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Characterization of the Major Secreted Zinc Metalloprotease-Dependent Glycerophospholipid:Cholesterol Acyltransferase, PlaC, of *Legionella pneumophila*

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Legionella pneumophila*, an intracellular pathogen causing a severe pneumonia, possesses distinct lipolytic activities which have not been completely assigned to specific enzymes so far. We cloned and characterized a gene, *plaC*, encoding a protein with high homology to PlaA, the major secreted lysophospholipase A of *L. pneumophila* and to other hydrolytic enzymes belonging to the GDSL family. Here we show that *L. pneumophila plaC* mutants possessed reduced phospholipase A and lysophospholipase A activities and lacked glycerophospholipid:cholesterol acyltransferase activity in their culture supernatants. The mutants' reduced phospholipase A and acyltransferase activities were complemented by reintroduction of an intact copy of *plaC*. Additionally, *plaC* conferred increased lysophospholipase A and glycerophospholipid:cholesterol acytransferase activities to recombinant *Escherichia coli*. Furthermore, PlaC was shown to be another candidate exported by the *L. pneumophila* type II secretion system and was activated by a factor present in the bacterial culture supernatant dependent on the zinc metalloprotease. Finally, the role of *plaC* in intracellular infection of *Acanthamoeba castellanii* and U937 macrophages with *L. pneumophila* was assessed, and *plaC* was found to be dispensable. Thus, *L. pneumophila* possesses another secreted lipolytic enzyme, a protein with acyltransferase, phospholipase A, and lysophospholipase A activities. This enzyme is distinguished from the previously characterized phospholipases A and lysophospholipases A by its capacity not only to cleave fatty acids from lipids but to transfer them to cholesterol. Cholesterol is an important compound of eukaryotic membranes, and an acyltransferase might be a tool for host cell modification to fit the needs of the bacterium.

Legionella pneumophila is a gram-negative bacterium which is found in freshwater environments, where it associates with amoebae. The inhalation of *Legionella*-containing aerosols can lead to an acute pneumonia, Legionnaires' disease. In the human lung the bacteria are able to replicate within alveolar macrophages and epithelial cells, causing tissue damage and eventually leading to lung failure. Some of the factors which promote intracellular replication are exported by or depend on other factors exported by the type II secretion system Lsp or the type IVB secretion system Dot/Icm (6, 22, 32, 39, 40). The type II secretion system is responsible for translocation of a variety of hydrolytic activities, because *L. pneumophila* type II secretion mutants show considerably reduced protease, acid phosphatase, lipase, lysophospholipase A (LPLA), phospholipase A (PLA), *p*-nitrophenylphosphorylcholine (p-NPPC) hydrolase, and nuclease activities in their culture supernatants (22, 39). Accordingly, substrates of the type II secretion system include the major zinc metalloprotease ProA or Msp, the major acid phosphatase Map, the lipase LipA, and the major secreted LPLA PlaA (3, 4, 17, 22). Studies with knockout mutants indicate that none of these proteins are essential for infection of amoebae or macrophages (3, 18, 47). However, the loss of one might easily be compensated for by enzymes fulfilling similar functions. Thus, the type II secreted factors necessary for intracellular replication are still unknown. Substrates of the type IVB secretion system may also include hydrolytic enzymes, because one recently identified substrate, SidB, shows homology to lipases and possesses three paralogs, SdbA to SdbC, in the *Legionella* genome (28). Although the major secreted lipase, PLA, and LPLA activities of *L. pneumophila* are dependent on the type II protein secretion system, there is evidence that some secreted hydrolytic enzymes might be exported differently, as *L. pneumophila* mutants lacking a functional type II secretion system still retain residual secreted lipolytic activities (3, 17, 39).

Phospholipases are hydrolytic enzymes which cleave ester bonds in phospholipids. These enzymes are categorized into four groups, groups A to D. PLAs cleave fatty acid residues from the sn-1 or sn-2 position of the glycerol backbone. LPLAs, a subgroup of the first group, hydrolyze phospholipids which have only one fatty acid residue. Phospholipases B cleave both fatty acid residues from the sn-1 and sn-2 positions. Phospholipases C (PLC) and D are phosphodiesterases that release phosphomonoesters and the alcohol, respectively. There is evidence that especially PLA and PLC are involved in

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TABLE 1. Overview of the *L. pneumophila* strains used in this study

L. pneumophila strain	Characteristic(s)	Antibiotic selection marker(s)	Reference
130b	Wild type		Strain ATCC BAA-74
plaC2 (from 130b)	*plaC* knockout	Km^r	This study
plaC4 (from 130b)	*plaC* knockout	Km^r	This study
plaC5 (from 130b)	*plaC* knockout	Km^r	This study
plaC8 (from 130b)	*plaC* knockout	Km^r	This study
130b(pMMB2002)		Cm^r	This study
plaC5(pMMB2002)		Km^r, Cm^r	This study
plaC5(pMY7)	*plaC* complementation	Km^r, Cm^r	This study
Δ*lspDE* (from 130b)	Type II secretion mutant	Km^r	39
Δ*proA* (from 130b)	Zinc metalloprotease mutant	Km^r	34
Δ*plaA* (from 130b)	Lysophospholipase A mutant	Km^r	18

bacterial pathogenesis. For example, ExoU, a type III secreted cytotoxin of *Pseudomonas aeruginosa*, has been recently identified as a PLA and LPLA (37, 43, 48). Furthermore, the PLCs of *Listeria monocytogenes* are virulence factors important for releasing the bacterium into the cytoplasm, where it can replicate (21), and are also necessary for cell-to-cell spread of the bacteria (44). *L. pneumophila* possesses several lipolytic enzymes, including a lipase LipA, a secreted LPLA PlaA, and a cell-associated PLA/LPLA PlaB (4, 18, 19). Additionally, *L. pneumophila* has been reported to export PLA and acyltransferase activities into its culture supernatant, which have not been assigned to any specific gene so far (16, 18). A screen of the *L. pneumophila* genome for putative lipolytic enzymes identified two more homologs of PlaA (18). The major secreted LPLA PlaA belongs to the family of GDSL hydrolases, which include lipases, PLAs, hemolysins, and acyltransferases (49). The proteins of this family possess five successive conserved blocks in which block I and block V contain the putative catalytic active serine, glycine, and histidine residues (49). The best-characterized homolog of PlaA is the glycerophospholipid:cholesterol acyltransferase (GCAT) SatA of *Aeromonas salmonicida*, a major secreted toxin (10, 26). *A. salmonicida* SatA was shown to form high-molecular-weight complexes with lipopolysaccharide which are toxic to fish (26). Additionally, SatA is known to be activated by a serine protease (23). Since the *Legionella* genome encodes two more proteins with the GDSL motif (18), we aimed to characterize them further. Here we demonstrate that one of these proteins, designated PlaC, possesses PLA and LPLA activities and is the major secreted GCAT of *L. pneumophila*. Furthermore, we show that PlaC is another candidate for type II secretion and that its activity depends on the major secreted zinc metalloprotease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *L. pneumophila* sg1 strain 130b was used for mutagenesis of the *plaC* gene and later served as the wild-type control. The *plaA* mutant, a 130b derivative, is defective in the major secreted LPLA activity (Table 1). The *lspDE* and *proA* mutants are two further derivatives of 130b; the *lspDE* mutant is defective in the type II secretion system, and the *proA* mutant contains a knockout of the zinc metalloprotease gene (Table 1). *L. pneumophila* was routinely grown on buffered charcoal-yeast extract (BCYE) agar for 2 or 3 days at 37°C (15) and subsequently was cultured in buffered yeast extract (BYE) broth at 37°C with shaking at 350 rpm. Bacterial growth was checked by determining the optical density at 660 nm (OD_{660}) with a Beckman spectrophotometer DU520 (Beckman Coulter, Unterschleißheim, Germany) after inoculation to an OD_{660} of 0.2 to 0.4. *Escherichia coli* strain DH5α, the host for new recombinant plasmids, was grown in Luria-Bertani (LB) agar or broth (5). In LB broth, *E. coli* was grown to an OD_{660} of 1.0 to 1.1, and isopropyl β-D-thiogalactopyranoside (Sigma Chemicals, Munich, Germany) was added to a final concentration of 1 mM in order to induce a P_{tac} or P_{lac} promoter on the respective vector and bacterial growth was continued until the OD_{660} was 2.0 to 2.1. When appropriate, media were supplemented with antibiotics at final concentrations suitable for *L. pneumophila* or *E. coli*, as follows: kanamycin, 25 μg/ml for *L. pneumophila* and 50 μg/ml for *E. coli*; chloramphenicol, 6 μg/ml for *L. pneumophila* and 30 μg/ml for *E. coli*; and ampicillin, 100 μg/ml for *E. coli*.

Preparations of culture supernatants and cell lysates. Culture supernatants for assessment of hydrolytic activities were obtained at the end of exponential growth (i.e., at an OD_{660} of 2.0 to 2.1) by centrifugation for 5 min at 5,000 × *g*. Cell lysates were produced as described previously (18, 19). In short, bacteria from the late exponential phase were pelleted by centrifugation as described above and then lysed by addition of 1/20 volume of the original culture volume of 10 mg/ml of lysozyme and 1 μl/ml of Triton X-100 in 40 mM Tris-HCl pH 7.5 (25°C) at 37°C for 30 min. After repeated passage through a 26-gauge needle, the lysates were finally resuspended in one-quarter of the original culture volume of 40 mM Tris-HCl (pH 7.5) (25°C) for *E. coli* lysates or were resuspended in the original culture volume of Tris-HCl and diluted 1:10 in Tris-HCl for *L. pneumophila* lysates. Culture supernatants and cell lysates either were tested immediately for enzymatic activities or were stored overnight at 4°C.

DNA techniques and sequence analysis. PCR was carried out using a T-gradient thermocycler (Biometra, Göttingen, Germany) and *Taq* DNA polymerase (New England Biolabs, Frankfurt am Main, Germany), Platinum *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany), or *Pfu* DNA polymerase (Fermentas GmbH, St. Leon-Rot, Germany). *E. coli* DH5α was employed for propagation of recombinant plasmid DNA. The following vectors were used: pGEMTeasy (backbone in plasmids pBH1, pBH5, and pAF10; Promega, Mannheim, Germany), pBCKS+ (backbone in plasmid pMY2; Stratagene, Heidelberg, Germany), and pMMB2002 (backbone in plasmid pMY3 and pMY7) (Table 2) (40). Foreign DNA was introduced into bacterial strains by electroporation by means of a Cell-Porator from Life Technologies (Paisley, Scotland) used according to the manufacturer's specifications. Electroporation of *E. coli* (*L. pneumophila*) was carried out using 200 DC volts (400 DC volts), 4 kΩ (4 kΩ), and 330 μF (330 μF). Both strands of plasmid DNA were sequenced by using a BigDye terminator cycle sequencing mixture (Applied Biosystems, Darmstadt, Germany) and an automated DNA sequencer at the sequencing facility of the Robert Koch-Institut. Primers were purchased from Tib Molbiol (Berlin, Germany). Nucleotide and translated protein sequences were analyzed using the DNASTAR package, the pedant website (http://pedant.gsf.de/), the SignalP program (36), and the *L. pneumophila* genome project website (http://genome3.cpmc.columbia.edu/~legion.) (12). Nucleotide sequences were also analyzed for promoters using the web-based program BPROM (www.softberry.com). Sequence database searches as well as protein alignments were performed by the BLAST algorithm (1).

Southern hybridization. Chromosomal DNA from *L. pneumophila* 130b wild type and *plaC* mutants, obtained by means of an E.Z.N.A. bacterial DNA kit from Peqlab (Erlangen, Germany), was digested with AvaI and AvaII and subjected to electrophoresis, and fragments were transferred to a nylon membrane (Roche Diagnostics, Mannheim, Germany) by means of a Bio-Rad vacuum blotter (Bio-Rad, Munich, Germany). The DNA probe, generated with primers gdsl2c1 (5′-TTATGATCCAAAACAACAGG-3′) and gdsl2e1 (5′-GAGGATC AATTTAGACAACTT-3′), containing the complete *L. pneumophila plaC* gene, was used as a *plaC*-specific probe. Likewise a kanamycin resistance gene probe was generated from pET28b (Novagen, San Diego, Calif.) using primers kan1

TABLE 2. Overview of *plaC* plasmid constructs used in this study

Plasmid	Construct	Size of insert (kbp)	Antibiotic selection marker(s)	Primers used for amplification
pAF10	pGEMTeasy + *plaC*[a,b]	1.559	Amp^r	gdsl2a1, gdsl2b1
pBH1	pGEMTeasy + *plaC*	1.383	Amp^r	gdsl2c1, gdsl2e1
pMY2	pBCKS + *plaC*	1.383	Cm^r	Derived from pBH1
pBH5	pGEMTeasy + *plaC*::Km^r[a,b]	2.375	Amp^r, Km^r	Derived from pAF10
pMY3	pMMB2002 + *plaC*	1.383	Cm^r	Derived from pMY2
pMY7	pMMB2002 + *plaC*[b]	1.559	Cm^r	gdsl2a1, gdsl2b1

[a] The cloned *plaC* sequence contains a point mutation at base pair position 30, where guanine is mutated to adenine.
[b] Contains a putative promoter region.

(5′-GACGCTCAGTGGAACGAAAAC-3′) and kan2 (5′-ATGTGCGCGGAA CCCCTATT-3′). All reagents needed for labeling and detection of the DNA probes were obtained from Roche Diagnostics (Mannheim, Germany). Detection was carried out by means of disodium 3-(4-methospiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate (CSPD), the chemiluminescent substrate for alkaline phosphatase. Hybridization was performed at 42°C overnight. Membranes were washed twice for 5 min at room temperature in low-stringency buffer (2× SSC, 0.1% sodium dodecyl sulfate [SDS] [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), followed by 15 min at 65°C in high-stringency buffer (0.1× SSC, 0.1% SDS). Hybridization and detection were carried out according to the manufacturer's instructions.

Gene cloning and *Legionella* mutant construction. Primers gdsl2c1 and gdsl2e1, based on the sequence found in the *L. pneumophila* Philadelphia-1 database (http://genome3.cpmc.columbia.edu/~legion.) (12), were used to amplify the *plaC* gene from strain 130b DNA. The resulting 1,383-bp PCR product begins 2 bp upstream of *plaC* and ends 79 bp after the stop codon. To assist with ascribing function to *plaC*, several recombinant plasmids were derived from pBH1, a *plaC*-containing plasmid, which was generated by cloning the PCR product obtained with primers gdsl2c1 and gdsl2e1 into plasmid pGEMTeasy. Next, the *plaC* gene from pBH1 was subcloned into the vector pBCKS by using SpeI and SacII, yielding pMY2. pMY2 was then used to clone the *plaC* gene into pMMB2002 after digestion with KpnI and SacI, resulting in pMY3 (Table 2). For disruption of the *plaC* gene, a 1,559-bp fragment was amplified from strain 130b using primers gdsl2a1 (5′-TTAACGGCATATTGGGTGAA-3′), which binds 148 bp upstream from the *plaC* open reading frame (ORF), and gdsl2b1 (5′-T GCTTAAAAACCGCTCTGGA-3′), which binds 110 bp after the end of the *plaC* ORF, and cloned into pGEMTeasy, resulting in pAF10. Next, pAF10 was restricted with HindIII and ligated with a kanamycin resistance gene (Km^r) cassette amplified from pET28b using primers kan1 and kan2, yielding pBH5 (Table 2). Plasmids were isolated from *E. coli* by alkaline lysis using either a Miniprep kit from QIAGEN (Hilden, Germany) or a Midiprep kit from Bio-Rad. To generate an *L. pneumophila plaC* mutant, plasmid pBH5 was brought into strain 130b, and the Km^r cassette was introduced into the chromosomal *plaC* gene by natural transformation and homolog recombination as described previously (18). PCR and Southern blot analysis were used to examine Km^r legionellae for the presence of the *plaC* mutation (42). For *trans* complementation of the *plaC* mutant, the PCR product obtained with primers gdsl2a1 and gdsl2b1 by amplification with *Pfu* polymerase was cloned into pMMB2002 restricted with SmaI, yielding pMY7. After ligation, pMY7 was directly transformed into an *L. pneumophila plaC* mutant by electroporation.

Enzymatic assay for lipolytic activities. Enzymatic activities were detected as described previously (18, 19). In short, different lipids were incubated with the same volume of bacterial culture supernatant or cell lysate in a mixture containing 6.7 mM substrate (1,2-dipalmitoylphosphatidylcholine [DPPC], 1,2-dipalmitoylphosphatidylglycerol [DPPG], 1-monopalmitoyllysophosphatidylcholine [MPLPC], 1-monopalmitoyllysophosphatidylglycerol [MPLPG], or 1-monopalmitoylglycerol [1-MPG]), 3 mM NaN_3, 0.5% (vol/vol) Triton X-100, and 40 mM Tris-HCl (pH 7.5) (25°C). 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, as indicated below for specific experiments. Free fatty acids (FFA) were determined by using an NEFA-C-kit (WAKO Chemicals, Neuss, Germany) according to the instructions of the manufacturer. Depending upon the nature of the experiment, BYE broth, LB broth, or 40 mM Tris-HCl (pH 7.5) (25°C) was incubated, treated like the cultures, and subsequently used as a negative control. When culture supernatants or cell lysates were assessed for GCAT activity, 50 μl per ml of cholesterol in ethanol (10 mg/ml) was added to a DPPG mixture prior to sonication. All lipids, including standards for thin-layer chromatography (TLC), were obtained from Sigma Chemicals 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 with a probe (Sonoplus; Bandelin, Berlin, Germany) three times for 15 s at cycle 4 (10%) with the power set to 65%. In order to activate PlaC in bacterial culture supernatants and cell lysates, 100-μl reaction mixtures (containing the lipid suspension and the bacterial product) were incubated with 20 μl of culture supernatant from *L. pneumophila plaC* mutants or partly purified zinc metalloprotease. Partly purified protease was obtained by the following method (17). *L. pneumophila* culture supernatant was concentrated 10-fold by ultrafiltration (exclusion pore size, 30 kDa; Millipore, Schwalbach, Germany) and subjected to anion-exchange chromatography using a Resource Q column material (Amersham Biosciences, Freiburg, Germany), followed by gel filtration using a prepacked HiLoad 26/60 Superdex 200 column (Amersham Biosciences) of protease-active fractions. Gel filtration fractions which contained only protease, as estimated by SDS-polyacrylamide gel electrophoresis and silver staining, with a protein concentration of approximately 2 μg/ml were stored at −20°C. $ZnCl_2$ at a final concentration of 12 mM was added to the fractions prior use in the activation assays.

Lipid extraction and thin-layer chromatography. For the detection of distinct apolar lipids, including cholesterol esters, reaction mixtures of lipids and cholesterol with culture supernatants or cell lysates and corresponding negative controls were subjected to lipid extraction (7). The lower chloroform phase was subsequently used for separation of lipids by TLC. For detection of cholesterol esters, silica gel plates (Merck, Darmstadt, Germany) were developed in tanks containing a petroleum ether-diethyl ether-glacial acetic acid (90:10:1, vol/vol/vol) solvent mixture (29). For visualization, the silica gel plates were then stained with naphthol blue black (Aldrich, Milwaukee, Wis.) (18, 38).

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 (80 nM phorbol-12-mystrate-13-acetate [P-8139; Sigma Chemicals, Munich, Germany]; incubation for 36 to 48 h), were used as hosts for in vitro infection by *L. pneumophila* (13, 33). The amoebae and the cell line were maintained and infected as previously described (13, 27, 33). To assess intracellular growth of *L. pneumophila*, wells containing amoebae or U937 cells at concentrations of 10^5/ml and 10^6/ml, respectively, were infected with wild-type bacteria or isogenic mutants at multiplicities of infection of 0.01 for amoebae and 1 for U937 cell infections (zero time). U937 macrophages were incubated for 2 h with the added bacteria in plain RPMI, and then monolayers were washed three times with plain RPMI to remove unbound bacteria and were incubated with RPMI containing 10% (vol/vol) fetal calf serum (PAA, Linz, Austria). At various times, coincubations of U937 cells and legionellae were treated with 10% (wt/vol) saponin (Sigma Chemicals, Munich, Germany) to lyse the host cells, and serial dilutions were plated on BCYE agar.

Nucleotide accession number. The *L. pneumophila* 130b *plaC* sequence has been deposited in the GenBank database at the National Center for Biotechnology Information under accession number AY745197.

RESULTS

Identification of a new *Legionella* gene that codes for a putative lipolytic enzyme. Since *L. pneumophila* secretes PLA and acyltransferase activities encoded by unidentified genes, we sought to identify potential candidates coding for these

	Organism	Accession No.	Name and Function	Δ	Block I	Δ
A)	*Legionella pneumophila*	AY745197	PlaC, GCAT	30	IVVFGDSLSDNG	108
B)	*Legionella pneumophila*	AF510106	PlaA, LPLA	23	IVVFGDSLSDNG	41
C)	*Aeromonas salmonicida*	AAG09804	SatA, GCAT	27	IVMFGDSLSDTG	37

	Block II	Δ	Block III	Δ	Block IV	Δ	Block V	Δ
A)	EVYLNKAFGGSW	53	YFIYSGSNDYI	34	AGARRFVIMGIPH	123	FWDEIHPT	30
B)	SHLLDYAFGGAG	34	FVIWIGANNYL	26	KGAKHILVLNLPD	104	FFDLVHPT	25
C)	LTIANEAEGGAT	37	VILWVGANDYL	26	NGAKQILLFNLPD	128	FWDQVHPT	24

FIG. 1. Partial alignment of amino acid sequences involving conserved blocks I to V from PlaC and PlaA of *L. pneumophila* 130b and SatA from *A. salmonicida*. The amino acid sequences from *L. pneumophila* PlaC and PlaA, as well as from *A. salmonicida* GCAT, were aligned using the program MEGALIGN (DNASTAR). Putative active site amino acids are underlined. The numbers under uppercase delta indicate the numbers of amino acids between blocks.

activities. To do this, the PlaA sequence was used in a BLAST search against the *L. pneumophila* genome (http://genome3.cpmc.columbia.edu/) (12). In this way, we identified two paralogs of PlaA containing the GDSL motif (18). One of these paralogs, designated PlaC for PLA protein C, is encoded by a 1,302-bp ORF and was predicted to represent a 433-amino-acid protein with a molecular mass of about 49,8 kDa and an isoelectric point of 5.27. Moreover, the PlaC protein was predicted to have a signal sequence of 22 amino acids at its N terminus and might therefore be secreted by the sec system and additionally might be a candidate for a type II secreted protein. PlaC showed 26% identity and 46% similarity to the paralog PlaA and 22% similarity and 40% identity to SatA of *A. salmonicida*, an orthologous GDSL protein with acyltransferase, PLA, and LPLA activities (10). The closest homolog of PlaC as evaluated by the expect value is a putative phospholipase/lecithinase/hemolysin from *Nostoc punctiforme* (24% identity, 40% similarity) also belonging to the GDSL family. In addition, PlaC contained all five conserved blocks characteristic of members of the GDSL family (Fig. 1). Noticeably, an alignment of PlaC with *L. pneumophila* PlaA and *A. salmonicida* SatA showed that the distance between the first two blocks in the PlaC sequence is more than twice as large as the distance in the other two sequences (Fig. 1). As PlaC seemed to be a potentially type II secreted hydrolytic enzyme, because of its high homology to lipolytic proteins and its predicted signal peptide, we were interested in further characterization. Next, we examined the genetic *plaC* locus. Two uncharacterized genes flank *plaC* (Fig. 2). Interestingly, ORF1 to ORF3 surrounding *plaC* encoded proteins which showed homology to different transferases. The downstream ORF1, which is oriented in the same direction as *plaC*, possesses homology to a putative glucosamine-fructose-6-phosphate aminotransferase of *P. aeruginosa* (gi 15600742 [46]). The oppositely oriented upstream ORF2 displays homology to a putative sulfur transferase of *Microbulbifer degradans* (gi 48862923). ORF3 is homologous to a putative thiopurine methyltransferase of *Photobacterium profundum* (CAG20091). A gene encoding a predicted transcriptional accessory protein is located farther downstream (ORF4), and this protein shows homology to the Tex protein of *Bordetella pertussis* involved in toxin expression (20) (Fig. 2). We suspect that *plaC* and ORF1 do not form an

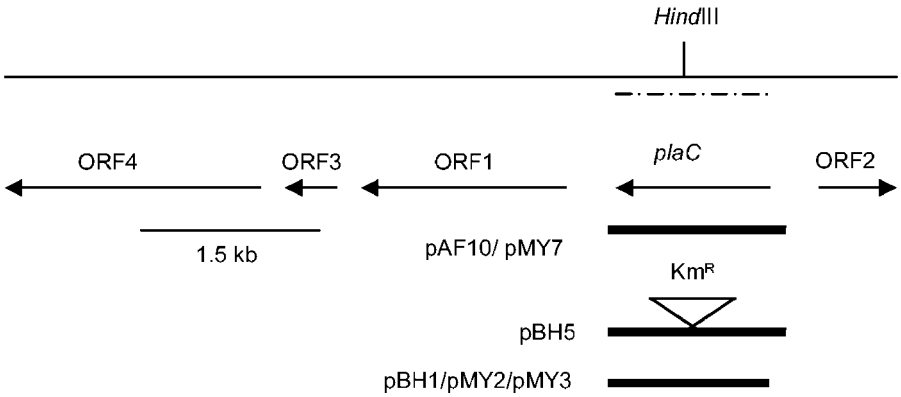

FIG. 2. *plaC* locus in *L. pneumophila* and recombinant *E. coli*. The upper line represents the *L. pneumophila* Philadelphia-1 chromosome region that contains the *plaC* gene, along with the location of the relevant HindIII restriction site which was utilized for introduction of a Km^r cassette. The dotted line below it illustrates the region from 130b which was sequenced. The arrows below this line depict the relative locations, sizes, and orientations of *plaC* and neighboring ORFs. The lines at the bottom represent the segments of *Legionella* DNA that were cloned into plasmid vectors. Plasmid pBH5 contained a Km^r gene cassette.

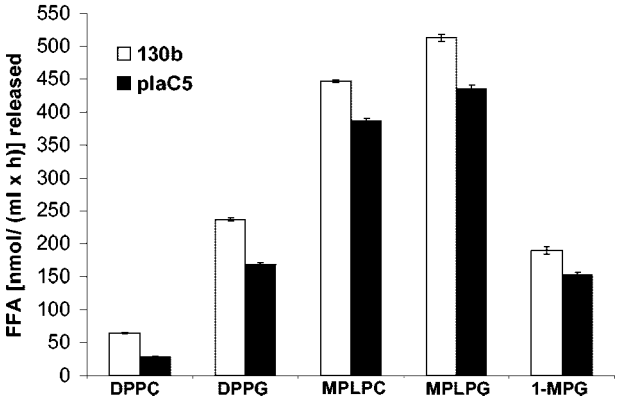

FIG. 3. Lipolytic activities of *L. pneumophila* wild type and a *plaC* mutant. Culture supernatants of the wild type and a *plaC* mutant were incubated with DPPC, DPPG, MPLPC, MPLPG, or 1-MPG for 5 h at 37°C. The release of FFA was quantified. Data are expressed as differences between the amount of FFA released by culture supernatants and the amount released by BYE broth. The results represent the means and standard deviations of duplicate cultures and are representative of three independent experiments. For all substrates, the lipolytic activities were significantly different from the wild-type activities ($P < 0.01$ for DPPC, DPPG, MPLPC and MPLPG, $P < 0.05$ for 1-MPG; Student's *t* test).

operon as they are separated by 367 bp and because there are −10 and −35 promoter sequences in the intergenomic region. Thus, *plaC* most probably is transcribed monocistronically.

Isolation of *L. pneumophila plaC* mutants. To investigate the enzymatic activities of PlaC in *L. pneumophila* and its importance for bacterial infection, knockout mutants with mutations in the *plaC* gene were generated. pBH5, which contained the *plaC* gene disrupted by a Km^r cassette (Table 2), was used to introduce a Km^r cassette into the chromosomal *plaC* gene of strain 130b by allelic exchange, taking advantage of the natural competence of *L. pneumophila* when it is grown at 30°C (18, 45). Four independent *plaC* mutants (plaC2, plaC4, plaC5, plaC8) were obtained. The mutation in the *plaC* gene was confirmed by PCR and Southern blot analysis (data not shown). In the following experiments, results are given mostly for plaC5, but plaC2 and plaC4 behaved similarly. plaC8 differed from the other clones regarding colony morphology and intracellular multiplication in host cells, which might have been due to a second-site mutation in a gene or genes other than *plaC* (data not shown).

Lipolytic activities of *L. pneumophila plaC* mutants. *L. pneumophila* possesses several secreted as well as cell-associated lipolytic activities (2, 4, 16, 17, 18, 19). As PlaA, the major secreted LPLA of *L. pneumophila*, is a paralog of PlaC, we tested the *L. pneumophila plaC* mutants for loss of lipolytic activities. Lipolytic activities were determined by measuring the amounts of free fatty acids released from substrates. Accordingly, culture supernatants and cell lysates of the *plaC* mutants were incubated with diacylphospholipids (DPPC and DPPG, substrates for PLA activity), lysophospholipids (MPLPC and MPLPG, substrates for LPLA activity), and a nonphospholipid (1-MPG, a substrate for lipase activity) and monitored for the release of FFA. The culture supernatants of the *plaC* mutants liberated less FFA from all the lipids tested (Fig. 3), implying that PlaC contributes to the exported PLA, LPLA, and lipase activities of *L. pneumophila*. That an LPLA can also cleave nonphospholipids and thereby show lipase activity has been shown previously for PlaA, because the *L. pneumophila plaA* mutant exhibited a 50%-reduced capacity to cleave 1-MPG (17, 18). On the other hand, the cell lysates of the wild type and *plaC* mutants released comparable amounts of FFA from the substrates (data not shown). In conclusion, our data show that PlaC is a PLA and LPLA which is transported into the *Legionella* culture supernatant.

Since PlaC showed homology to SatA from *A. salmonicida*, we wanted to determine whether it is responsible for the GCAT activity observed in culture supernatants of *L. pneumophila* (18). In previous experiments, it was established that DPPG is the preferred substrate of *L. pneumophila* GCAT (18). In the presence of GCAT, a fatty acid residue from a phospholipid donor molecule is transferred to cholesterol, resulting in the formation of a cholesterol ester which can be detected, for example, by TLC. Indeed, whereas the culture supernatant of the *L. pneumophila* wild type incubated with DPPG and cholesterol showed GCAT activity, the culture supernatants of the *plaC* mutants lacked GCAT activity (Fig. 4A), indicating that *plaC* is essential for the secreted GCAT activity of *L. pneumophila*. Interestingly, under the same reaction conditions, the *plaC* mutant also generated reduced amounts of an additional unidentified spot (Fig. 4A). Since this spot occurs only when cholesterol is added to the lipid reaction mixture, it likely represents an unknown cholesterol derivative (data not shown). Unlike the culture supernatants, the bacterial cell lysates of both the wild type and *plaC* mutants, under the reaction conditions used, did not possess GCAT activity (data not shown). Thus, PlaC not only contributes to PLA and LPLA activities but is also the major secreted GCAT of *L. pneumophila*.

Lipolytic activities of *trans*-complemented *plaC* mutants. Next we assessed whether the introduction of *plaC* in *trans* could restore the missing lipolytic activities of the *L. pneumophila plaC* mutants, particularly the missing GCAT activity. To that end, culture supernatants of the wild type and the *plaC* mutant harboring either the empty pMMB2002 vector or pMY7 (Table 2) were assessed for GCAT activity by incubation with cholesterol and DPPG. As expected, the wild type showed GCAT activity, as indicated by the formation of the cholesterol ester, which was missing in the *plaC* mutant harboring the empty pMMB2002 vector (Fig. 4B). In contrast, the *plaC* mutant harboring pMY7 with intact *plaC* showed GCAT activity, confirming that *plaC* is indeed responsible for the secreted GCAT activities of *L. pneumophila* (Fig. 4B). Next, culture supernatants of the wild type as well as the *plaC* mutant harboring pMMB2002 or pMY7 were assessed for PLA, LPLA, and lipase activity by monitoring the release of FFA from the respective substrates. Again, the culture supernatant of plaC5 harboring the empty pMMB2002 vector showed a reduced capacity to cleave FFA from DPPC, MPLPC, and 1-MPG compared to the wild type (Fig. 5). In contrast, plaC5 harboring pMY7 exhibited a considerable increase in the release of FFA from DPPC and 1-MPG, verifying that *plaC* contributes to the secreted PLA and lipase activities of *L. pneumophila* (Fig. 5). The LPLA activity could not be restored by *trans* complementation (Fig. 5). It is possible that the overexpression of *plaC* might result in the formation of partially

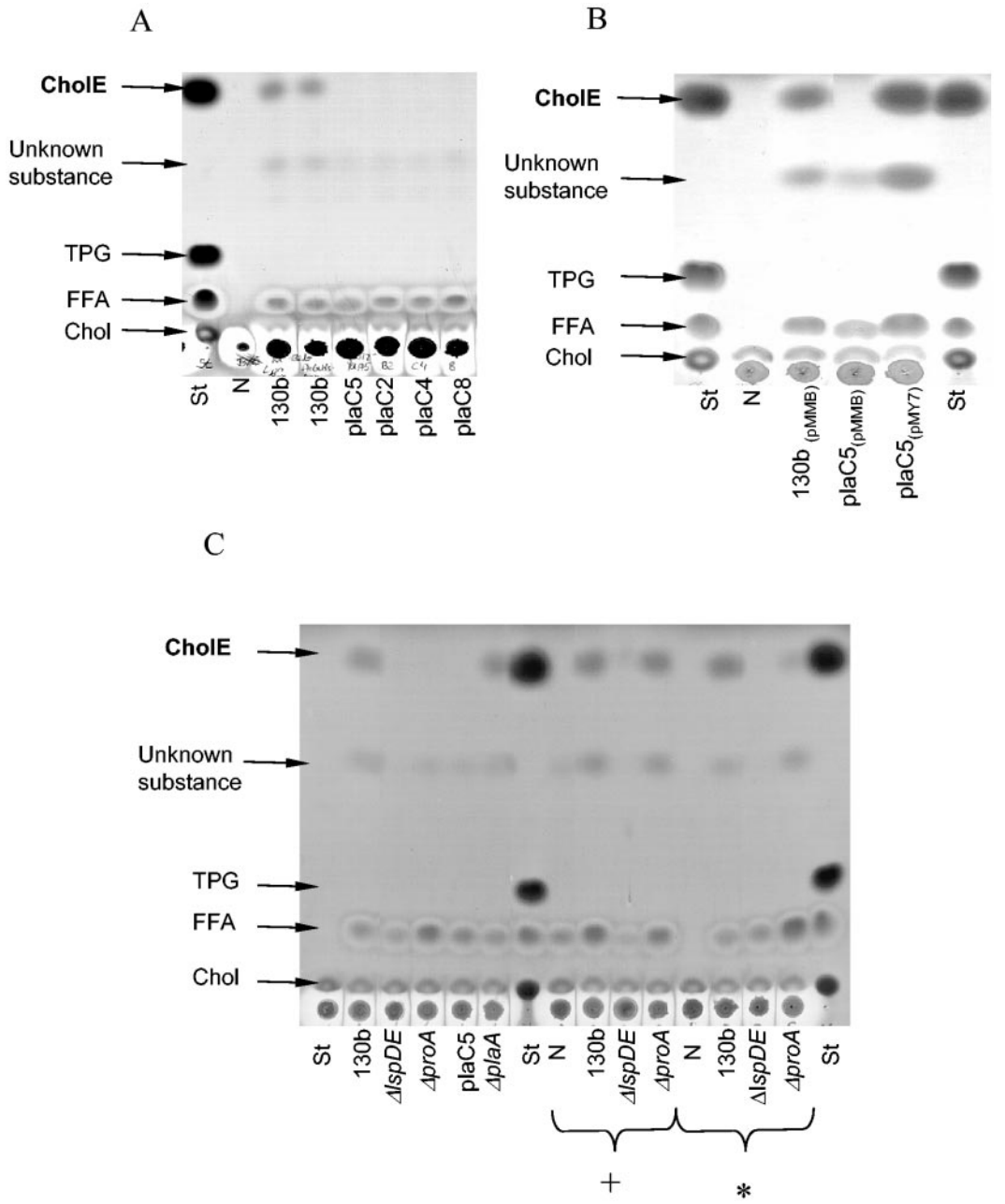

FIG. 4. TLC analysis of GCAT activity of *L. pneumophila* wild type and different mutants. Culture supernatants of wild-type strain 130b (duplicate supernatants) and *plaC* mutants plaC5, plaC2, plaC4, and plaC8 were incubated with a mixture of DPPG and cholesterol for 23 h at 37°C, and then lipids were extracted and applied to a TLC (A). Culture supernatants of the wild type harboring the empty pMMB2002 vector [$130b_{(pMMB)}$], the *plaC* mutant plaC5 harboring the empty pMMB2002 vector [$plaC5_{(pMMB)}$], and plaC5 harboring pMY7 [$plaC5_{(pMY7)}$] were incubated with a mixture of DPPG and cholesterol for 23 h at 37°C, and then lipids were extracted and applied to a TLC (B). Culture supernatants of wild-type strain 130b, a *lspDE* mutant (Δ*lspDE*), a *proA* mutant (Δ*proA*), *plaC* mutant plaC5, and a *plaA* mutant (Δ*plaA*) were either incubated with a mixture of DPPG and cholesterol or added to a lipid mixture incubated with supernatant from a *plaC* mutant (plus sign) or partly purified ProA (asterisk) for 20 h at 37°C, and then lipids were extracted and applied to a TLC (C). An apolar solvent mixture was employed for separation of the apolar lipids, particularly the cholesterol ester. A mixture of BYE and the lipids was also incubated and served as a negative control (lane N). For qualitative identification of the lipid spots, lanes St containing lipid standards were used. Abbreviations: CholE, cholesterol ester; TPG, tripalmitoylglycerol; Chol, cholesterol.

folded PlaC with restricted activities. Taken together, our complementation data demonstrate that *plaC* contributes to *L. pneumophila* PLA and lipase activities and is essential for the GCAT activity of *L. pneumophila*.

Secretion and activation of PlaC by *L. pneumophila*. Since the *L. pneumophila plaC* mutants showed reduced lipolytic activities in their culture supernatants and since the PlaC protein possessed a predicted signal sequence and might therefore

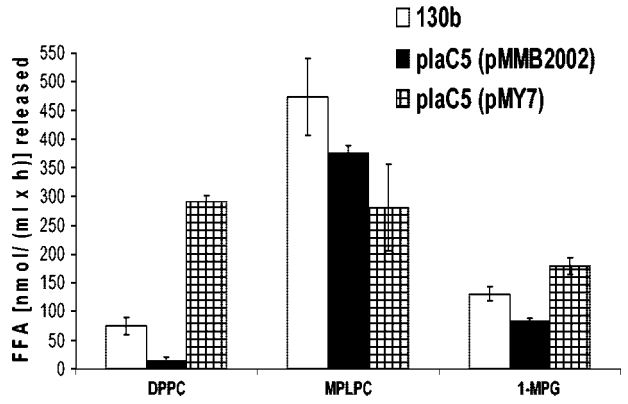

FIG. 5. Lipolytic activities of *L. pneumophila* wild type, a *plaC* mutant, and a complemented strain. Culture supernatants of the wild type, *plaC* mutant plaC5 harboring the empty pMMB2002 vector, and plaC5 harboring pMY7 were incubated with DPPC, MPLPC, or 1-MPG for 5 h at 37°C. The release of FFA was quantified. Data are expressed as differences between the amount of FFA released by culture supernatants and the amount released by BYE broth. The results represent the means and standard deviations of duplicate cultures and are representative of three independent experiments. For all substrates except MPLPC, the lipolytic activities were significantly different from those of the plaC5 mutant ($P < 0.01$; Student's *t* test).

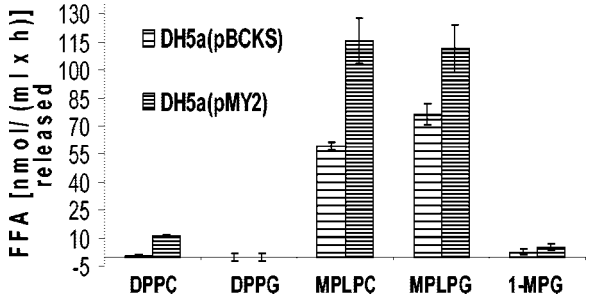

FIG. 6. Lipolytic activities of recombinant *E. coli* containing *L. pneumophila plaC*. Fourfold-concentrated cell lysates of *E. coli* containing pBCKS or its derivative pMY2 were mixed with DPPG, DPPC, MPLPC, MPLPG, and 1-MPG. After 23 h of incubation at 37°C, the release of FFA was quantified. Data are expressed as differences between the amount of FFA released by cell lysate and the amount released by Tris-HCl buffer. The results represent the means and standard deviations of duplicate cultures and are representative of three independent experiments. For all substrates except DPPG the lipolytic activities were significantly different from those of *E. coli* carrying the empty vector ($P < 0.01$; Student's *t* test).

be a type II secreted protein, we examined a type II secretion mutant (*lspDE*) for secretion of PlaC as represented by its GCAT activity. The culture supernatant of the type II secretion mutant showed considerably decreased acyltransferase activity (Fig. 4C). This indicates either that PlaC is exported by the type II system or that its activity is dependent on a type II secreted effector. Since the *lspDE* mutant also lacks the type II secreted zinc metalloprotease (Msp or ProA) which has been shown to contribute to the PLA and LPLA activities of *L. pneumophila* culture supernatants (2, 18, 22, 39), we also tested the culture supernatant of an isogenic *proA* mutant for GCAT activity. We found that culture supernatants of the *proA* mutant had also lost GCAT activity, suggesting that ProA is directly or indirectly involved in PlaC activation (Fig. 4C). To exclude the possibility that the lack of GCAT activity in the culture supernatant of the *lspDE* mutant was simply due to the absence of ProA, we coincubated the culture supernatant of the *lspDE* mutant with (i) the supernatant of a *plaC* mutant which still contained protease activity and other type II secreted activities but lacked GCAT activity and (ii) partially purified chromatography fractions containing ProA. For both incubations, the wild type showed GCAT activity, whereas the type II secretion mutant did not show GCAT activity, confirming that PlaC itself is a candidate for a type II secreted effector (Fig. 4C). In contrast, the GCAT activity could be recovered from culture supernatants of the *proA* mutant by incubation with the culture supernatant of a *plaC* mutant or partially purified ProA (Fig. 4C). In conclusion, it was shown that PlaC is another candidate for export by the type II secretion system of *L. pneumophila* and is dependent on the presence of the zinc metalloprotease.

Enzymatic activities of *E. coli* clones harboring the *plaC* gene. *L. pneumophila plaC* mutants showed reduced PLA, LPLA, and lipase activities and a loss of acyltransferase activity in their culture supernatants. Since *L. pneumophila* possesses several lipolytic enzymes which may influence each other, we investigated the enzymatic activities of PlaC in recombinant *E. coli* clones. To do this, the recombinant *E. coli* harboring pMY2 (Table 2) was assayed for PLA, LPLA, lipase, and acyltransferase activities in both culture supernatant and cell lysates. Indeed, compared to the clone containing the empty pBCKS vector, the cell lysates from the *E. coli* clone harboring pMY2 showed an increased capacity to cleave fatty acids from MPLPC and MPLPG, indicating that PlaC, like PlaA, is an LPLA (Fig. 6). There was a small increase in the amount of FFA released from DPPC, which was not consistently reproducible in additional experiments; since no increased FFA release could be observed for DPPG, it is not yet clear whether PlaC also expresses PLA activity in *E. coli*. No increased release of fatty acids from any of the substrates tested could be detected in the culture supernatant, indicating that PlaC is not exported into the culture supernatant by *E. coli* (data not shown).

Additionally, we wanted to assess whether GCAT activity could also be detected in *E. coli* clones harboring *plaC*. To do this, culture supernatants and cell lysates of the *E. coli* clones were assayed for acyltransferase activity. *E. coli* clones containing only pBCKS or pMMB2002 did not show acyltransferase activity in their culture supernatants and cell lysates. In contrast, GCAT activity could be detected in cell lysates but not in culture supernatants of *E. coli* clones containing *plaC* in pMMB2002 (pMY3) (Fig. 7). *E. coli* clones carrying the *plaC* gene in the pBCKS vector (pMY2) did not show GCAT activity in their culture supernatants or in bacterial lysates (Fig. 7). As described above, we discovered that in *L. pneumophila* the GCAT activity of PlaC is dependent on a factor secreted by *L. pneumophila*, which could either be the zinc metalloprotease ProA itself or a factor dependent on ProA. We therefore additionally investigated the effect of culture supernatant of the *plaC* mutant (showing no GCAT activity but showing protease activity) on the acyltransferase activity of *plaC* cloned into *E. coli*. In order to stimulate PlaC activity, culture supernatants and cell lysates of *E. coli* clones harboring *plaC*, as well

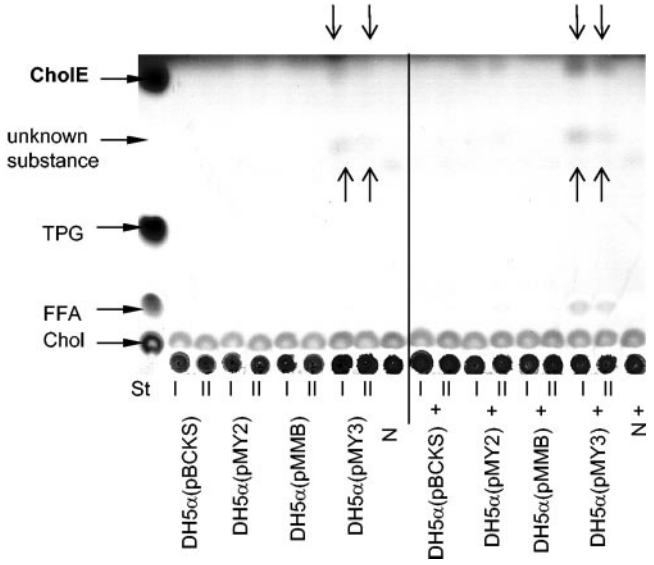

FIG. 7. Acyltransferase activities of recombinant *E. coli* DH5α containing *L. pneumophila plaC* after addition of putative PlaC activating factors of *L. pneumophila*. Fourfold-concentrated cell lysates of *E. coli* containing pBCKS [DH5α(pBCKS)] or its derivative pMY2 [DH5α(pMY2)], as well as pMMB2002 [DH5α(pMMB)] or its derivative pMY3 [DH5α(pMY3)], were incubated with a mixture of DPPG and cholesterol or added to the lipid mixture incubated with supernatant from a *plaC* mutant (plus sign) for 20 h at 37°C, and then lipids were extracted and applied to a TLC. A mixture of Tris-HCl and the lipids was also incubated and served as a negative control (lanes N and N+). Lanes St contained lipid standards used for qualitative identification of the lipid spots. The observations depicted here were made on two more occasions. I and II indicate duplicate cultures. The arrows indicate the presence of cholesterol ester or the unknown substance related to cholesterol. Abbreviations: CholE, cholesterol ester; TPG, tripalmitoylglycerol; Chol, cholesterol.

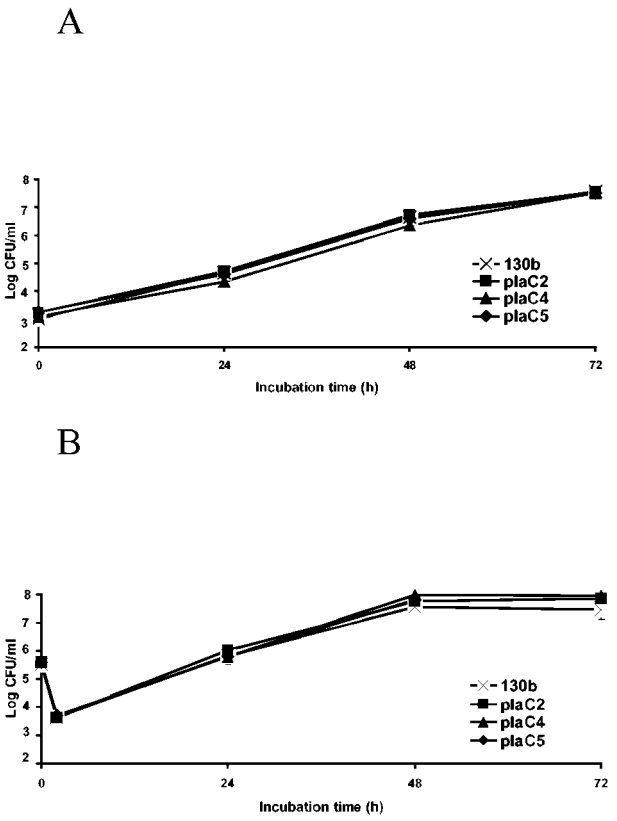

FIG. 8. Intracellular infection by wild-type and *plaC* mutant *L. pneumophila*. Strains 130b and *plaC* mutants plaC5, plaC2, and plaC4 were used to infect monolayers of *A. castellanii* amoebae (A) and cultures of U937 macrophages (B) at multiplicities of infection of 0.01 and 1, respectively. At various times postinoculation, the numbers of bacteria were quantified by plating aliquots on BCYE agar. The results represent the means and standard deviations of triplicate samples and are representative of three independent experiments.

as the respective clones harboring the empty vector, were coincubated with culture supernatant of an *L. pneumophila plaC* knockout mutant, DPPG, and cholesterol. Under these conditions, the cell lysates but not the culture supernatants of recombinant *E. coli* clones containing pMY2 or pMY3 both showed increased GCAT activities, confirming that *plaC* actually codes for an acyltransferase (Fig. 7 and data not shown). Taken together, our data corroborate the conclusion that *plaC* confers LPLA and GCAT activity on *E. coli* and that the latter activity can be enhanced by a factor present in *L. pneumophila* culture supernatant.

Role of *plaC* during intracellular infection of *A. castellanii* amoebae and U937 macrophages. Since phospholipases are also known as virulence factors that support intracellular survival of bacteria, we wanted to assess the importance of PlaC for infection of amoebae and macrophages. To determine whether *plaC* is essential for *L. pneumophila* to infect its natural host, *A. castellanii* amoebae were inoculated with wild-type *L. pneumophila* and *plaC* mutants, and the CFU were counted at various times. The *plaC* mutants showed the same increase in CFU during the whole infection period as the wild type (Fig. 8A); namely, both the *plaC* mutants and the *L. pneumophila* wild type multiplied about 100,000- to 1,000,000-fold by 72 h postinoculation. Additionally, we investigated the ability of the *plaC* mutants to infect U937 macrophages. Again, the *L. pneumophila* wild type and *plaC* mutants displayed comparable replication rates (Fig. 8B). In summary, our studies show that *plaC* is not essential for infection of and replication within *A. castellanii* amoebae and U937 macrophages.

DISCUSSION

In the present investigation, we identified the gene for the major secreted glycerophospholipid:cholesterol acyltransferase of *L. pneumophila* designated *plaC*. We demonstrated that PlaC possesses PLA, LPLA, lipase, and GCAT activities and is a candidate for a type II exported effector. Furthermore, we showed that PlaC requires activation which is either directly or indirectly dependent on the zinc metalloprotease ProA. This study presents evidence that PlaC is closely related to *A. salmonicida* SatA. Both proteins display GCAT activity as well as PLA and LPLA activities (10). PlaC has only weak phospholipase A activity when it is expressed in *E. coli*. The difference in substrate specificity between *plaC* cloned into *E. coli* and *plaC* in *L. pneumophila* may be caused by activating proteins, chaperones, or cofactors present in *Legionella* but not in *E. coli*. Furthermore, *A. salmonicida* SatA has been shown to possess broad substrate specificity, accepting a variety of phos-

pholipids as acyl donors but preferring short-chain or unsaturated fatty acids (9). The preferred acyl acceptor of *A. salmonicida* SatA is cholesterol, but other steroids with a planar ring system and β-hydroxyl group at the 3 position are suitable as well; even aliphatic alcohols were found to act as acyl acceptors (9). Whether PlaC accepts a similar variety of substrates has yet to be determined. Moreover, *A. salmonicida* SatA requires activation by the AspA serine protease (23, 50). *L. pneumophila* PlaC also requires an unidentified factor which enhances its activity. We have shown that this activating factor is present in the *L. pneumophila* culture supernatant and is dependent on the zinc metalloprotease ProA. However, *L. pneumophila* ProA does not belong to the family of serine proteases, like *A. salmonicida* AspA. Since *A. salmonicida* SatA and PlaC are homologous to each other on the protein level and show similar substrate preferences, we favor the hypothesis that a serine protease is the more likely activating factor of PlaC which can be processed by ProA. Apart from ProA, no other proteases have been characterized on the molecular level for *L. pneumophila* so far. Nevertheless, a BLAST search of the *L. pneumophila* genome with the AspA protease of *A. salmonicida* showed that *L. pneumophila* indeed possesses a putative serine protease with homology to *A. salmonicida* AspA (25% identity, 42% similarity).

PlaC belongs to the family of GDSL hydrolases comprising plant and prokaryotic lipases, phospholipases, acyltransferases, and hemolysins. So far no crystal structure of a GDSL enzyme has been solved, but the structures of some GDSL-like enzymes, such as the *Streptomyces scabies* esterase which forms a Ser-His dyad (51) and the mammalian platelet-activating factor acetylhydrolase PAF-AH(Ib) which forms a Ser-His-Asp triad (25), have been elucidated. It was shown for the *Aeromonas hydrophila* GCAT, which also is a member of the GDSL family, that the active site serine belonging to a catalytic triad is located in block I near the N terminus and is embedded in the conserved motif G-X-S-X-S (8, 24, 25). The other two members of the active site were proposed to be aspartic acid in block III located in the motif G-X-N-D and histidine in block V located in the motif F-X-D-X-X-H-P (8). However, elucidation of the crystal structure of several GDSL-like enzymes, including the above-mentioned platelet-activating factor PAF-AH(Ib) and *Aspergillus aculeatus* rhamnogalacturonan acetylesterase (25, 35), revealed that the active site aspartic acid is located in block V in close proximity to the histidine residue rather than in block III. An alignment of PlaC with SatA of *A. salmonicida* showed that the PlaC sequence not only contains all five conserved blocks but also possesses the three members of the putative catalytic triad in blocks I and V embedded in the respective conserved motifs (Fig. 1). Interestingly, both PlaC and *A. salmonicida* GCAT possess the conserved aspartic acid motif in block III, whereas PlaA lacks this motif. In contrast to SatA of *A. salmonicida* and PlaC, which are acyltransferases with additional PLA and LPLA activity, PlaA possesses only LPLA activity and lacks GCAT activity (18). The conserved aspartate has been found to stabilize the conformation of the enzyme by participating in several hydrogen bonds (35). However, since it is present in most of the diverse GDSL enzymes and is missing only in some (e.g., the esterase of *S. scabies* and the phospholipase from *Vibrio vulnificus* [14, 18]), it is difficult to predict its effect on the enzymatic activity without data on the acyltransferase activity.

Furthermore, we noticed that the distance between blocks I and II in the PlaC sequence is more than twice as large as the distance in most GDSL enzymes (Fig. 1) (8, 14, 18). In the case of rhamnogalacturonan acetylesterase of *A. aculeatus*, the oxyanion of the transition state is stabilized by hydrogen bonds from the main chain NH groups of the active site serine in block I, the conserved glycine residue in block II, and the side chain amide of the conserved asparagine in block III (35). Thus, a large distance between blocks I and II, like that present in PlaC, may allow entrance of a larger molecule and might indicate that PlaC could be able to hydrolyze substrates more voluminous than phospholipids and lysophospholipids. Another possibility is that the loop formed between blocks I and II could be involved in the multimerization of the enzyme, as in the case of PAF-AH(Ib), in which the N-terminal α helix preceding the first β strand is involved in dimer formation of the enzyme (25). The possibility that PlaC might be a multimeric enzyme is suggested by the fact that after gel filtration of *L. pneumophila* culture supernatant, the GCAT activity eluted at a considerably higher molecular mass than the molecular mass of the monomer (data not shown). *A. hydrophila* GCAT is also found in a high-molecular-weight complex (>500 kDa), as estimated by gel filtration. This complex originates from association of the enzyme with lipopolysaccharide-containing outer membrane vesicles rather than from a multimeric form (29, 30, 31).

Moreover, we have shown that PlaC is not required for intracellular infection of *A. castellanii* and U937 macrophages. Likewise, it was shown that an *A. salmonicida satA* mutant does not display attenuated virulence. In spite of being a potent toxin, the enzyme is dispensable for establishment of lethal acute furunculosis in Atlantic salmon (50). Since *L. pneumophila* possesses at least three GDSL enzymes (18), it is possible that the loss of one of these enzymes might be compensated for by one or both of the others. There are other indications which link GCAT activity to bacterial virulence. For instance, the GCAT of *Staphylococcus aureus* and *Staphylococcus epidermidis*, termed FAME, is a virulence factor which neutralizes bactericidal fatty acids by binding them to cholesterol (11). Another putative acyltransferase belonging to the GDSL family is SseJ of *Salmonella enterica* serovar Typhimurium, which is a type III translocated virulence factor and localizes with the *Salmonella*-containing vacuolar membrane (41). SseJ is considered to be necessary for modification of the vacuolar membrane, which is enriched in cholesterol (41). Likewise, PlaC might be a way for *L. pneumophila* to modify host cell membranes as cholesterol is present in eukaryotic membranes but not in the membrane of the bacterium.

In summary, we have shown that PlaC is a new candidate for a type II exported protein which is dependent on the zinc metalloprotease for its activity. PlaC displays PLA, LPLA, and lipase activities and is essential for the GCAT activity of *L. pneumophila*. Finally, it was shown that PlaC is dispensable for intracellular infection. Identification of the activating factor of PlaC and the combined role of PlaC and PlaA during intracellular infection are subjects for further investigation. GCAT activity in gram-negative bacteria has been described previously only for the family *Vibrionaceae*, in particular *Aeromonas*

sp., and *S. aureus* is the only member of gram-positive bacteria for which GCAT activity has been reported (30). Thus, PlaC is the first GCAT of a gram-negative bacterium that does not belong to the family *Vibrionaceae* and the first GCAT to be described for an intracellular pathogen.

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