologous proteins (expect values$<6\times10^{-6}$) (gi52841784, gi52842443, gi52840382, gi52841361) with a predicted function in lipid metabolism, i.e. acyl coenzyme A ligases. Consequently, it seems probable that *L. pneumophila* encodes one or more paralogous proteins which might have replaced the function of Aas. Therefore, a more specific approach for the detection of the acyltransferase activity of Aas is required, e.g. the incorporation of labelled fatty acids (e.g. fluorescent labelled) into lysophospholipids preferably in the *plaB/ aas* double mutant. On the other hand it might be possible that Aas requires a different acceptor molecule for its acyltransferase activity and/or different reaction condtions. The observation that compared to the 130b wild type *L. pneumophila aas* mutants have a small reduction (approximately 10 %, see Fig. 4.68) in their ability to hydrolyze mono-and diacylphospholipids indicates that Aas might act as a weak PLA/LPLA in the absence of an acceptor or might somehow be required for the activity of PlaB. Therefore, it might also be possible that Aas does not have acyltransferase activity but only contributes to PLA and LPLA acivities although the presence of the conserved catalytic acyltransferase domains strongly suggests otherwise. A very notable feature of

L. pneumophila Aas is its contribution to the detoxification of lysophosphatidylcholine. *L. pneumophila aas* mutants were approximately 100-fold more susceptible to the cytolytic effects of this lysophospholipid than the wild type (Fig. 4.72). Aas could neutralize lysophospholipids by turning them to diacylphospholipids by acylation or alternatively by hydrolyzing them to glycerophosphorylcholine as has been shown for *L. penumophila* PlaA (75). Whether *E. coli* Aas might play a similar role in detoxification has not been investigated so far but it has been already suggested (87). The analysis of the cell membranes of the wild type and the *L. pneumophila aas* mutants following incubation with 0.2 mM MPLPC showed that in contrast to the wild type, the *aas* mutants accumulated fatty acids in their membrane (Fig. 4.73). This finding indicates that the increased amounts of fatty acids formed by the hydrolysis of MPLPC through the action of *L. pneumophila* lysophospholipases A were taken up by the bacteria. While the wild type seems to effectively dispose of the excess fatty acids, the *L. pneumophila aas* mutants accumulate these suggesting that Aas might act as an important pathway for the utilization of exogenous fatty acids. Indeed, it has been shown that *E. coli* is able to incorporate fatty acids and lysophospholipids which are present outside of the cell by the action of Aas (102).

5.5.3.3 Role of Aas in intracellular infection

Finally, the role of Aas during *L. pneumophila* intracellular infection was assessed in the three host models U937 macrophages, A549 epithelial cells, and *A. castellanii* amoebae, and Aas was found to be dispensable, because *L. pneumophila aas* mutants showed the same increase in CFU in all three hosts during 72 h of infection as the wild type (Fig. 4.74, 4.75). Since A549 epithelial cells are known to secrete surfactant lipids which are cleaved to lysophospholipids by the action of *L. pneumophila* PLA it was hypothesized that the lack of Aas might indirectly affect the viability of the bacteria by the higher susceptibility of the *L. pneumophila aas* mutants to MPLPC (117, 142). Yet, no infection defect of the *L. pneumophila* 130b *aas* mutants was observed in the A549 infection model. The amount of surfactant produced by the used A459 cells has not been determined so that it might have been too low to result in the accumulation of sufficient cytolytic MPLPC. In spite of the dispensability of *L. pneumophila* Aas in the three employed infection models, it is likely that the enzymatic functions of Aas might be of more impact *in vivo*, because an unbalanced lipid composition of the bacterial membrane can render the bacterium more susceptible to host defence mechanisms.

5.6 Conclusion

The present study identified seven so far uncharacterized *L. pneumophila* proteins and investigated their phospholipase A, lysophospholipase A, and lipase activities. Five of the seven enzymes were found to possess such lipolytic activities. Although only one of the newly identified *L. pneumophila* phospholipases A, which is PatA, contributed to the bacterial infection process, the high number of predominantly secreted phospholipases A and lysophospholipases A indicates an important role for this class of enzymes in the bacterial life cycle. These enzymes might not only serve the bacterium to derive nutrients for a favourable growth but may also enable *L. pneumophila* to adapt to diverse hosts and environmental niches, as it is capable to colonize a variety of protozoa and different mammalian cells (macrophages, epithelial cells). As a conclusion, this study has provided a deeper understanding of the major *L. pneumophila* phospholipid degrading enzymes and a basis for future more detailed investigations of these important enzymes.