2. Introduction

2.1 Importance of *Legionella*

Legionella is a genus of Gram-negative bacteria ubiquitous to fresh-water environments. They are facultative intracellular bacteria which can replicate in protozoa and mammalian cells (67). Today, there are 48 identified species with more than 70 serogroups. Notably, the species which is most frequently associated with human disease is *Legionella pneumophila* which consists of 16 serogroups (12, 67, 198). An infection occurs, when *Legionella*-containing aerosols are inhaled. Then, the bacteria can cause two kinds of disease. The mild form with flu-like symptoms has been termed as Pontiac-fever. The severe form is a potentially fatal atypical pneumonia which is referred to as Legionnaire's disease (198). A very important factor for the establishment of an infection is the immune status of the host. The bacteria are normally cleared by an immune-competent host. However, if the immune status is impaired, e.g. as is the case for elderly persons, persons with diabetes or chronic lung diseases, there is a high risk for the development of a disease (67, 198). In the year 2004, the statistical incidence of Legionellosis in Europe amounted to 8.2 cases per 1 million inhabitants. For Germany in particular, 475 cases of Legionellosis were reported in 2004. However, the number of actual cases is presumed to be much higher. Based on the assumption that 6 % of all ambulant cases of pneumonia are caused by *Legionella*, the actual number of Legionellosis would amount to 30000 cases per year in Germany (Epidemiologisches Bulletin, 48, 2005).

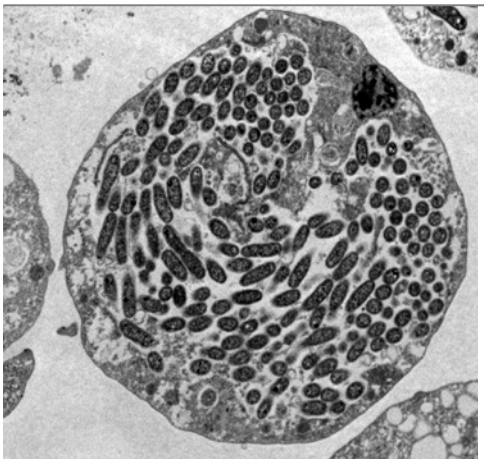

FIG.1.1. *Hartmannella vermiformis* infected with *L. pneumophila*. Electron-microscopic picture (Flieger et al. 2003 Bundesgesundheitsblatt 46:694-699). Magnification: ×2400.

2.2 Intracellular life cycle of *Legionella pneumophila*

Legionella pneumophila is an inhabitant of fresh-water reservoirs. Due to its complex nutrition requirements, e.g. amino acids as a carbon source, *L. pneumophila* is dependent on the association with other micro-organisms in biofilms or intracellular growth in host cells. Under laboratory conditions, *L. pneumophila* is grown in a broth containing yeast extract as a source for amino acids, L-cysteine hydrochloride, ferric pyrophosphate, and α-ketoglutarate. The optimal temperature for bacterial growth in laboratory media is about 35 °C and the optimal pH is 6.9±0.4 with generation times of 4-6 h (61, 66). In biofilms, however, the bacteria are able to survive temperatures above 60 °C and a pH up to 8. When experiencing lack of nutrients in culture broth, *L. pneumophila* accumulates the stringent response signal (p)ppGpp which leads to exit from the exponential growth phase and entry into the stationary growth phase and to the expression of virulence traits associated with motility, cytotoxicity, resistance to osmotic shock, and sensitivity to sodium (13, 34, 84). This phenotype promotes the transmission of the bacteria into host cells. In the aquatic environment, *Legionella* utilizes protozoa as host cells for deriving nutrients and protection from environmental stress factors. An example for the bacterium's ability to grow inside protozoan cells is pictured in *figure* 1.1 which displays a *Hartmannella vermiformis* amoebal cell filled with *L. pneumophila*. Since man-made water reservoirs, e.g. cooling towers and plumbing systems, provide good growing conditions for *L. pneumophila*, they frequently allow transmission of the bacteria to the human host (12, 60). When the bacteria enter the human lung they are taken up by alveolar macrophages and are able to replicate in them. Notably, the intracellular life cycles in the protozoan and the mammalian hosts are very similar (182). Figure 1.2 illustrates the intracellular life cycle of *L. pneumophila*. The internalization by macrophages takes place by the actin-dependent pathway of phagocytosis which is either mediated by complement receptors CR1 and CR3 in the presence of opsonins or by the attachment through *L. pneumophila* adhesin (80, 95, 162). Normally, a phagosome undergoes a maturation process which involves fusion events with early and late endosomal compartments leading to the formation of a phagolysosome. *L. pneumophila*, however, resides in a specialized phagosome that evades fusion with lysosomes and acidification during the first 8 h of the infection process (97, 98). Instead, the *Legionella*-phagosome associates small early secretory vesicles that are derived from the endoplasmic reticulum (ER) (96). The phagosome also interacts with mitochondria and becomes completely enclosed by rough ER membrane 4-6 h post infection (46, 96, 199). At this time (6-10 h post infection), the bacteria start to replicate (1, 65, 99). Notably, an infection study with murine bone marrow-derived macrophages by

Sturgill-Koszycki and Swanson showed that replicative *L. pneumophila* (16 h post infection) can reside in an acidic environment that possesses lysosomal markers such as cathepsin D, indicating a fusion of the replication-vacuole with the lysosome and acidification of the phago-lysosome (197). At 24 h post infection, the number of bacteria increases 50-100-fold and *L. pneumophila* eventually mediates lysis of the host cell and the bacteria infect new host cells (133).

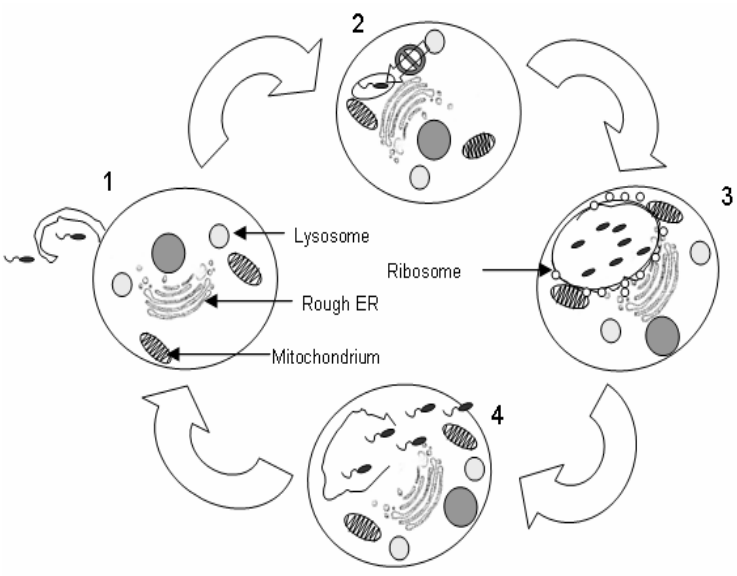

FIG.1.2. Aspects of the intracellular life cycle of *L. pneumophila*. Motile *L. pneumophila* enter the host cell (macrophage or amoebae) by conventional or coiling phagocytosis (1). The *Legionella*-phagosome evades fusion with lysosomes and recruits vesicles from rough endoplasmtic reticulum (ER) and interacts with mitochondria (2). *L. pneumophila* switches to the non-motile replicative form and replicates in large numbers in a phagosome that is studded with ribosomes (3). After exhaustion of the cell, bacteria switch back to transmissible phenotype, mediate disruption of the phagosome as well as the cell membrane and free *L. pneumophila* start a new

2.3 Virulence factors of *L. pneumophila*

Many bacterial factors which contribute to the infection of amoebae and macrophages by *L. pneumophila* have been described. These include the two *L. pneumophila* protein secretion systems, the type II (Lsp) and the type IVB (Dot/Icm) secretion system (114, 178). *L. pneumophila* which are defective in the type II secretion apparatus show a growth defect in the amoebal host and display attenuated growth in the macrophage infection model, suggesting a role for type II secreted effectors in bacterial pathogenesis (82, 118, 163, 164). The most important role for *L. pneumophila* pathogenesis, however, is attributed to a set of 25 genes distributed in two chromosomal regions. These genes were designated *icm* (intracellular multiplication) or *dot* (defect in organelle trafficking) and are structural components or components required for the assembly of a type IV secretion system (7, 179, 213).

L. pneumophila additionally possesses a putative type I (Lss) secretion system which still awaits further characterization (106).

2.3.1 Type IV secretion systems of *L. pneumophila*

Type IV secretion systems are related to bacterial conjugation systems and are utilized by bacteria for the delivery of macromolecules such as protein-DNA complexes and proteins across the bacterial inner and outer membranes into the cytoplasm of host cells. They are categorized into two groups. Type IVA systems resemble the *Agrobacterium tumefaciens* Vir system (VirB1-B11 and VirD4) and the pKM101 Tra system while the prototype of the type IVB secretion system is the *L. pneumophila* Icm/Dot system and the IncI plasmids Tra/Trb system (178). *L. pneumophila* possesses three type IV secretion systems, two type IVA secretion systems (the Tra system which is located on a pathogenicity island and the Lvh system, both of which are specific to strain Philadelphia-1) and the above mentioned type IVB secretion system Icm/Dot (28, 172, 180). Unlike the Icm/dot system, the *L. pneumophila* Lvh secretion system was found to be dispensable for infection of amoebae and macrophages (180). However, another study demonstrated that the Lvh type IVA secretion system, represented by the probable pilin-protein LvhB2, is required for infection of macrophages at 30 °C (160). The close relationship between the type IVA and type IVB secretion systems is indicated by the fact that IcmE and DotB are homologuos to *A. tumefaciens* VirB10 and VirB11 and furthermore by the fact that components of the Lvh secretion system and the Icm/Dot system can replace each other for conjugation of the RSF1010 plasmid (180). The *L. pneumophila icm/dot* genes are localized in two separate genomic regions (21, 127, 169). Region I contains seven genes (*icmV, W*, and *X*, and *dotA*, *B*, *C*, and *D*) (7, 22, 27, 127). Region II contains 18 genes (*icmT*, *S*, *R*, *Q*, *P*, *O, N*, *M*, *L*, *K*, *E, G*, *C*, *D*, *J*, *B, F*, and *H*) (7, 154, 179, 181, 224). The *L. pneumophila* Icm/Dot system was found to contribute by a variety of mechanisms to bacterial pathogenicity some of which are illustrated in Fig. 1.3. First, the Icm/Dot type IVB secretion system enhances bacterial uptake by phagocytosis and pinocytosis (90, 214). Subsequently it contributes to pore formation in the host membrane thereby facilitating the translocation of effector proteins (110). Moreover, the *L. pneumophila* Icm/Dot secretion system is involved in the induction of apoptosis during early stages of the infection in macrophages by activation of caspase-3 (137, 223). It also directs the formation of the *Legionella*–phagosome, characterized by the recruitment of early secretory vesicles and thinning of the phagosome membrane in the early infection process, followed by the recruitment of rough ER later on and inhibition of phagosome-lysosome fusion (108, 206,

219). Finally, an egress pore is formed which releases the bacteria from the host cell, and this second pore formation is likewise mediated by the Icm/Dot apparatus (133, 135).

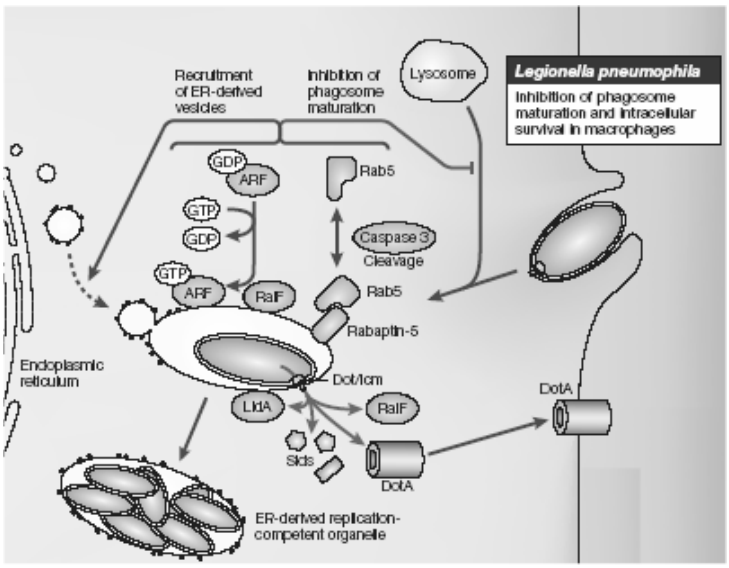

FIG.1.3. Role of the type IVB secretion system in intracellular replication of *L. pneumophila* (183). (www.nature.com/nrmicro/poster/secretion/). Important substrates of the *L. pneumophila* Icm/Dot apparatus are depicted (DotA, RalF, Sids, LidA) as well as effects mediated by the type IVB secretion system, e.g. induction of apoptosis, recruitment of ER-derived vesicles, and inhibition of phagosome maturation.

Most of the *icm*/*dot* genes (e.g. *dotA*, *dotB*, *icmT*, *icmGCDJBF*) are found to be essential for infection of amoebae and/or macrophages (134, 154, 184). Interestingly, DotA is not only a structural component of the *L. pneumophila* type IVB secretion system but is also translocated by this system (141, 165). Recently, a number of effector proteins translocated by the *L. pneumophila* Icm/Dot apparatus have been identified: RalF is a guanine nucleotide exchange factor which recruits the host protein ARF-1 to the *Legionella*-phagosome membrane (140). A further effector is LidA which is involved in the recruitment of ER-derived vesicles to the *Legionella*-vacuole, LepA and LepB are essential for the non-lytic release of the bacterium from amoebae via an exocytic pathway (37, 50, 56). Furthermore, a screen which looked for interaction partners of DotF identified a group of eight proteins named Sid (substrate of Icm/Dot transporter) with so far unknown function (123). Interestingly, none of these substrates are essential for the infection of macrophages or amoebae. This might indicate a functional redundancy among the substrates of the Icm/Dot secretion system. Three other proteins have been identified as type IVB translocated effectors, VipA, VipD, and VipF which lead to vacuolar mistrafficking in yeast (187). Since VipD possesses homology to a group of lipolytic enzymes, it has been identified in this work, too, and its enzymatic properties have been analyzed.

2.3.2 Type II secretion system

The type II secretion system of Gram-negative bacteria transports proteins across the outer membrane. The prerequisite transport across the inner bacterial membrane occurs either via the secretory (sec) or via the twin-arginine translocation (tat) pathway. The sec pathway transports proteins in an unfolded state across the cytoplasmic membrane while the tat pathway transports already folded proteins. For both pathways, an N-terminal signal sequence is required which in case of the tat pathway comprises a twin arginine motif. So far, *L. pneumophila* is the only intracellular pathogen that possesses a type II secretion system with a role in virulence. The *L. pneumophila* type II secretion system, Lsp for *Legionella* secretion pathway, is encoded by 12 genes located on five different genomic regions (82, 163). LspD is located in the outer bacterial membrane and builds the secretin that translocates the substrate across the outer membrane. This ATP-dependent process is facilitated by the cytoplasmic ATPase LspE. Since the functional Type II apparatus requires pseudopilins (encoded by *lspG*, *H*, *I*, *J*, *K*) which are processed by the prepillin peptidase PilD, this enzyme is also required for a functional type II apparatus (118, 163). PilD additionally processes components of the *L. pneumophila* type IV pili which are upregulated at lower temperatures and contribute to bacterial competence regarding DNA transformation and to adherence to host cells (120, 185, 193, 194). The relationship between the *L. pneumophila* type II secretion system, the type IV pilus system, and PilD is depicted in figure 1.4. *L. pneumophila lsp* type II secretion mutants do not grow in *Acanthamoeba castellanii* and *Hartmannella vermiformis* amoebae and are partially defective for growth in U937 macrophages as well as in the A/J mouse model (82, 163, 164). Since lack of type IV pili does not lead to reduced infectivity in either amoebal and macrophage host cells or in the A/J mouse model, the reduced virulence of *L. pneumophila pilD* mutants in the same infection models can be fully attributed to the lack of a functional type II secretion system in these mutants (118, 163, 164, 193).

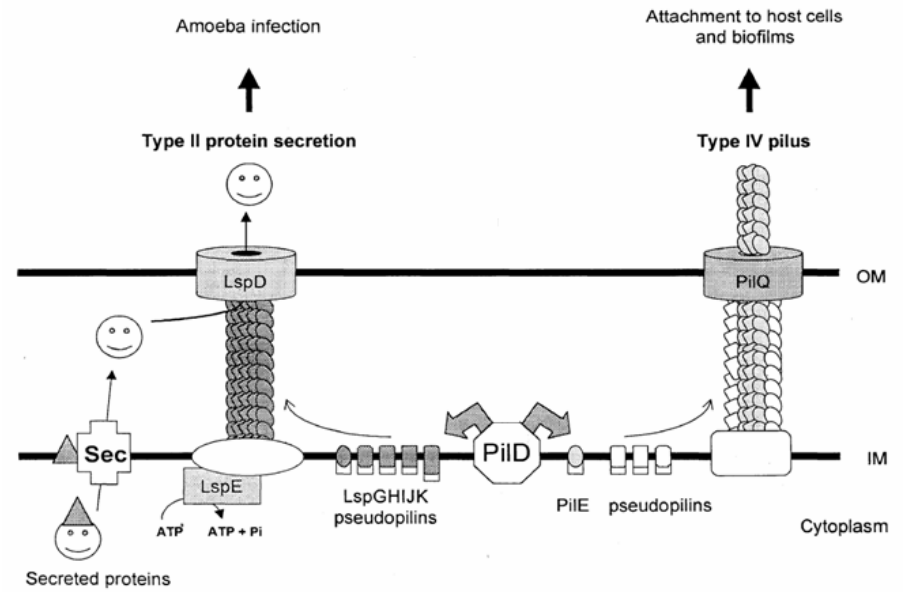

FIG.1.4.The type II secretion system and the type IV pilus in *L. pneumophila* (163). Illustrated are the components of the *L. pneumophila* type II secretion system and type IV pilus. Both consist of pseudopilins which are processed by the prepilin pepdidase PilD.

A number of type II translocated enzymatic activities have been identified which include lipase and esterase activities, phospholipase A (PLA) and lysophospholipase A (LPLA) activities, RNase, phosphatase, and protease activities (9, 163, 164). The protease activity is caused by the most abundant *L. pneumophila* type II-secreted enzyme, the zinc metalloprotease ProA or Msp (82). *L. pneumophila* ProA is a protease that requires zinc as a cofactor and belongs to the family of bacterial neutral proteases that includes *Pseudomonas aeruginosa* elastase and similar to this enzyme comprises a glutamate in its active site (23, 59, 130). Furthermore, ProA was shown to cleave different substrates including collagen, gelatine, and casein and showed cytotoxic and hemolytic properties (48, 155, 156). ProA also degrades or inactivates host cell proteins including human α-1-antitrypsin, TNF-α, IL-12, and CD4 (49, 88, 129). Notably, purified ProA leads to lesions in guinea-pig lungs that resemble those caused by an *L. pneumophila* infection and inhibits the formation of superoxide anions by monocytes and the activity of natural killer cells (18, 48, 159). *proA* was also identified among several *icm*/*dot* genes to be required for *L. pneumophila* virulence in the guinea pig model by a signature-tagged mutagenesis screen (62). Earlier studies, however, had come to a different result.

There ProA was found to be dispensable for the infection of amoebae, macrophages and in the guinea-pig model, although low-doses of infection showed a delayed death of animals infected with an *L. pneumophila proA* mutant compared to wild type (24, 131, 200). Further type II secreted proteins include the major acid phosphatase Map, PlcA which is a protein with phospholipase C-like activity, the lipases LipA and LipB, and the LPLA PlaA (8-10, 75, 163, 164). Since none of these enzymes are essential for infection of amoebae or macrophages, the major type II secreted virulence factor of *L. pneumophila* still remains to be identified. As *L. pneumophila* type II secretion mutants show highly reduced phospholipase A activity, the lack of phospholipase(s) A might contribute to the reduced virulence of this mutant.

2.3.3 Other virulence factors

A number of further *L. pneumophila* virulence factors contributing to the infection of protozoan and/or macrophage host cells have been identified by now. One group of such virulence factors are surface proteins which are often involved in adherence to and entry into the host cells but their lack also leads to attenuated intracellular growth. An important member of this group is the *L. pneumophila* major outer membrane protein (MOMP) which is a porin shown to mediate phagocytosis by binding to complement components necessary for the attachment to the monocyte receptors CR1 and CR3 (20, 79, 111, 149). Also belonging to this group are the cell surface expressed peptidyl-prolyl cis/trans isomerase Mip (macrophage infectivity potentiator) and the cell-associated toxin RtxA, a cytotoxic protein belonging to a group of toxins which possess repeats of the amino acid motif RTX (40-44, 71). Consistent with the observation that the transmissive phenotype (associated with cytotoxicity and motility) of *L. pneumophila* is induced with its entry into the stationary phase a second group of virulence factors comprises regulators of the stationary phase phenotype which are the two component system LetA/S and the alternative sigma factor RpoS (13, 83, 85). Accordingly, enzymes which are induced or upregulated in the stationary phase have also been found to promote virulence, e.g. the flagellum protein FlaA essential for invasion of host cells or the *L. pneumophila* periplasmic and cytoplasmic catalase-peroxidases, designated KatA and KatB, respectively which protect the bacterium from oxidative damage by hydrogen peroxide (14, 58). Moreover, proteins which are involved in iron acquisition are also essential for intracellular and extracellular growth under iron-limiting conditions (89, 119, 144, 152, 153, 161, 211, 212).

Table 1.1 Overview of selected *L. pneumophila* virulence factors

Name	Description	Mode of action
Lsp	Type II secretion system	Exports metalloprotease, lipase, phospholipase A, lysophospholipase A, phospholipase C-like activity, RNase, and phosphatase activities
Dot/Icm	Type IVB secretion system	Exports factors facilitating different steps of the infection process: DotA, RalF, LidA, LepA, LepB, Sid proteins, VipA, VipD, VipF
MOMP	Porin	Promotes phagocytosis by binding complement components
Mip	Peptidyl-prolyl cis/trans isomerase	Promotes intracellular multiplication by an unknown mode
RtxA	Toxin	Promotes adherence, entry and pore formation by an unknown mode
LetA/S	Two component system	Induces stationary phase phenotype
RpoS	Alternative sigma factor	Induces stationary phase phenotype
FlaA	Flagellum protein	Promotes invasion
KatA, KatB	Catalase-peroxidases	Protection against hydrogen peroxide, e.g. generated during oxidative burst
Factors involved in bacterial iron acquisition	Heme utilization, factors involved in iron transport, ferric reductases	Factors mediating iron uptake: -cytochrome c maturation genes -transporter of iron loaded peptides encoded by the *irAB* locus -legiobactin (siderophore) -FrgA: iron-regulated homologue of aerobactin synthetases -ferrous iron transport system encoded by the *feoAB* locus

2.4 *L. pneumophila* lipids and pathways of lipid biosynthesis

2.4.1 Membrane phospholipids of *L. pneumophila*

Lipids are a diverse class of molecules which consists of fatty acids and their naturally-occurring derivatives (esters or amides), and substances related biosynthetically or functionally to these compounds (W. W. Christie, www.lipidlibrary.co.uk).

According to their backbone, naturally-occurring fatty acid derivatives can be divided into four major groups, the glycerolipids, the glycerophospholipids, the sterol lipids, and the sphingolipids. A special type of lipids, exclusively found in Gram-negative bacteria are the lipopolysaccharides (LPS) which are build up from a covalently bound lipid (termed lipid A) and a heteropolysaccharide. The outer membrane of Gram-negative bacteria has an outer leaflet which is predominantly build up from LPS. The inner leaflet of the outer membrane consists of a glycerophospholipid bilayer. Glycerophospholipids are also the major structural and functional components of the bacterial inner membrane. The most abundant phospholipid in the *L. pneumophila* cellular envelope under laboratory growth conditions is phosphatidylcholine (PC), followed by phosphatidylethanolamine (PE), cardiolipin (diphosphatidylglycerol), phosphatidylmonomethylethanolamine, phosphatidylglycerol (PG), and phosphatidyldimethylethanolamine (70). The fatty acids of the *L. pneumophila* membrane phospholipids are solely branched-chain fatty acids ranging from C14 to C20, with C16 being the most abundant fatty acid (70, 138). Notably, the phospholipid compositions of other Gram-negative bacteria are different from that of *L. pneumophila*. The membrane phospholipids of *Escherichia coli* and *Aeromonas hydrophila*, for example, are mainly constituted by PE, PG, Cardiolipin, and lack PC (55, 100, 218).

2.4.2 Pathways of bacterial lipid biosynthesis

The pathways and enzymes for the biosynthesis of membrane phospholipids are well studied for *E. coli* (104) and the pathways are illustrated in figure 1.5. The precursor for all phospholipids in bacteria is phosphatidic acid (PA). As a first step, PA is activated by binding to cytidylmonophosphate (CMP) resulting in the formation of cytidyldiphosphate (CDP)-diacylglycerol by CDP-diacylglycerol synthetase. Then there are two pathways in *E. coli*. One leads to the formation of the aminophospholipid PE via the decarboxylation of phosphatidylserine which is generated by the condensation of serine with CDP-diacylglycerol. The second pathway leads to the generation of the acidic phospholipid PG by the condensation of CDP-diacylglycerol with glycerol-3-phosphate followed by dephosphorylation of the resulting PG phosphate. Two PG molecules are condensed to cardiolipin.

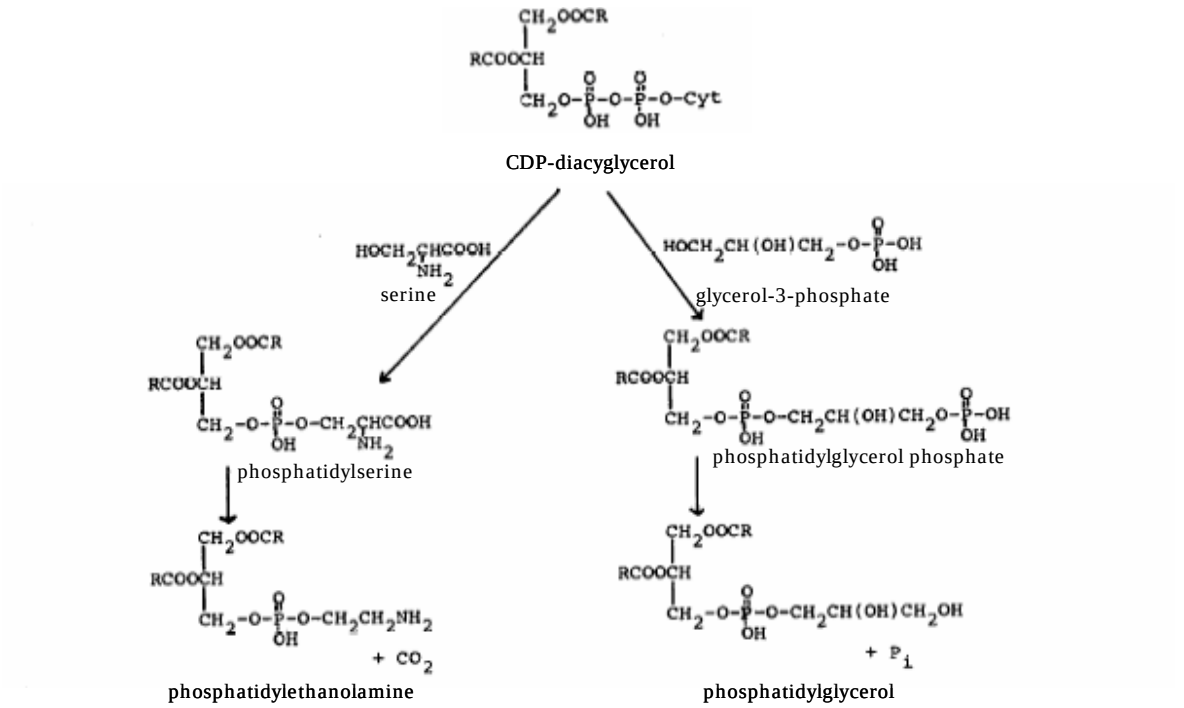

FIG.1.5. Pathways of phospholipid biosynthesis in *E. coli* adopted from (109). CDP-diacylglycerol is either transformed to phosphatidylethanolamine by condensation with serine followed by decarboxylation or to phosphatidylglycerol by the condensation with glycerol-3-phosphate followed by dephosphorylation.

Although PC is the dominant phospholipid of eucaryotic membranes only about 10 % of bacteria, principally intracellular replicating bacteria, possess PC in their cell envelopes and *L. pneumophila* belongs to this small bacterial group (70, 128). There are two pathways for PC biogenesis. In one pathway, CDP-diacylglycerol and free choline are condensed to PC. In the second pathway, PC is generated by three successive methylations of PE via the intermediates monometylphosphatidylethanolamine and dimethylphosphatidylethanolamine (128). Both pathways for PC synthesis have been found in *L. pneumophila* (128). Furthermore, bacteria are also able to synthesize phospholipids from lysophospholipids. *E. coli*, for example, possesses a bifunctional enzyme with acyltransferase activity, designated Aas, which can bind a free fatty acid moiety to its acyl carrier protein subunit and subsequently transfer it to the C1 position from 2-acyl-lysophosphatidylethanolamine (LPE) and thereby generate PE. The primary role of Aas is to act as a salvage pathway for the resynthesis of PE from 2-acyl-LPE taken up from the medium or generated by transacyl reactions or the activity of PLAs (51, 105). Notably, an *L. pneumophila* protein was identified and initially characterized in this study which possesses homology to the Aas protein of *E. coli*.

2.5 Bacterial lipolytic enzymes and their role in virulence

2.5.1 Characteristic features of lipases

Phospholipases are hydrolytic enzymes which cleave ester bonds in phospholipids. These enzymes are categorized into four groups A to D (see figure 1.6). PLA cleave fatty acid residues from the sn-1 or sn-2 position of the glycerol backbone. LPLA, a subgroup of the first group, hydrolyze phospholipids which have only one fatty acid residue. Phospholipases B cleave both fatty acid residues from sn-1 and sn-2 position. Phospholipases C (PLC) and D are phosphodiesterases releasing phosphomonoesters or the alcohol, respectively.

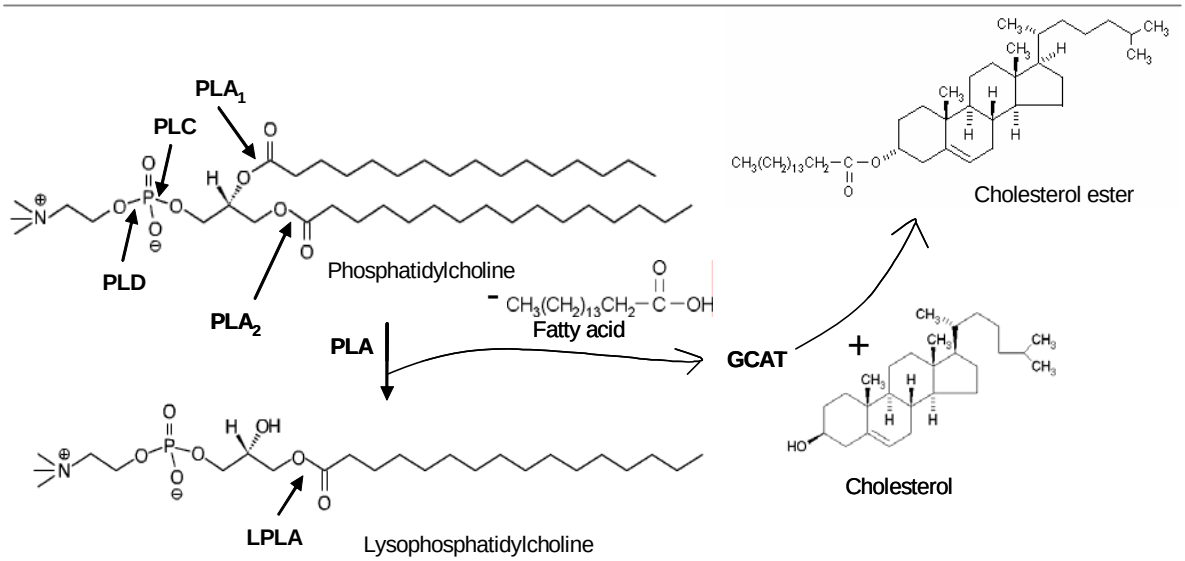

FIG.1.6. Categories of phospholipases. Phospholipases A (PLA) hydrolyze the carboxylester bonds at sn-1 and sn-2 positions (PLA_1 and PLA_2, respectively). The resulting lysophospholipid is cleaved by lysophospholipases A (LPLA). The released fatty acid is transferred by acyltransferases to an acceptor molecule, e.g. to cholesterol by the glycerophospholipid:cholesterol acyltransferase (GCAT). Phospholipases C and D hydrolyze either side of the phosphodiester bond of the phospholipid, respectively.

PLAs and PLBs, being carboxylesterases, possess many characteristics of classical lipases which are defined as carboxylesterases of acylglycerolesters with a preference for long chain (chain length ≥ 10 carbon atoms) acyl residues. One feature of many but not all lipases is the interfacial activation. Interfacial activation is the enhanced activity of a lipase when the lipid substrate forms micelles, bilayers, or an emulsion and thereby provides an interface to the aquatic environment. The reason for the interfacial activation is a helix or loop in the three dimensional structure of many lipases, referred to as the lid, which screens the catalytic site

from the substrate and only opens at contact to an interface. Moreover, in spite of little sequence homology, most lipases have a common three dimensional fold, the α/β-hydrolase fold (147, 176). The α/β-hydrolase fold consists of eight almost parallel ß-sheets (the second ß-sheet is anti parallel) in the center of the hydrolase which are surrounded on both sides by α-helices (147). Interestingly, this fold is also adopted by many other hydrolytic enzymes, e.g. acetylcholine esterases or serine carboxypeptidases. Enzymes which adopt the α/ β-hydrolase fold have a common catalytic site. Their catalytic site consists of three amino acids:

-a nucleophilic serine, cystein or aspartate

-an acidic amino acid: aspartate or glutamate, and

-a catalytic active histidine

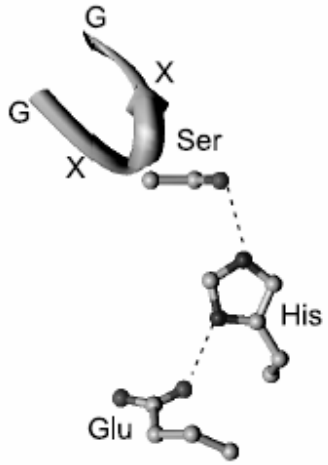

FIG.1.7. The members of the catalytic triad of the Ca*ndida rugosa* lipase. Ca*ndida rugosa* lipase, protein domain database entry 1crl; taken from Schulz, 2001 (177). Serine 209, glutamate 341, and histidine 449 as well as the hydrogen bonds between serine-histidine and histidine-glutamate are shown. Illustrated is also the nucleophilic elbow (G-X-S-X-G) which puts the nucleophilic serine into an ideal position for the reaction.

Lipases are often serine hydrolases and their catalytic triad usually consists of a nucleophilic serine, a glutamate/aspartate, and a histidine. The nucleophilic serine is embedded in a highly conserved penta-peptide, also called the lipase motif: Gly-X-Ser-X-Gly. This arrangement of the preceding ß-sheet, the nucleophile, and the following α-helix is called the nucleophilic elbow, because of the sharp turn between the ß-sheet and α-helix. The mechanism of lipid hydrolysis which is illustrated in figure 1.8 is similar to that of serine proteases and involves nucleophilic attack of a carbonyl carbon by the hydroxyl group of the serine. The imidazole ring in histidine functions as a base, enhancing the nucleophilicity of the serine. The aspartic acid residue, although not directly involved in the active site, can be seen in X-ray structures to form a hydrogen bond with the histidine, suggesting that the acetate group of Asp functions to pass on protons amongst the members of the triad.

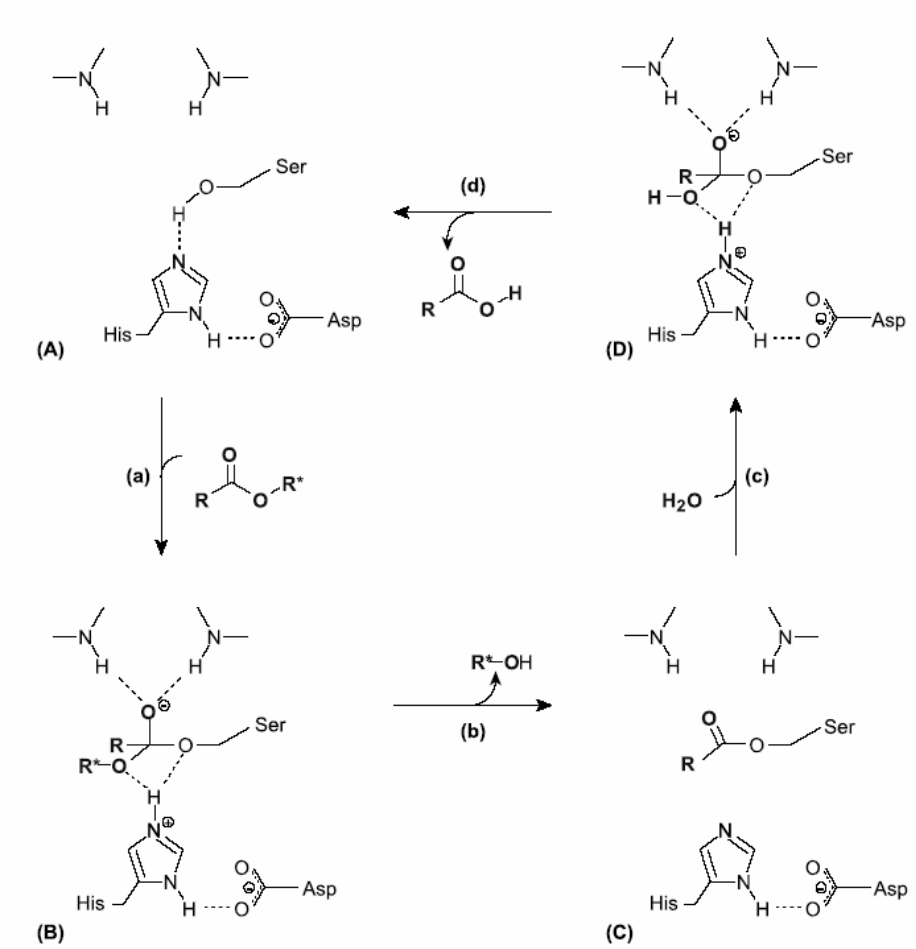

FIG.1.8. Mechanism of substrate hydrolysis by lipases (177). The nucleophilic attack of the carbonyl carbon by the hydroxyl group of the active site serine leads to the formation of a tetrahedral intermediate which is stabilized by hydrogen bonds to the backbone amides of amino acid residues (often glycine) building the oxyanion hole (step a). The active site histidine transfers a proton to the cleaved alcohol residue (step b). The carbonyl atom of the remaining acyl-enzyme is attacked by a nucleophilic water molecule (step c) leading to the generation of a second tetrahedral intermediate followed by the release of the fatty acid residue and regeneration of the enzyme (step d).

2.5.2 Classification of bacterial lipases

Arpigny and Jaeger classified bacterial lipolytic enzymes into eight families, whereby family I consists of true lipases, family II is build up by a group possessing the consensus sequence GDSL which embeds the active site serine (11). Members of the family of GDSL hydrolases initially defined by Upton and Buckley possess five blocks of amino acid homology and adopt a α/β-tertiary fold which is different from the α/β-hydrolase fold (132, 209). Moreover, instead of a triad, some of the enzymes belonging to the GDSL family, e.g. *Streptomyces scrapies* esterase, possess a catalytic dyad which consists of Ser and His, located in block I and IV, respectively

(11, 215). Family III comprises a special group of lipases which are homologues to the intracellular and plasma isoforms of the human platelet-activating factor-acteylhydrolase (PAF-AH) and do not possess a lid structure (11, 216). Family IV is the hormone-sensitive lipase (HSL) family, a group of bacterial lipases showing striking sequence similarity to the mammalian HSL. Family V comprises lipases that show homology to bacterial non-lipolytic enzymes which also have the α/β-hydrolase fold, e.g. epoxide hydrolases, dehalogenases. Family VI consist of very small esterases (23-26 kDa), family VII is a group of larger lipases possessing homology to eucaryotic acetylcholine esterases and intestine/liver carboxylesterases, and family VIII finally, consists of lipases which show homology to class C ß-lactamases with a conserved Ser-X-X-Lys motif. In this study, a new group of bacterial proteins (mostly with unknown function) has been identified in the genomes of a large number of Gram-positive and Gram-negative bacteria the members of which possess conserved domains of amino acid homology to potato patatin (16). Patatin is a plant protein with phospholipase A activity involved in parasite defence and signal transduction (6, 94, 196).

2.5.3 *L. pneumophila* lipases

So far, three enzymes with carboxylesterase activity have been identified and characterized in *L. pneumophila*. Two lipases designated LipA and LipB, and a lysophospholipase A, designated PlaA (10, 75). *L pneumophila lipA* mutants showed a reduced activity to release fatty acids from 1-MPG and triacylglycerols (tricaprylin, tripalmitin, triolein) in their culture supernatant indicating that LipA possesses lipase activity. *L. pneumophila* LipA is predicted to adopt the α/β-hydrolase fold and shows homology to Lip3 (gi45597348) of *Pseudomonas aeruginosa* (Expect Value: 1×10^{-11}, Identity: 24 %, Similarity: 42 %). Therefore, LipA probably belongs to family I of the lipase families defined by Arpigny and Jaeger (10, 11, 145). *L. pneumophila* LipB is a rather small (28 kDa) protein which is also predicted to adopt the classical α/β-hydrolase fold. It possesses parts of the conserved regions of amino acid homology to pancreatic lipase-like enzymes (NCBI conserved domain database). *L. pneumophila* LipB mutants showed only a weak reduction in their capacity to hydrolyse tricaprylin and the same capacity as the wild type to hydrolyse mono- or diacylglycerols in their culture supernatants (10). Remarkably, a protein with sequence similarity to LipB has been identified in *L. pneumophila*, designated PlaB, and this study has contributed to its characterization with regard to its lipolytic activities (76). The third enzyme, PlaA is a lysophospholipase A belonging to the family of GDSL-hydrolases which includes lipases, PLAs, hemolysins, and acyltransferases (11, 30, 75). *L. pneumophila* PlaA was

purified from *L. pneumophila* culture supernatant and was identified by N-terminal protein sequencing. PlaA was found to be secreted in a type II-system dependent way and was responsible for most of the secreted lysophosphatidylglycerol- and lysophosphatidylcholine-hydrolyzing activities as well as for a part of the 1-monopalmitoylglycerol (1-MPG)-hydrolyzing activity of *L. pneumophila* (73, 75). The best characterized protein with homology to PlaA is the glycerophospholipid:cholesterol acyltransferase (GCAT) SatA of *Aeromonas salmonicida*, a major secreted toxin (33, 115). Apart from its GCAT activity which transfers fatty acids from phospholipids to cholesterol resulting in the formation of lysophospholipids and cholesterol ester (see figure 1.6), SatA was also shown to possess PLA and LPLA activities (32, 33). Since *L. pneumophila* also possesses GCAT activity, PlaA was suspected to be responsible for this activity (75). However, the culture supernatant of *L. pneumophila plaA* mutants still retains its ability to transfer fatty acids from PG to cholesterol (75). Interestingly, the *L. pneumophila* genome encodes two paralogs of PlaA containing the GDSL motif, suggesting that one of them might cause the secreted GCAT activity of *L. pneumophila*. Both paralogs of PlaA have been investigated in this study and one of them has indeed been identified as the major *L. pneumophila* GCAT (15).

2.5.4 Role of phospholipases in bacterial virulence

Phospholipases are considered as potential bacterial virulence factors, because they facilitate lysis of host cell membranes permitting bacterial egress and mediate bacterial cytotoxicity (Fig. 1.9) (175). The two *Listeria monocytogenes* phospholipases C for instance, enable the bacterium to exit the phagosome and spread from cell to cell (81, 190). *Clostridium perfrigens*, the causative agent of gas gangrene, secretes a toxin which has been identified as a phospholipase C causing membrane disruption and interfering with host signalling pathways (170). The *Aeromonas salmonicida* SatA is a GCAT and a PLA which acts as a hemolysin and cytotoxin (115). Interestingly, PLA activity is presumed to contribute to the pathogenesis of *Rickettsia* sp. although the corresponding gene has not yet been identified (220, 221). Furthermore, it has been shown that ExoU, a type III secreted cytotoxin of *Pseudomonas aeruginosa* PA103, possesses PLA and LPLA activities (150, 173, 174, 201). ExoU causes acute lung injury which could be linked to its PLA activity (4, 69, 148). ExoU contains conserved amino acid domains, including the catalytic sites, of patatin, a potato storage glycoprotein with lipid acylhydrolase activity (68, 148, 157, 174). Mutagenesis studies have revealed that these conserved domains are essential for the PLA/LPLA activity of ExoU as well as for its cytotoxicity

towards eukaryotic cells (68, 157). A recent study by Shohdy and colleagues identified three new substrates of the type IVB secretion system (Dot/Icm) one of which designated VipD, displayed homology to the *P. aeruginosa* PLA/LPLA ExoU (187). Therefore, some of the effects of the *L. pneumophila* type IVB secretion system during the bacterial infection process might partially be attributed to translocated PLAs which might mediate the remodeling and lysis of the phagosomal membrane and might create reaction products capable of interfering with the signal transduction of the host (136, 187, 206) (see figure 1.9). Moreover, it has been shown that secreted *L. pneumophila* phospholipases A readily hydrolyze lung surfactant, a phospholipid monolayer essential for the stability of the alveoli (74). The resulting formation of cytolytic lysophosphatidylcholine (> 60 % of the human lung surfactant consists of phosphatidylcholine) could also lead to the induction of apoptosis or mediate inflammation by upregulation of CD54 (ICAM-1) on (alveolar) endothelial cells (101, 112). Notably, *L. pneumophila* is able to protect itself against the harmful effect of lysophosphatidylcholine by the action of PlaA (75). Since *L. pneumophila* possesses a remarkable amount and variety of phospholipase degrading or modifying activities representing important bacterial tools to survive both inside and outside of hosts, their characterization will promote our understanding of the life cycle of *L. pneumophila*.

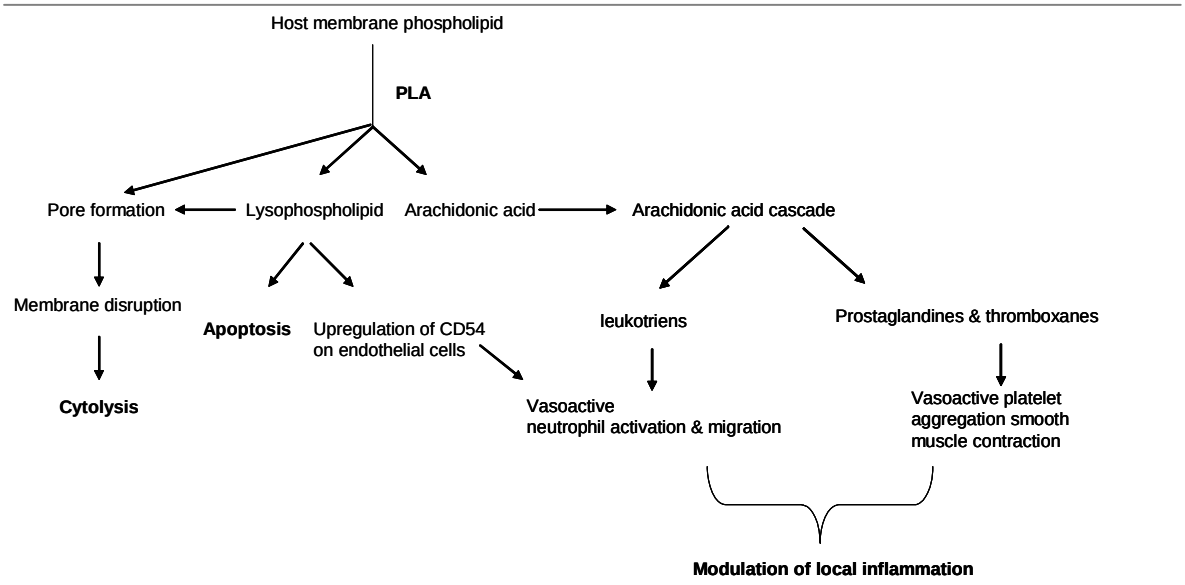

FIG.1.9. Signaling pathways induced by the second messengers released by phospholipases A, modified from Schmiel and Miller, 1999 (175). The hydrolysis of host phospholipids could release cytolytic lysophospholipids and arachidonic acid, a typical eukaryotic fatty acid. In addition to their cytolytic effect, lysophospholipids can induce apoptosis or mediate inflammation. Arachidonic acid can trigger the arachidonic acid cascade and lead to immune modulation. PLC activity can also trigger the arachidonic acid cascade via the release of diacylglycerol (not shown). Arrows do not necessarily indicate a direct induction.