Cloning and Characterization of the Gene Encoding the Major Cell-Associated Phospholipase A of *Legionella pneumophila*, *plaB*, Exhibiting Hemolytic Activity

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**Legionella pneumophila**, the causative agent of Legionnaires’ disease, is an intracellular pathogen of amoebae, macrophages, and epithelial cells. The pathology of *Legionella* infections involves alveolar cell destruction, and several proteins of *L. pneumophila* are known to contribute to this ability. By screening a genomic library of *L. pneumophila*, we found an additional *L. pneumophila* gene, *plaB*, which coded for a hemolytic activity and contained a lipase consensus motif in its deduced protein sequence. Moreover, *Escherichia coli* harboring the *L. pneumophila plaB* gene showed increased activity in releasing fatty acids predominantly from diacylphospholipids, demonstrating that it encodes a phospholipase A. It has been reported that culture supernatants and cell lysates of *L. pneumophila* possess phospholipase A activity; however, only the major secreted lysophospholipase A PlaA has been investigated on the molecular level. We therefore generated isogenic *L. pneumophila plaB* mutants and tested those for hemolysis, lipolytic activities, and intracellular survival in amoebae and macrophages. Compared to wild-type *L. pneumophila*, the *plaB* mutant showed reduced hemolysis of human red blood cells and almost completely lost its cell-associated lipolytic activity. We conclude that *L. pneumophila plaB* is the gene encoding the major cell-associated phospholipase A, possibly contributing to bacterial cytotoxicity due to its hemolytic activity. On the other hand, in view of the fact that the *plaB* mutant multiplied like the wild type both in U937 macrophages and in *Acanthamoeba castellanii* amoebae, *plaB* is not essential for intracellular survival of the pathogen.
A study, activity still remain to be characterized on the molecular level. Here, we identify the gene for the major cell-associated phospholipase A, which was found by screening a genomic *L. pneumophila* library for new hemolysis genes. Furthermore, we examine its contribution to the hemolytic activity of the bacterium and its significance in intracellular infection.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *L. pneumophila* sg1 strain Corby (33) was used to construct an expression library in *E. coli* coli (30) for mutagenesis of the *Legionella* plaB gene and later served as a wild-type control. *Legionella* strains used for Southern blot analysis were *L. pneumophila* sg1 strain Philadelphia I (ATCC 33152), *L. pneumophila* sg1 strain Mspl9 (7), *L. pneumophila* sg1 strain 685 (7), *L. pneumophila* sg3 strain Bloomington (ATCC 33155), *L. pneumophila* sg3 strain U22 (7), *L. pneumophila* sg6 strain Los Angeles (ATCC 33156), *L. pneumophila* sg6 strain Chicago-2 (ATCC 33215), *L. pneumophila* sg6 sg6 (patient isolate) (7), *L. pneumophila* type strains (sg7, sg10, sg12, and sg13) (kindly provided by P. C. Lück, Dresden, Germany), *L. anisa* (21), *L. dnonii* (ATCC 33279), *L. erythri* (51), *L. garmanni* (ATCC 33297), *L. backalea* sg1 and sg2 (ATCC 33250 and ATCC 35999, respectively), *L. serralta* (ATCC 43119), *L. longbeachae* (ATCC 33486, respectively), *L. micdadei* (ATCC 33218), *L. oakhridense* (ATCC 33761), and *Sarcobacter* (*Legionella*) tyhicum (PCM 2298; from the Polish Culture of Microorganisms).

*L. pneumophila* was routinely grown on buffered charcoal-yeast extract (BCYE) agar for 2 days at 37°C (19). For extracellular growth, *L. pneumophila* was cultured in buffered yeast extract broth (BYE) at 37°C with shaking at 350 rpm. Bacterial growth was monitored by determining the optical density at 660 nm (OD660) of the culture with a Beckman spectrophotometer DUS520 (Beckman Coulter, Unterschleißheim, Germany), following inoculation to an OD660 of 0.2 to 0.3. *E. coli* strain DH5α, the host for new recombinant plasmids, was grown in Luria-Bertani (LB) broth or agar (6). An *E. coli* (EIEC 12860, a clinical isolate provided by Helge Karch; serotype O:124) cyA knockout mutant was kindly provided by Christian Hüttermann (University of Würzburg, Würzburg, Germany). When appropriate, media were supplemented with antibiotics at final concentrations suitable for *L. pneumophila* or *E. coli*; kanamycin at 25 or 50 μg/ml, respectively; chloramphenicol at 6 or 30 μg/ml, respectively; and ampicillin at 100 μg/ml.

**Preparation of culture supernatants and cell lysates.** Culture supernatants for assessment of hydrolytic activities were obtained at the end of exponential growth at 37°C, 2.2 to 2.3 × 10^9/ml by centrifugation for 5 min at 5,000 × g. For the generation of cell lysates, bacteria from the late-exponential phase were pelleted by centrifugation as described above and then lysed as described previously, except that the lysate was repeatedly passed through a 22-gauge needle (25). Culture supernatants and cell lysates were either tested immediately for enzymatic activities or stored overnight at 4°C.

**DNA techniques and sequence analysis.** An expression library of *L. pneumophila* Corby was constructed as described previously (30). *E. coli* DH5α was used for the propagation of recombinant plasmid DNA. The following vectors were used: pUC18 (backbone in plasmids pKHL102, pCL102-1, pCL102-2, pCL102-3, pKH190, pKH194, and pSY-CABD) or pUC19 (Amerham Biosciences, Freiburg, Germany), pBCKS+ (backbone in plasmid pKHK192; Stratagene, Heidelberg, Germany), and pBOD20 (backbone in plasmid pHK195) (44). Genomic and plasmid DNA were prepared according to standard protocols (49). PCR was carried out using a TRIO-Thermoblock or a T-Gradient thermocycler (Biometra, Tuttingen, Germany) or Taq polymerase (Perkin-Elmer, Weitersheim, Germany). Foreign DNA was introduced into bacterial strains by electroporation.

**Lipid extraction and TLC.** For detection of polar lipids, silica gel plates (Merck, Darmstadt, Germany) were developed in tanks containing a solvent mixture of chloroform-methanol-water in a 65:25:4 (vol/vol) ratio. Substrates in reaction mixtures of lipids with cell lysates or corresponding negative controls were subjected to lipid extraction (9, 22). The lower chloroform phase was subsequently used as a negative control. Lipid extraction and TLC.

**Hemolysis assays.** For detection of hemolytic activity of *L. pneumophila* on *E. coli* strains on blood agar media, 30 ml of blood/liter was added to BCYE (without charcoal) or LB agar medium, respectively. Bacterial cultures were inoculated onto human or sheep blood agar plates and incubated for 48 h at 37°C. *E. coli* wild-type and mutant strains, or complemented *L. pneumophila* strains were prepared for quantitative liquid hemolysis assays in the following manner. *E. coli* logarithmic-growth-phase cultures were set to an OD660 of 1, 2 mM isosporyl-β-thiogalactopyranoside (IPTG) was added, and bacteria were incubated for 2 h. *L. pneumophila* strains were grown on BCYE agar at 37°C for 1 day. Subsequently, bacteria were resuspended in phosphate-buffered saline (PBS) and adjusted to an OD660 of 1. A 200-μl volume of *E. coli* or *L. pneumophila* bacteria was mixed with 800 μl of a suspension of 0.5 ml of human red blood cells in 40 ml of PBS. To test for the hemolytic activity of *L. pneumophila*, sample mixtures were centrifuged (at 800 × g for 2 min) prior to incubation. After incubation for 20 h (E. coli) or 7 h (*Legionella*) at 37°C, samples were centrifuged (at 800 × g for 2 min), and the OD660 of the supernatant was determined.

**Enzymatic assay for lipolytic activities.** Enzymatic assay for lipolytic activities was performed as described previously (22, 25) with minor modifications. Briefly, different phospholipids or lipids were incubated with bacterial culture supernatants or cell lysates in a mixture containing 6.7 mM lipid substrate (1-monopalmitoyllysophosphatidylethanolamine [MPLP], 1-monopalmitoyllysophosphatidylglycerol [MPLG], 1-monopalmitoylglycerol [1-MPG], 1,2-dipalmitylphosphatidylethanolamine [DPPC], 1,2-dipalmitylphosphatidylglycerol [1,2-DG], or tripalmitoylphosphatidylglycerol [TPG]), 3 mM NaCl, 0.5% (vol/vol) Triton X-100, and 20 mM Tris-HCl (pH 7.2). All lipids, including standards for thin-layer chromatography (TLC), were obtained from Sigma Chemicals (Munich, Germany) or Avanti Polar Lipids, Inc. (Alabaster, Ala.). Prior to incubation, the lipid substrates were vortexed for 15 min at 37°C and then exposed to ultrasonication (Sonopuls, Bandelin, Berlin, Germany) three times, for 15 s each time, at cycle 4 × 10% with the power set to 65%. Incubations with bacterial products were performed at 37°C with continuous agitation at 100 rpm for overnight incubations and at 170 rpm for various shorter times, which are given in descriptions of specific experiments. Levels of free fatty acids (FFA) were determined by use of the NEFA-C kit (Wako Chemicals, Neuss, Germany) according to the manufacturer’s instructions. Depending on the nature of the experiment, BYE broth, LB broth, or 40 mM Tris-HCl (pH 7.2) (25°C) was incubated, treated like the cultures, and subsequently used as a negative control.

**Lipid extraction and TLC.** For detection of distinct polar and apolar lipids, reaction mixtures of lipids with cell lysates or corresponding negative controls were subjected to lipid extraction (9, 22). The lower chloroform phase was dried under N2 and redissolved to electrophoresis. For TLC, silica gel plates (Merck, Darmstadt, Germany) were developed in tanks containing a solvent mixture of chloroform-methanol-water in a 65:25:4 (vol/vol) ratio.
FIG. 1. plaB locus in L. pneumophila and recombinant E. coli. (Top) Diagram represents the L. pneumophila chromosome region that contains the phospholipase A gene plaB, along with the locations of the relevant restriction enzyme sites (B, BamHI; H, HindIII; Ps, PstI; S, Sau3A1; ScI, SacI; ScII, SacII). (Center) Horizontal arrows depict the relative location, size, and orientation of plaB and neighboring ORFs. (Bottom) Lines represent the segments of Legionella DNA that were cloned into plasmid vectors. Plasmids pKH194 and pKH195 contained a Km' gene cassette. Plus and minus signs indicate whether recombinant E. coli exhibited hemolytic activity on human red blood agar plates.

RESULTS

Identification of a new L. pneumophila gene encoding a hemolytic protein. To isolate new L. pneumophila genes responsible for hemolytic activity, a total of 8,000 E. coli clones of an L. pneumophila expression library were screened for hemolytic activity on human red blood agar plates. Three clones showing hemolytic activity were obtained. After retransformation, plasmids pKHL82 and pKHL102, continuously displaying hemolytic activity, were found to be identical by restriction analysis. Subclones (pCL102-1, pCL102-2, pCL102-3, pKH190, and pKH192) containing different segments of the Legionella DNA present in pKH102 were generated and tested for hemolytic activity (Fig. 1). Hemolysis of human blood agar was mediated by E. coli clones with plasmid pKHL102, pCL102-3, pKH190, or pKH192 but not by clones containing pCL102-1 or pCL102-2 (Fig. 1 and 2A). This suggests that the genetic region coding for the hemolysin is located downstream from the HindIII cleavage site of the L. pneumophila sequence present in pKH102. In pKH102, only one complete open reading frame (ORF) (insert nucleotides 918 to 2342) was identified and designated plaB. It was completely (pKH190 and pKH192) or approximately two-thirds (pCL102-3) present in all clones showing hemolytic activity. Hemolysis of E. coli containing pKH190 was not caused by induction of the latent E. coli hemolysin CylA (equivalent to SheA) (16), because an E. coli cylA knockout mutant harboring pKH190 was still hemolytic (data not shown). Inserting a Km' cassette into the SacII restriction site (nucleotide position 1080 within the ORF) of plaB present in pKH190 abolished the hemolytic activity of the corresponding recombinant E. coli clone, confirming that the ORF identified was responsible for the hemolytic phenotype (Fig. 1 and 2A).

Nucleotide sequence accession number. The L. pneumophila Cotby plab sequence has been deposited in GenBank at the National Center for Biotechnology Information (NCBI) under accession no. AJ565849.
under all conditions used (data not shown). To quantitatively confirm the results from the hemolysis plate assays, E. coli harboring pBCKS+ or pKH192 was tested for hemolysis of human red blood cells in a liquid hemolysis assay. As shown in Fig. 2B, recombinant E. coli containing pKH192 was more hemolytic than E. coli harboring pBCKS+. The L. pneumophila plaB gene sequence was predicted to encode a protein of 474 amino acids. As examined by the BlastP algorithm, the deduced protein sequence showed significant homology to three proteins: a hypothetical protein from a Nostoc sp. (gi17230632), a hypothetical protein from Pseudomonas aeruginosa (gi15598123), and the putative lipase LipB from L. pneumophila (gi21666984) (2). All three proteins showed homology to the N-terminal half of L. pneumophila PlaB. LipB of L. pneumophila contains a lipase consensus sequence [((L/I/V)-X-(L/I/V/F/Y)-(L/I/V/M/S/T)-G-(H/Y/W/V)-S-X-G-(G/S/T/A/C)] (PROSITE accession no. PS00120) (4), which we found was substantially conserved in the Legionella hemolysis protein (78-FACHTGSTG-89). Neither LipB nor the hemolysin protein shares the first conserved glycine residue with the lipase consensus sequence; both contain a tyrosine residue instead, suggesting that both proteins may possess biochemical properties distinct from those of other lipases. Indeed, since an L. pneumophila lipB mutant released smaller amounts of fatty acids from tricaprylin but not from lipase substrates such as 1-MPG or 1,2-DG (4), there is still some ambiguity as to whether LipB should be considered a lipase. Using the conserved-domain search tool from the NCBI BLAST page, we found that the N-terminal ~270 amino acids mainly revealed regions homologous to hydrolytic enzymes, such as the lipA domain of predicted acetyltransferases and hydrolases with the α/β-hydrolase fold (COG1075), the PldB domains of lysophospholipase A (COG2267), and the lipase class 2 domain (pfam01674). Interestingly, the homologous Nostoc sp. protein, but not the P. aeruginosa protein or Legionella LipB, also shared homology with the C-terminal region of Legionella PlaB.

The protein sequence of L. pneumophila PlaB indicated similarities to lipolytic enzymes. Since the majority of the secreted lipid-hydrolyzing activity is exported depending on the type II protein secretion machinery (23, 48), we examined the protein sequence of the L. pneumophila hemolysis protein for a signal sequence. The protein was not predicted to have an N-terminal signal peptide, as tested with SignalP and the PSORT server, but was calculated to be located in the bacterial cytoplasm (41, 42).

Two uncharacterized genes, orf1 and orf2, flanked the L. pneumophila hemolysis gene (Fig. 1). The closest homolog of the orf1 protein product was the DlrA protein of Dictyostelium discoideum, and the closest homologs of the orf2 protein product were several hypothetical proteins from Mesorhizobium loti, Brucella melitensis, and Agrobacterium tumefaciens. Both the upstream orf1 gene and the downstream orf2 gene were oriented in the opposite direction from the hemolysis gene, suggesting that the hemolysis gene message is monocistronic. Farther upstream, two genes encoding proteins with homology to enzymes of the fatty acid oxidation complex were found.

Analysis of different Legionella strains for the presence of plaB genes. Since the species L. pneumophila has been most frequently associated with the development of Legionnaires’ disease, researchers often seek to identify virulence factors which are present only in L. pneumophila and not in less pathogenic species. Therefore, we were also interested in whether the gene coding for PlaB was present in Legionella species other than L. pneumophila. To investigate this, genomic DNAs of 16 L. pneumophila strains and 15 non-L. pneumophila strains were treated with restriction enzymes and examined by Southern blot analysis. We found that under low-stringency conditions, the plaB gene probe did hybridize exclusively to DNA from the L. pneumophila strains tested, and not to DNA from non-L. pneumophila species (data not shown). This indicates that the plaB gene may be restricted to L. pneumophila. However, even though a growing number of L. pneumophila-specific genes have been identified, in some cases the failure of cross-hybridization may arise from the high DNA diversity found in different Legionella strains.

Enzymatic activities of E. coli clones containing the L. pneumophila plaB gene. Since the protein sequence of the newly characterized L. pneumophila plaB gene showed sequence homology to lipolytic hydrolases, we tested both culture supernatants and cell lysates of recombinant E. coli clones containing the plaB gene for fatty acid release from various lipid substrates, in particular from diacylglycerophospholipids (DPPG and DPPC) to check for phospholipase A activity, from 1-monoacylphospholipids (lysophospholipids) (MPLPG and MPLPC) to examine for lysophospholipase A activity, and

FIG. 2. Hemolysis of human red blood cells by recombinant E. coli containing L. pneumophila plaB. (A) Human blood agar was inoculated with recombinant E. coli harboring different plasmids and then incubated for 48 h at 37°C. Plasmids contained the following inserts: pKH190 and pKH192, intact plaB; pKH194, disrupted plaB; pUC19, empty vector; phlyCABD, hemolysin-encoding operon of E. coli 536 in pUC18 (compare Fig. 1). (B) Recombinant E. coli containing either plain pBCKS+ or pBCKS+ with L. pneumophila plaB (pKH192) was grown to the logarithmic-growth phase and subsequently treated with IPTG. Afterwards, human red blood cells were incubated with the bacteria for 20 h and then quantitatively assessed for hemolysis. Results are means and standard deviations from triplicate cultures and are representative of three independent experiments.
from monoacylglycerol (1-MPG) to test for lipase activity. Both culture supernatants and cell lysates of the hemolytic *E. coli* clones containing either pKH190 or pKH192 released significantly more FFA from all of the tested lipid substrates than *E. coli* harboring the corresponding control vectors (Fig. 3). Clones containing *plaB* hydrolyzed predominantly the diacyl- and lysophospholipid substrates, showing that the respective protein product is both a phospholipase A and a lysophospholipase A. Moreover, the clone harboring pKH194 and its inactivated *plaB* gene did not liberate increased amounts of FFA from the different lipids (Fig. 3). Because the *Legionella plaB* gene cloned into *E. coli* predominantly possessed phospholipase A and lysophospholipase A activity, we designated the gene *plaB*, for phospholipase A gene B.

**Isolation of *L. pneumophila plaB* mutants.** In order to determine the degree to which *plaB* is responsible for phospholipase A-lysophospholipase A activity in *Legionella* and whether PlaB is a protein associated with the bacterial cell or transported into the culture supernatant, we constructed two sets of *L. pneumophila plaB* mutants. The first mutants were generated by cloning the interrupted *plaB* gene of pKH194 into pBOC20 and by subsequent allelic exchange forced by the counterselectable *sacB* gene present in pBOC20 (11, 43). Two *plaB* mutants (*plaB37* and *plaB60*) were obtained and confirmed by

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**FIG. 3.** Lipolytic activities of recombinant *E. coli* containing *L. pneumophila plaB*. Culture supernatants (A) and cell lysates (B) of *E. coli* containing pBCKS or its derivative pKH192, or pUC18 or its derivative pKH190 or pKH194, were mixed with DPPG, DPPC, MPLPG, MPLPC, or 1-MPG. In some cases, culture supernatants or cell lysates were diluted prior to incubation with lipids as indicated. After a 2-h (A) or 50-min (B) incubation at 37°C, the release of FFA was quantified. Data are expressed as differences between the amount of FFA released by the culture supernatant or cell lysate and the amount released by uninoculated LB broth or Tris-HCl buffer, respectively. Results are means and standard deviations of duplicate cultures and are representative of three independent experiments.
PCR and Southern blot analysis (data not shown). The second set of mutants was generated by using pKH194 without further cloning steps and with allelic exchange to introduce a Kmr cassette into the chromosomal plaB gene of strain Corby. Two plaB mutants (plaB1 and plaB4) were obtained following two separate DNA transformations and allelic exchange selections. PCR and Southern blot analysis confirmed the mutations in plaB (data not shown). All of the subsequent experiments involving enzymatic and hemolytic activities, except genetic complementation, were performed with plaB37, plaB60, plaB1, and plaB4 with comparable results, although in most cases data for plaB60 and in some cases also for plaB1 are presented. In host cell infection assays, all four mutants were tested in amebae, whereupon mutants plaB1 and plaB4 behaved similarly, but mutants plaB37 and plaB60 were found to contain a second-site mutation in a uncharacterized gene (data not shown). The plaB1, plaB37, and plaB60 mutants were examined for intracellular growth in U937 macrophages and yielded comparable results. Furthermore, a plaB mutant was generated in L. pneumophila strain 130b (ATCC strain BAA-74, also known as Wadsworth or AA100) by the second mutagenesis method. This mutant behaved similarly to L. pneumophila Corby mutants plaB1 and plaB4 in the enzymatic as well as the host cell infection assays (data not shown).

To assess the importance of plaB for the extracellular growth of L. pneumophila, we compared the growth of strain Corby

FIG. 4. Lipolytic activities of wild type, plaB mutant, and genetically complemented L. pneumophila strains. Culture supernatants (A) and cell lysates (B) of wild type, plaB mutant, and genetically complemented L. pneumophila strains were incubated with DPPG, DPPC, MPLPG, MPLPC, or 1-MPG for 2 h (A) or 30 min (B) at 37°C, and then the release of FFA was quantified. In some cases, culture supernatants or cell lysates were diluted with lipids as indicated prior to incubation. Data are expressed as differences between the amount of FFA released by the culture supernatant or cell lysate and the amount released by uninoculated BYE broth or Tris-HCl buffer, respectively. Results are means and standard deviations from duplicate cultures and are representative of three independent experiments.
Lipolytic activities of an L. pneumophila plaB mutant. L. pneumophila has been shown to secrete several lipolytic activities into the culture supernatant as well as to possess lipolytic activities associated with the bacterial cell (3, 4, 22, 23, 25, 40). To assess the plaB mutant with respect to lipid hydrolysis, we tested both culture supernatants and bacterial-cell lysates for their abilities to release FFA from DPPG, DPPC, MPLPG, MPLPC, and 1-MPG. The supernatants of wild-type L. pneumophila as well as those of mutants plaB60 and plaB1 showed comparable hydrolysis of DPPG, DPPC, MPLPG, MPLPC, and 1-MPG, suggesting that PlaB is not secreted into the culture supernatant of L. pneumophila (Fig. 4A). Next, we examined whether plaB contributes to the cell-associated lipolytic activities of L. pneumophila. Indeed, the cell-associated activities hydrolyzing DPPG, DPPC, MPLPG, and MPLPC were dramatically reduced in the plaB mutants (Fig. 4B).

These data show that plaB is the gene for the major cell-associated phospholipase A-lyso phospholipase A activity of L. pneumophila. The ability of plaB60 to fully release FFA from DPPG, DPPC, MPLPG, and MPLPC was restored after trans-complementation with plaB on plasmid pKH192 (Fig. 4C). The activities of both the complemented wild type and complemented plaB60 against DPPG, DPPC, MPLPG, and MPLPC were more than 10-fold higher than wild-type activities, a result that is likely due to multiple copies of plaB.

To confirm that the plaB mutant had altered lipolytic activities, we used TLC to examine the pattern of phospholipid cleavage caused by cell lysates. In agreement with our FFA release data from the colorimetric assay, strain Corby cell lysates almost completely hydrolyzed the DPPG and DPPC substrates and liberated large amounts of FFA (Fig. 5A and B). Only minor amounts of lysophospholipids were detected, corroborating that the cell-associated lipolytic activity of L. pneumophila exhibits both phospholipase A and lysophospholipase A activity. Smaller amounts of FFA were also observed for 1-MPG incubations with L. pneumophila wild-type cell lysates; however, no detectable amounts of FFA were released from 1,2-DG or TG, indicating that the cell-associated lipolytic activity of L. pneumophila preferentially hydrolyzes phospholipids (Fig. 5C).

The phenotype of the L. pneumophila plaB mutant was fully complemented by a plasmid copy of plaB (Fig. 5). In summary,
was determined by OD \textsubscript{415} readings. When the bacteria were examined whether the lytic activity of \textit{L. pneumophila} we examined whether the wild-type (Corby), \textit{plaB} mutant (\textit{plaB60}), and genetically complemented \textit{L. pneumophila} strains were grown for 24 h at 37°C on BCYE agar plates. Afterward, the bacteria were added to human red blood cells, centrifuged onto the cells or not, incubated for 7 h at 37°C, and then quantitatively assessed for hemolysis. SDS served as a positive control, which was able to lyse 100% of the red blood cells. As a negative control, PBS was added to the cells instead of bacteria. Results are means and standard deviations from triplicate cultures and are representative of three independent experiments.

\textit{L. pneumophila} \textit{plaB} is a cell-associated phospholipase A and lyso phospholipase A that preferentially cleaves phospholipids (both diacylphospholipids and lysophospholipids) and, to a lesser extent, also hydrolyzes the nonphospholipid 1-MPG.

**Hemolytic activities of an \textit{L. pneumophila} \textit{plaB} mutant.** Next, we examined whether the \textit{plaB} gene contributes to the hemolytic activity of \textit{L. pneumophila}. \textit{L. pneumophila} harboring \textit{pKH192}, which contains the \textit{plaB} gene, caused faster and more prominent hemolysis on human blood agar plates than the wild-type strain (data not shown). To confirm and quantitatively examine this observation, we additionally used a hemolysis assay, where the different bacterial strains were added to human red blood cells and, after 7 h of incubation, hemolysis was determined by OD\textsubscript{415} readings. When the bacteria were centrifuged onto red blood cells, wild-type \textit{L. pneumophila} harboring \textit{pBCKS}+ lysed approximately 50% of the cells (Fig. 6). A \textit{plaB} mutant containing \textit{pBCKS}+ lysed less than 40% of the red blood cells, demonstrating that \textit{L. pneumophila} \textit{PlaB} contributes to the hemolytic activity of the bacterium but is, as expected, not the only \textit{L. pneumophila} protein mediating hemolysis. Overexpression of \textit{plaB} in \textit{L. pneumophila} led to two-fold-higher hemolysis of red blood cells relative to that by the wild type strain. Additionally, an intact \textit{plaB} gene reintroduced on a plasmid into an \textit{L. pneumophila} \textit{plaB} mutant compensated for the loss of hemolytic activity. The prominent hemolytic activity of \textit{L. pneumophila} has been shown to be contact dependent (36). In agreement with this finding, we observed that centrifugation of the bacteria onto the red blood cells potentiated hemolysis and made the hemolysis defect of the \textit{plaB} mutant detectable (Fig. 6). In conclusion, we have shown that \textit{plaB} is partially responsible for the hemolytic phenotype of \textit{L. pneumophila}.

**Intracellular infection by \textit{L. pneumophila} \textit{plaB} mutants.** After enzymatically profiling the \textit{plaB} knockout mutation in \textit{L. pneumophila}, we investigated the importance of the hemolytic phospholipase A \textit{PlaB} for the intracellular infection of \textit{A. castellanii} amoebae and U937 macrophages by \textit{L. pneumophila}. To that end, we compared the abilities of \textit{L. pneumophila} Corby and \textit{plaB} mutants to grow in amoebae. Whereas strain Corby and mutants \textit{plaB1} and \textit{plaB4} revealed the typical pattern of intracellular growth, in which bacterial numbers increased about 10,000- to 100,000-fold by 48 h postinoculation, the numbers of mutants \textit{plaB37} and \textit{plaB60} increased only 100- to 1,000-fold (Fig. 7A and data not shown). Complementation experiments with mutants \textit{plaB37} and \textit{plaB60} clarified that the defect in intracellular infection was caused by a mutation other than that in \textit{plaB} (data not shown). The nature of this mutation was not further studied here. To determine the role of \textit{PlaB} in U937 cell infection, macrophage cultures were inoculated with wild-type or \textit{plaB} mutant \textit{L. pneumophila} and the numbers of bacteria were recorded at different times. Comparable numbers of wild-type and mutant bacteria were recovered from the macrophage cocultures (Fig. 7B). Taken together, our data...

![Graph showing OD\textsubscript{415} readings for different strains with and without centrifugation.](http://iai.asm.org/)

![Graph showing growth of wild-type and \textit{plaB} mutant strains in A. castellanii and U937 cells.](http://iai.asm.org/)
indicate that plaB is not required for intracellular infection by L. pneumophila.

**DISCUSSION**

In this investigation, we identified the gene for a new hemolytic protein of L. pneumophila, the major cell-associated phospholipase A PlaB. L. pneumophila PlaB releases fatty acids from a variety of phospholipids and lysophospholipids, and also, to a lesser extent, from the nonphospholipid 1-MPG, but not in detectable amounts from the respective diacyl- and triacylglycerols, showing that it is a phospholipase A with only a low affinity for typical lipase substrates. The finding that enzymes hydrolyzing predominantly phospholipids can also degrade nonphospholipids has been reported for other enzymes. For example, the L. pneumophila lysophospholipase A PlaA primarily hydrolyzes lysophospholipids but can also release FFA to a lesser extent from 1-MPG (25). Furthermore, the Staphylococcus hyicus lipase turned out to preferentially degrade phospholipids over nonphospholipids (57). L. pneumophila PlaB contains a short region of similarity to a lipase consensus motif which is also conserved in lipase family I, including lipases from P. aeruginosa (accession no. D50587) and Bacillus subtilis (M74010) and the phospholipase A from S. hyicus (X02844) (5). Enzymes of this family possess a catalytic triad characterized by the nucleophile agent serine, embedded in the consensus sequence GXXG, aspartate, and histidine (5). Interestingly, the first glycine residue of the putative nucleophile-containing motif is not conserved in L. pneumophila PlaB. It is also not conserved in L. pneumophila LipB; both of these proteins contain a tyrosine residue instead (4). The lipid substrates hydrolyzed by LipB and its location in the bacterial cell still need to be elucidated, but since PlaB and LipB show sequence homology in one of their putative catalytic domains, it seems reasonable to test LipB for phospholipase A activity. This is also supported by the observation that outer membrane phospholipases of several gram-negative bacteria, such as E. coli, Helicobacter pylori, and Salmonella enterica serovar Typhimurium, also comprise a primary sequence motif with the catalytically active serine where the first glycine is replaced by a histidine residue, which serves as one of the other members of the catalytic triad (15). The two other important catalytic domains of lipase family I, containing the residues aspartate (motif NDGLV) and histidine [motif (N/D)HLD], were not found to be recognizably conserved in PlaB. Interestingly, the motif HPT, in a region where the catalytically active histidine would be expected, was found in PlaB. This motif is conserved in lipolytic enzymes of the GDSL family, for example, in L. pneumophila lysophospholipase PlaA (5, 25). The amino acid environment of the catalytically active histidine, in particular, has been shown to be important for expression of either lipase or phospholipase A activity. For example, S. hyicus phospholipase shares high conservation of the catalytic domains with other lipases of family I, with one important exception. The amino acid adjacent to the catalytically active histidine is a serine, a more polar amino acid, instead of the hydrophobic residues leucine, valine, etc., present in most other lipases. Replacement of serine 356 by valine decreased phospholipase activity more than 10-fold (56); additionally, the lipase of Bacillus thermocatenulatus was converted into a phospholipase A by replacement of the leucine adjacent to the active-site histidine with serine (34). The question of which sequential or structural determinant defines the phospholipase activity of L. pneumophila PlaB needs further elucidation, for example, by site-directed mutagenesis and by solution of the three-dimensional structure.

In contrast to L. pneumophila LipB, PlaB contains a C-terminal extension of about 200 amino acids adjacent to the potential catalytic active domain. At the moment, it is difficult to even speculate on the possible functions of this domain. However, it is noteworthy that the L. pneumophila plaB mutants constructed in this study were defective in cell-associated phospholipase A activity although the Km' cassette disrupting the plaB gene was placed after the putative catalytic domains, suggesting that the C terminus might be important for activation, stability, or proper transport of PlaB. Indeed, the cytotoxic activity of the type III secreted P. aeruginosa cytotoxin ExoU, recently found to be a phospholipase A (45, 50), has been suggested to depend on the C-terminal region adjacent to the catalytic domain, since a mutant containing a transposon insertion 88 nucleotides from the exoU stop codon secretes a stable protein but is defective in cell killing (29). Furthermore, several type I secreted bacterial proteins, such as the E. coli hemolysin HlyA or the Erwinia chrysanthemi metallopeptase PrtG, contain their signal for transport via an ABC transporter in their C termini (8). A putative type I secretion system has recently been identified in L. pneumophila (32). Since an L. pneumophila plaB mutant showed reduced red blood cell lysis activity upon bacterial contact with the target cell, we believe that PlaB is located in the outer bacterial membrane and is presented to the external environment. Interestingly, PlaB seems not to contain a recognizable signal sequence, suggesting that it is not sec-dependently transported to the periplasm. Further experiments are necessary to explore the mechanisms involved in PlaB export and activation.

L. pneumophila PlaB shares several properties with the outer membrane phospholipase A (OMPLA) of several enteropathogenic bacteria such as E. coli or Salmonella serovar Typhimurium and other gram-negative bacteria such as H. pylori or Neisseria meningitidis. As shown here for PlaB, OMPLA also hydrolyzes phospholipids, lysophospholipids, and nonphospholipids; under laboratory conditions, the enzyme is not essential for the growth rate; and in C. coli and H. pylori, OMPLA has been found to be hemolytic (15, 18, 27). Whether other characteristics—(i) involvement of a nucleophilic serine in catalysis, (ii) activation of OMPLA upon bacterial disintegration, (iii) regulation of OMPLA activity by dimerization, (iv) requirement of calcium ions for activity, or (v) contribution to bacterial pathogenesis—are true for PlaB, as they are for H. pylori OMPLA, remains to be determined (15, 18).

We have shown that L. pneumophila PlaB is a phospholipase A contributing to the total cytotoxic activity of the pathogen. Other bacterial factors involved in the destruction of eukaryotic cells, such as the RtxA toxin (12) or the icm/dot gene products (1, 36, 40), have already been characterized. However, especially in the case of the icm/dot genes, which code for a type IV protein secretion system (52, 58), it is possible that these determinants do not represent the structural genes actually accounting for the cell-lysing agent. The genes might rather code for proteins important for toxin translocation.
In conclusion, we have shown that L. pneumophila pldB is the gene for the major cell-associated phospholipase A exhibiting hemolytic activity on human red blood cells. The hemolytic activity of PlaB could be one factor among others contributing to bacterial cytotoxicity, but it is not essential for intracellular infection of A. castellanii amoebae or U937 macrophages. Future experiments will be designed to examine the catalytic active residues necessary for phospholipase and hemolytic activities as well as the impact of the different protein domains of PlaB on its activation and transport.

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