

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 diacylphospho- and lysophospholipids, 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.

Legionella pneumophila is an inhabitant of fresh water, where it intracellularly colonizes protozoa (20). When bacteria-laden aerosols are inhaled by humans, *L. pneumophila* exploits alveolar macrophages and epithelial cells for its multiplication, leading to a severe pneumonia characterized by destruction of alveolar cells (60). The cytopathology of Legionnaires' disease involves several cytotoxic or hemolytic factors produced by *L. pneumophila*, for example, the zinc metalloprotease ProA, the legiolysin Lly, and several pore-forming toxins, one of which is an RTX (repeats in structural toxin) protein (12, 26, 31, 35, 36, 47, 61). The zinc metalloprotease ProA is the major extracellular protease of *L. pneumophila* and its export depends on the *L. pneumophila* type II protein secretion system (28, 37). The enzyme hydrolyzes a broad spectrum of protein substrates and confers hemolytic as well as cytolytic activities (47, 53). Additionally, ProA has been shown to contribute to bacterial pathogenesis in a guinea pig model of pneumonia (38). Another hemolytic, but not cytotoxic, protein is the *L. pneumophila* legiolysin Lly, which is also responsible for color production and fluorescence of the bacterium (61). Pore-forming activities of *L. pneumophila* confer contact-dependent hemolytic and cytotoxic activities toward a variety of cells, especially at high bacterial numbers (12, 31, 36). The *L. pneumophila* RTX toxin RtxA has been shown to contribute to cytotoxicity, to pore formation, and to the initial steps of infection, namely, adherence and entry of the bacterium into

host cells (12, 13). Pore formation is furthermore an indispensable step in bacterial egress after bacterial intracellular multiplication in both amoebae and macrophages (1, 40). Pore formation in *L. pneumophila* depends on the integrity of several genes of the *icm/dot* locus (e.g., *dotA*, *icmQ*, *icmR*, and *icmT*), responsible for the expression of a type IV protein secretion machinery involved in pathogenesis (14, 36, 40).

In addition to proteases and pore-forming toxins, bacterial lipolytic enzymes are known to confer hemolytic and cytotoxic activities by the destruction of cell membrane lipids and/or generation of membrane-perturbing reaction products, as shown for *Clostridium perfringens* alpha-toxin, a phospholipase C, or *Campylobacter coli* PldA, a phospholipase A (27, 54, 59). *L. pneumophila* also possesses a variety of lipolytic activities, including lipase, phospholipase A, and lysophospholipase A activities, secreted into the culture medium as well as associated with the bacterium (3, 4, 22, 23, 25). So far, research has focused primarily on activities secreted into the extracellular environment. The lipase LipA and the putative lipase/esterase LipB hydrolyze esters of palmitic or caprylic acid, respectively, but are not essential for the cytopathogenicity and intracellular survival of *L. pneumophila* in U937 macrophages and *Hartmannella* amoebae (4). The lysophospholipase A PlaA, specific for lysophospholipid hydrolysis, detoxifies lysophosphatidylcholine, a pore-forming agent which can be generated, for example, by the action of *Legionella* phospholipase A on lung surfactant (24, 25). *plaA* knockout mutants are not impaired in their intracellular infection of macrophages and amoebae (25). The secreted phospholipase A activities hydrolyzing diacylphospholipids, as well as the cell-associated phospholipase

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A 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 *Escherichia 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 Msp19 (7), *L. pneumophila* sg1 strain 685 (7), *L. pneumophila* sg3 strain Bloomington (ATCC 33155), *L. pneumophila* sg3 strain U22 (7), *L. pneumophila* sg4 strain Los Angeles (ATCC 33156), *L. pneumophila* sg6 strain Chicago-2 (ATCC 33215), *L. pneumophila* 664 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. dumoffii* (ATCC 33279), *L. erythra* (51), *L. gormanii* (ATCC 33297), *L. hackeliae* sg1 and sg2 (ATCC 33250 and ATCC 35999, respectively), *L. israelensis* (ATCC 43119), *L. longbeachae* sg1 and sg2 (ATCC 33462 and ATCC 33484, respectively), *L. micdadei* (ATCC 33218), *L. oakridgensis* (ATCC 33761), and *Sarcobium* (*Legionella*) *lyticum* (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 (BYE) broth 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 DU520 (Beckman Coulter, Unterschleißheim, Germany), following inoculation to an OD₆₆₀ of 0.2 to 0.3. *E. coli* strain DH5 α , 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) *cytA* knockout mutant was kindly provided by Christian Hüttinger (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 μ g/ml, respectively; chloramphenicol at 6 or 30 μ g/ml, respectively; and ampicillin at 100 μ g/ml.

Preparation of culture supernatants and cell lysates. Culture supernatants for assessment of hydrolytic activities were obtained at the end of exponential growth (OD₆₆₀: 2.2 to 2.3) by centrifugation for 5 min at 5,000 \times 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* DH5 α 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 *phlyCABD*) or pUC19 (Amersham Biosciences, Freiburg, Germany), pBCKS+ (backbone in plasmid pKH192; Stratagene, Heidelberg, Germany), and pBOC20 (backbone in plasmid pKH195) (44). Genomic and plasmid DNAs were prepared according to standard protocols (49). PCR was carried out using a TRIO-Thermoblock or a T-Gradient thermocycler (Biometra, Göttingen, Germany) and AmpliTaq polymerase (Perkin-Elmer, Weiterstadt, Germany) or *Taq* DNA polymerase (New England Biolabs, Frankfurt am Main, Germany). Foreign DNA was introduced into bacterial strains by electroporation with a Bio-Rad (Munich, Germany) gene pulser according to the manufacturer's specifications. *E. coli* or *L. pneumophila* strains were electroporated at 2.0 or 2.3 kV, respectively, 200 or 100 Ω , respectively, and 25 mF. Both strands of plasmid DNA were sequenced with infrared dye-labeled primers by using an automated DNA sequencer (LI-COR-DNA 4000; MWG-Biotech, Ebersberg, Germany). Nucleotide and translated protein sequences were analyzed by using the Genetics Computer Group package, the PEDANT website (<http://pedant.gsf.de/>), SMART (<http://smart.embl-heidelberg.de>), the SignalP program (42), and the *L. pneumophila* genome project web page (<http://genome3.cpmc.columbia.edu/~legion/>). Sequence database searches as well as protein alignments were performed by the BLAST algorithm (2).

Southern hybridization. Chromosomal DNA from various *Legionella* strains was digested with SacII and HindIII and then subjected to electrophoresis, and fragments were transferred to a nylon membrane (Pall, Dreieich, Germany) by capillary blotting. The DNA probe, generated from primers *pla1* (5'-GAATTC

AGTATAAAATAATCTAATATC-3'; located 70 bp in front of the start codon) and *pla2* (5'-TCTAGATTAATCTATCTTTTCCAGTTG-3'; ending with the stop codon of *plaB*), containing the complete *L. pneumophila* *plaB* gene, was used as a *plaB*-specific probe. DNA probes were labeled and detected by using the nonradioactive enhanced chemiluminescence detection kit (ECL; Amersham Biosciences). Hybridization was performed at 42°C overnight. Membranes were washed twice, for 10 min each time, at 42°C with wash buffer I (2 \times SSC [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate]-0.1% sodium dodecyl sulfate [SDS]) and twice, for 5 min each time, at room temperature with wash buffer II (2 \times SSC). Detection was carried out as described previously (30).

Gene cloning and *Legionella* mutant construction. Two different strategies were used for *L. pneumophila* *plaB* mutant generation. For both, a plasmid containing the disrupted *plaB* gene and allelic exchange were used to introduce a Km^r insertion mutation into the chromosome of strain Corby. The complete *plaB* gene was amplified by primers *pla1* and *pla2*, cloned into pUC18, yielding pKH190, and sequenced. A Km^r cassette was then inserted into the SacII restriction site of the *plaB* gene of pKH190, resulting in pKH194 (see Fig. 1). This construct was subcloned into pBOC20, yielding pKH195. *L. pneumophila* *plaB* knockout mutants were generated by electroporation of pKH195 into *L. pneumophila* Corby and allelic exchange. Screening for mutants obtained by double crossover was performed as described recently (17). Integration of the Km^r cassette into the chromosomal *plaB* gene was verified by Southern blot analysis (data not shown). The second method for mutant generation involved the introduction of plasmid pKH194 into *L. pneumophila* Corby and of the Km^r cassette into the chromosomal *plaB* gene by natural transformation and homolog recombination (25). PCR and Southern blot analysis were used to examine Km^r legionellae for the presence of the *plaB* mutation (49). For complementation studies, the complete *plaB* gene of pKH190 was subcloned into vector pBCKS+, resulting in plasmid pKH192 (see Fig. 1). pBCKS+ and pKH192 were introduced into wild-type and mutant *L. pneumophila* by electroporation.

Hemolysis assays. For detection of hemolytic activity of *L. pneumophila* or *E. coli* strains on blood agar media, 30 ml of blood/liter was added to BCYE- (without charcoal) or LB agar medium, respectively. Bacterial strains were inoculated onto human or sheep blood agar plates and incubated for 48 h at 37°C. Recombinant *E. coli* and wild-type, mutant, 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 OD₆₀₀ of 1, 2 mM isopropyl- β -D-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 OD₆₀₀ of 1. A 200- μ l volume of *E. coli* or *L. pneumophila* bacteria was mixed with 800 μ l 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 \times 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 \times g for 2 min), and the OD₄₁₅ of the supernatant was determined.

Enzymatic assay for lipolytic activities. Enzymatic activities were detected 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-monopalmitoyllysophosphatidylcholine [MPLPC], 1-monopalmitoyllysophosphatidylglycerol [MPLPG], 1-monopalmitoyllycylglycerol [1-MPG], 1,2-dipalmitoylphosphatidylglycerol [DPPG], 1,2-dipalmitoylphosphatidylcholine [DPPC], 1,2-dipalmitoyllycylglycerol [1,2-DG], or tripalmitoyllycylglycerol [TG]), 3 mM Na₂S₂O₈, 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 (Sonoplus; Bandelin, Berlin, Germany) three times, for 15 s each time, at cycle 4 \times 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 subsequently used for separation of lipids by 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/vol)

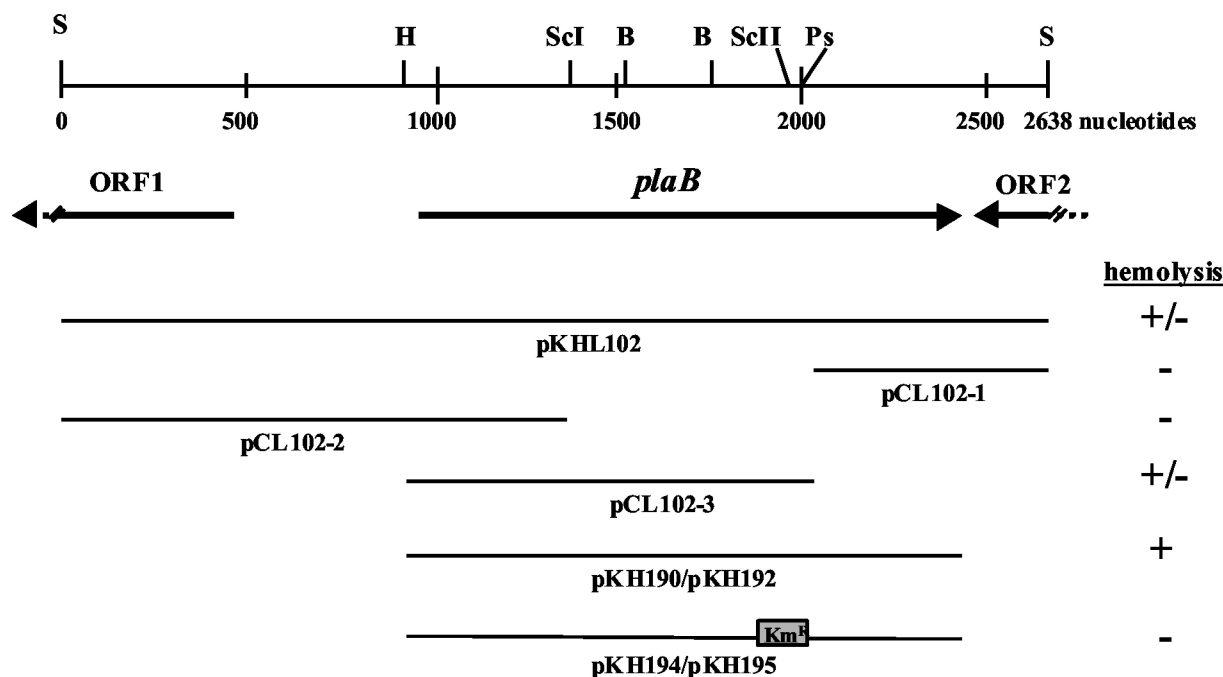


FIG. 1. *plaB* locus in *L. pneumophila* and recombinant *E. coli*. (Top) Diagram represents the *L. pneumophila* chromosome region that contains the phospholipase A gene *plaB*, along with the locations of the relevant restriction enzyme sites (B, BamHI; H, HindIII; Ps, PstI; S, Sau3A1; ScI, SacI; ScII, 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^r gene cassette. Plus and minus signs indicate whether recombinant *E. coli* exhibited hemolytic activity on human red blood agar plates.

ratio (22, 55). A mixture of *n*-hexane–diethyl ether–glacial acetic acid in a 70:30:4 (vol/vol/vol) ratio was used for separation of apolar lipids (22). For visualization, silica plates were then stained with naphthol blue black (Aldrich, Milwaukee, Wis.) (25, 46).

Intracellular infection of U937 cells and *Acanthamoeba castellanii* amoebae. *A. castellanii* amoebae and U937 (CRL-1593.2; American Type Culture Collection, Manassas, Va.), a human cell line that differentiates into macrophage-like cells upon treatment with phorbol esters (incubation for 36 to 48 h with 80 nM phorbol-12-myristate-13-acetate [P-8139; Sigma Chemicals]), were used as hosts for in vitro infection by *L. pneumophila* (10, 39). Amoebae and U937 cells were maintained and infected as previously described (10, 37, 39). To assess the intracellular growth of *L. pneumophila*, wells containing amoebae or U937 cells at a concentration of 10^5 or 10^6 /ml, respectively, were infected with wild-type bacteria or isogenic mutants at a multiplicity of infection of 0.01 for amoebal and 1 for U937 cell infections (time point, 0 h). U937 macrophages were incubated for 2 h with the added bacteria in plain RPMI; then monolayers were washed three times with plain RPMI to remove unbound bacteria and were subsequently incubated with RPMI containing 10% (vol/vol) fetal calf serum (PAA, Linz, Austria). At various time points, cocultures of U937 cells and legionellae were treated with 10% (wt/vol) saponin (Sigma Chemicals) for lysis of the host cells, and serial dilutions were plated on BCYE agar. The number of bacteria within the amoebal coculture was determined by plating serial dilutions on BCYE agar.

Nucleotide sequence accession number. The *L. pneumophila* Corby *plaB* sequence has been deposited in GenBank at the National Center for Biotechnology Information (NCBI) under accession no. AJ565849.

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 hemo-

lytic 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 pKHL102 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 pKHL102 (Fig. 1). In pKHL102, 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^r 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). It was also noted that clones harboring plasmids with *L. pneumophila* *plaB* did not show hemolytic activity on sheep blood agar and were also not hemolytic when human blood agar was incubated at 30 instead of 37°C (data not shown). Independently of blood cell origin and incubation temperature, *E. coli* containing the *E. coli* hemolysin locus *hlyCABD* on plasmid pUC18 was able to cause hemolysis un-

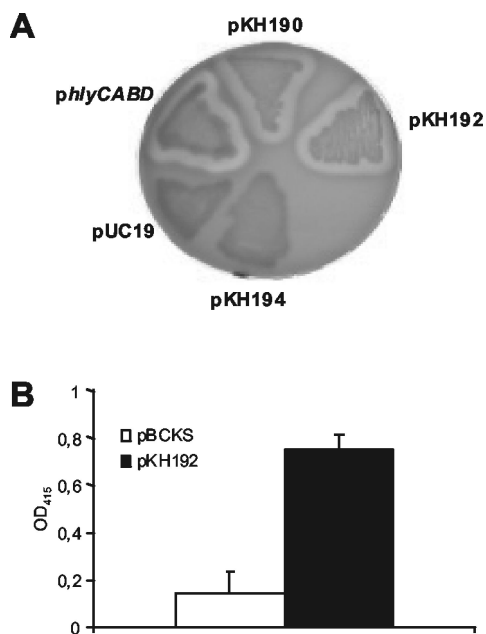


FIG. 2. Hemolysis of human red blood cells by recombinant *E. coli* containing *L. pneumophila* *plaB*. (A) Human blood agar was inoculated with recombinant *E. coli* harboring different plasmids and then incubated for 48 h at 37°C. Plasmids contained the following inserts: pKH190 and pKH192, intact *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.

der 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* hemolysin protein (78-FACITHSTGG-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* hemolysin 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* hemolysin 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 hemolysin gene, suggesting that the hemolysin 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

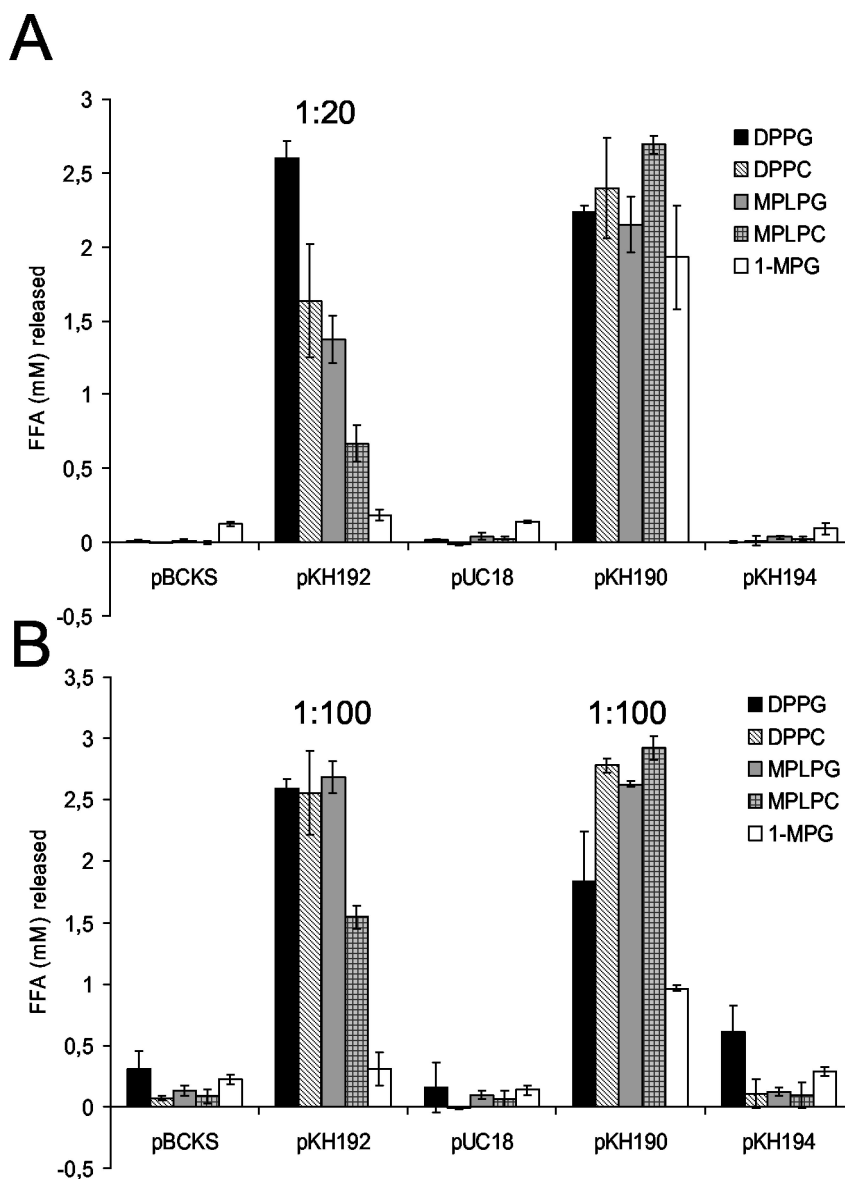


FIG. 3. Lipolytic activities of recombinant *E. coli* containing *L. pneumophila* *plaB*. Culture supernatants (A) and cell lysates (B) of *E. coli* containing pBCKS or its derivative pKH192, or pUC18 or its derivative pKH190 or pKH194, were mixed with DPPG, DPPC, MPLPG, MPLPC, or 1-MPG. In some cases, 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.

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 phospho-

lipase 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

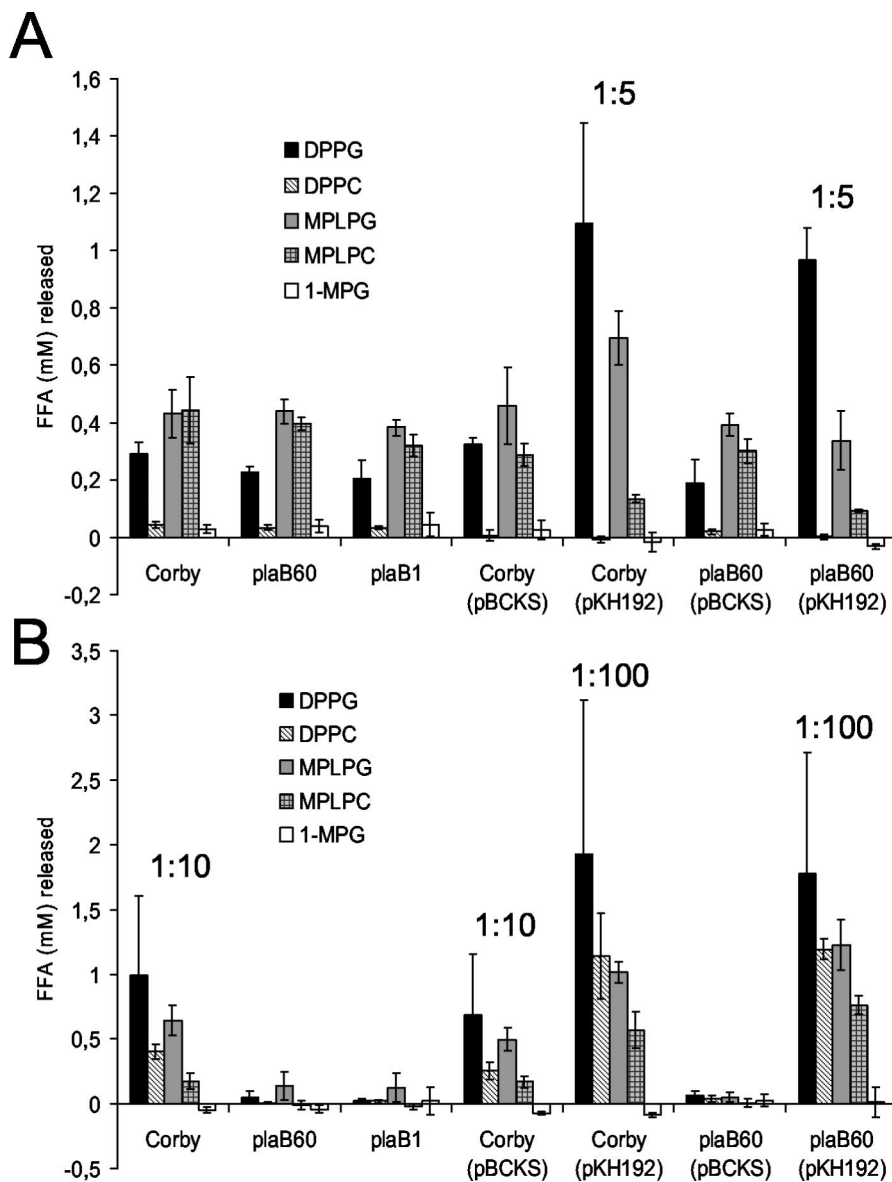


FIG. 4. Lipolytic activities of wild type, *plaB* mutant, and genetically complemented *L. pneumophila* strains. Culture supernatants (A) and cell lysates (B) of wild type, *plaB* mutant, and genetically complemented *L. pneumophila* strains were incubated with DPPG, DPPC, MPLPG, MPLPC, or 1-MPG for 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.

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 Km^r 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 amoe-

bae, whereupon mutants *plaB1* and *plaB4* behaved similarly, but mutants *plaB37* and *plaB60* were found to contain a second-site mutation in an 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

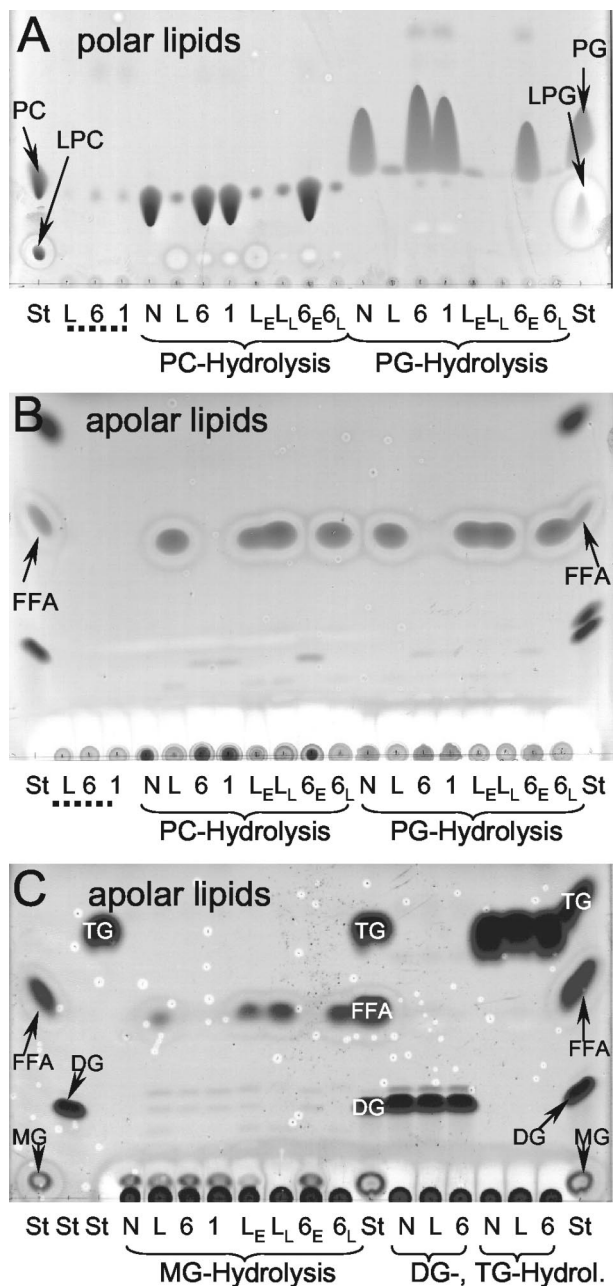


FIG. 5. TLC analysis of lipid hydrolysis by cell lysates of wild-type, *plaB* mutant, and genetically complemented *L. pneumophila* strains. Cell lysates of wild-type *L. pneumophila* (L), *plaB* mutants *plaB60* (6) and *plaB1* (1), or genetically complemented *L. pneumophila* (wild type and *plaB60* mutant harboring the empty vector [L_E and 6_E, respectively]) and wild type and *plaB60* mutant harboring pKH192, containing intact *plaB* [L_L and 6_L, respectively]) were incubated with DPPC (PC) or DPPG (PG) (A and B) or with 1-MPG (MG), 1,2-DG (DG), or TG (C) for 24 h at 37°C, and then lipids were extracted and subjected to TLC. A polar or apolar solvent mixture was used for the separation of the polar (A) or apolar (B and C) lipids, respectively. A mixture of Tris-HCl buffer and the lipids was also incubated and served as a negative control (N). Furthermore, bacterial-cell lysates not incubated with any lipid were extracted and served as the bacterial lipid background control (designated by dotted lines). In all incubations, samples were examined for degradation of the lipid substrate (for DPPC and DPPG, polar TLC [A]); for 1-MPG, 1,2-DG, and TG, apolar TLC [C]), enrichment of FFA (apolar TLC [B and C]), and in the case of DPPC and DPPG, incubations for enrichment of the respective

and the *plaB* mutants in BYE broth, the standard medium for culturing legionellae. As measured by the OD₆₆₀ of the cultures, the *plaB* mutants grew comparably to the wild type throughout the logarithmic- and stationary-growth phases when incubated at 37°C with shaking. Furthermore, the mutants grew normally on BCYE agar, the standard *Legionella* medium. Thus, these observations indicate that *plaB* is not required for normal extracellular growth in liquid or solid bacteriological media (data not shown).

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-lysophospholipase 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. 4). 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). On the other hand, cell lysates of *plaB* mutants did not release detectable amounts of FFA from any of the substrates examined (Fig. 5), showing that *plaB* is responsible for the cell-associated lipolytic activities of *L. pneumophila*. The phenotype of the *L. pneumophila plaB* mutant was fully complemented by a plasmid copy of *plaB* (Fig. 5). In summary,

lysophospholipid (polar TLC [A]). For qualitative identification of the lipid spots, lanes containing lipid standards (St) were included. The observations depicted here were made on one more occasion.

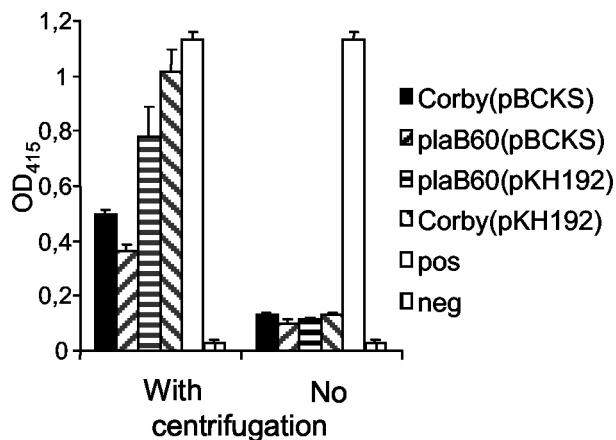


FIG. 6. Hemolysis of human red blood cells by recombinant wild-type, *plaB* mutant, and genetically complemented *L. pneumophila* strains. Wild-type (Corby), *plaB* mutant (*plaB60*), and genetically complemented *L. pneumophila* strains were grown for 24 h at 37°C on BCYE agar plates. Afterward, the 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.

L. pneumophila PlaB is a cell-associated phospholipase A and lysophospholipase A that preferentially cleaves phospholipids (both diacylphospholipids and lysophospholipids) and, to a lesser extent, also hydrolyzes the nonphospholipid 1-MPG.

Hemolytic activities of an *L. pneumophila plaB* mutant. Next, we examined whether the *plaB* gene contributes to the hemolytic activity of *L. pneumophila*. *L. pneumophila* harboring pKH192, which contains the *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 OD₄₁₅ readings. When the bacteria were centrifuged onto red blood cells, wild-type *L. pneumophila* harboring pBCKS+ lysed approximately 50% of the cells (Fig. 6). A *plaB* mutant containing pBCKS+ lysed less than 40% of the red blood cells, demonstrating that *L. pneumophila* PlaB contributes to the hemolytic activity of the bacterium but is, as expected, not the only *L. pneumophila* protein mediating hemolysis. Overexpression of *plaB* in *L. pneumophila* led to two-fold-higher hemolysis of red blood cells relative to that by the wild type strain. Additionally, an intact *plaB* gene reintroduced on a plasmid into an *L. pneumophila plaB* mutant compensated for the loss of hemolytic activity. The prominent hemolytic activity of *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 *plaB* mutant detectable (Fig. 6). In conclusion, we have shown that *plaB* is partially responsible for the hemolytic phenotype of *L. pneumophila*.

Intracellular infection by *L. pneumophila plaB* mutants. After enzymatically profiling the *plaB* knockout mutation in

L. pneumophila, we investigated the importance of the hemolytic phospholipase A PlaB for the intracellular infection of *A. castellanii* amoebae and U937 macrophages by *L. pneumophila*. To that end, we compared the abilities of *L. pneumophila* Corby and *plaB* mutants to grow in amoebae. Whereas strain Corby and mutants *plaB1* and *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 *plaB37* and *plaB60* increased only 100- to 1,000-fold (Fig. 7A and data not shown). Complementation experiments with mutants *plaB37* and *plaB60* clarified that the defect in intracellular infection was caused by a mutation other than that in *plaB* (data not shown). The nature of this mutation was not further studied here. To determine the role of PlaB in U937 cell infection, macrophage cultures were inoculated with wild-type or *plaB* mutant *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

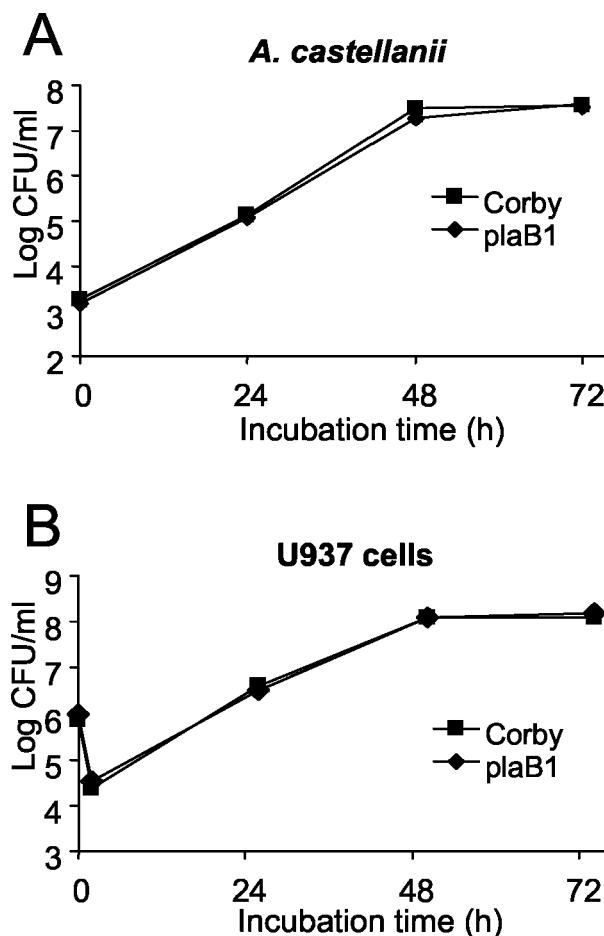


FIG. 7. Intracellular infection by wild-type and *plaB* mutant *L. pneumophila*. Strains Corby and *plaB1* were used to infect monolayers of *A. castellanii* amoebae (A) or cultures of U937 macrophages (B) at a multiplicity of infection of 0.01 or 1, respectively. At various time points postinoculation, bacteria were quantitated by plating aliquots on BCYE agar. Results are means and standard deviations from triplicate samples and are representative of three independent experiments.

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 nucleophilic agent serine, embedded in the consensus sequence GX SXG, 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 phos-

pholipase 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^r 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* metalloprotease 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 plaB* 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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