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Characterization of the Gene Encoding the Major Secreted Lysophospholipase A of *Legionella pneumophila* and Its Role in Detoxification of Lysophosphatidylcholine

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We previously showed that *Legionella pneumophila* secretes, via its type II secretion system, phospholipase A activities that are distinguished by their specificity for certain phospholipids. In this study, we identified and characterized *plaA*, a gene encoding a phospholipase A that cleaves fatty acids from lysophospholipids. The *plaA* gene encoded a 309-amino-acid protein (PlaA) which had homology to a group of lipolytic enzymes containing the catalytic signature GDSL. In *Escherichia coli*, the cloned gene conferred trypsin-resistant hydrolysis of lysophosphatidylcholine and lysophosphatidylglycerol. An *L. pneumophila plaA* mutant was generated by allelic exchange. Although the mutant grew normally in standard buffered yeast extract broth, its culture supernatants lost greater than 80% of their ability to release fatty acids from lysophosphatidylcholine and lysophosphatidylglycerol, implying that PlaA is the major secreted lysophospholipase A of *L. pneumophila*. The mutant's reduced lipolytic activity was confirmed by growth on egg yolk agar and thin layer chromatography and was complemented by reintroduction of an intact copy of *plaA*. Overexpression of *plaA* completely protected *L. pneumophila* from the toxic effects of lysophosphatidylcholine, suggesting a role for PlaA in bacterial detoxification of lysophospholipids. The *plaA* mutant grew like the wild type in U937 cell macrophages and *Hartmannella vermiformis* amoebae, indicating that PlaA is not essential for intracellular infection of *L. pneumophila*. In the course of characterizing *plaA*, we discovered that wild-type legionellae secrete a phospholipid cholesterol acyltransferase activity, highlighting the spectrum of lipolytic enzymes produced by *L. pneumophila*.

Legionella pneumophila is a gram-negative organism which naturally multiplies within aquatic protozoa. Following inhalation by humans, the bacterium also infects lung macrophages and epithelial cells, causing Legionnaires' disease. *L. pneumophila* secretes a variety of potentially destructive enzymes, including a zinc metalloprotease, acid phosphatases, multiple lipases and phospholipases A (PLAs), a phospholipase C-like activity, and an RNase (2, 4, 13, 28, 34). These enzymatic activities, which are secreted via the bacterial type II secretion system, might hydrolyze major cell constituents and thus be important for the development of the disease (2, 28, 35, 45).

Recently, several secreted PLA activities have been found in the genus *Legionella*. Phospholipases are divided into several subgroups depending on their specificity for hydrolysis of ester bonds at different locations in the phospholipid molecule (49). PLAs cleave long-chain fatty acids from the glycerol backbone of phospholipid molecules, whereas phospholipases B release fatty acids both from the sn-1 and sn-2 positions of the glycerol backbone, and phospholipases C and D generate water-soluble compounds as well as 1,2-diacylglycerol or phosphatidic acid, respectively. PLAs can affect phospholipids and/or lysophospholipids having different polar head groups with varying degrees of specificity. Furthermore, they confer a positional specificity for the fatty acid in the sn-1 or sn-2 position and, moreover, for the length and saturation of the fatty acid bound in these positions. The first PLA activity of *L. pneumophila* hydrolyzes phospholipids containing both fatty acids producing lysophospholipids (25–27) (Fig. 1). The second PLA activity, a lysophospholipase A, preferentially liberates fatty acids from lysophospholipids having only one remaining fatty acid (25, 26, 28) (Fig. 1). Both PLA and lysophospholipase A activities are dependent upon the *L. pneumophila* type II secretion apparatus (2, 28, 46). Since *Legionella* type II protein secretion promotes intracellular survival and virulence and since PLAs are known to contribute to the pathogenesis of fungi and other bacteria (19, 20, 30, 35, 46, 50), we sought to characterize the genetic basis of *L. pneumophila* lysophospholipase A activity. Here, we identify the gene encoding the major lysophospholipase A of *L. pneumophila* and then describe its contribution to hydrolysis of lipids, detoxification of lysophosphatidylcholine, and intracellular infection of macrophages and amoebae.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Virulent *L. pneumophila* strain 130b (ATCC strain BAA-74, also known as Wadsworth or AA100) was used for mutagenesis of the *Legionella plaA* gene and later served as the wild-type control (22). Strains NU258 and NU259, direct derivatives of 130b, contain insertion mutations in the *Legionella lspDE* and *lspG* genes, respectively (46). The *proA* mutant AA200, another 130b derivative, is defective for expression of the *L. pneumophila* zinc metalloprotease (38). *L. pneumophila* was routinely grown on buffered charcoal-yeast extract (BCYE) agar for 2 days at 37°C (21). For detection of lipolytic activities, *L. pneumophila* was also grown on egg yolk agar, which contained the same constituents as BCYE agar except that 5% (vol/vol) egg yolk was added and starch was substituted for charcoal (6, 7, 14). For detection of

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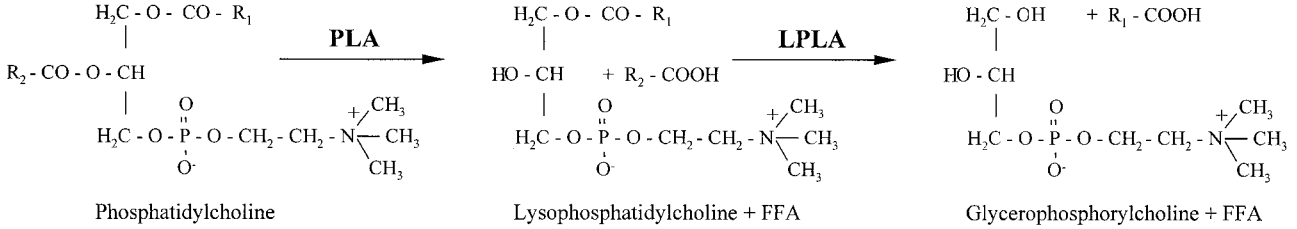

FIG. 1. Model of the two-step hydrolysis of phosphatidylcholine by secreted PLA and secreted lysophospholipase A (LPLA) activities of *L. pneumophila*.

proteolytic activity, bacterial strains were grown on casein agar, which contained the same ingredients as BCYE agar except that 1% (wt/vol) casein was added and charcoal was replaced by starch (54). In order to monitor extracellular growth in liquid media, *L. pneumophila* was cultured in buffered yeast extract (BYE) broth at 37°C with shaking at 250 rpm. Bacterial growth was monitored by determining the optical density of the culture at wavelength 660 nm (OD_{660}; Beckman spectrophotometer DU500), following inoculation to an OD_{660} of 0.2 to 0.3. In order to assess *L. pneumophila* susceptibility to lysophosphatidylcholine, bacteria were grown in BYE broth until mid-log phase, and then 1-monopalmitoyllysophosphatidylcholine (MPLPC) was added to the cultures at final concentrations of 0.05, 0.1, and 0.2 mM. Growth of the bacteria was monitored by determination of the OD_{660}, and after 4 or 16 h of incubation with the lipid, bacteria were plated onto BCYE for determination of CFU. Untreated controls were grown and plated in parallel to the treated cultures. *Escherichia coli* strains NovaBlue and DH5α, hosts for new recombinant plasmids, were grown in Luria-Bertani (LB) broth or agar (5). When appropriate, media were supplemented with antibiotics at final concentrations suitable for *L. pneumophila* (or *E. coli*) as follows: kanamycin, 25 μg/ml (50 μg/ml); chloramphenicol, 6 μg/ml (30 μg/ml); and ampicillin (only for *E. coli*; 100 μg/ml).

Preparation of culture supernatants and cell lysates. Culture supernatants for assessment of hydrolytic activities were obtained at the end of exponential growth (i.e., OD_{660} of 2.2 to 2.3) by centrifugation for 5 min at 5,000 × *g*. Ten- or 20-fold-concentrated culture supernatants were prepared by isopropanol precipitation as described earlier (26). In short, 2 volumes of precooled isopropanol was added to 1 volume of culture supernatant, mixed, and incubated at −20°C for 10 min. The proteins were subsequently separated by centrifugation at 5,000 × *g* for 30 min at 4°C. The protein pellet was dissolved in the appropriate volume of 20 mM Tris-HCl (pH 7.5, 26°C). For the generation of cell lysates, bacteria from late exponential phase were pelleted by centrifugation as described above and then lysed by addition of a 1/20 volume of the original culture volume of 10 mg of lysozyme/ml and 1 μl of Triton X-100/ml at 37°C for 30 min. After repeated passage through a 26-gauge needle, the lysate was rediluted to its initial culture volume. Culture supernatants and cell lysates were tested immediately for enzymatic activities.

PCR and DNA sequence analysis. Genomic DNA of *L. pneumophila* was isolated as previously described (43). Primers plaa-d1 (5′-GCATCATCCAGCT TCTTGTC-3′) and plaa-e1 (5′-CTGGCTTCACAGACGCAACC-3′), based on the sequence found in the incomplete *L. pneumophila* database (http://genome3.cpmc.colombia.edu/~legion/), were used to amplify the *plaA* gene from strain 130b DNA. The 1,151-bp PCR product begins 355 bp upstream of *plaA* and ends at bp 796 within the open reading frame (ORF). To isolate an intact copy of *plaA*, the amplified fragment was labeled with digoxigenin (Boehringer Mannheim, Indianapolis, Ind.) and used as a probe to screen a 130b library by colony blot hybridization. The 130b genomic library consisted of 3- to 6-kb *Sau*3AI-restricted DNA cloned into pBR322 (31). A double-stranded sequence of cloned *L. pneumophila* DNA was determined using the BigDye terminator cycle sequencing mix (PE Applied Biosystems, Foster City, Calif.). Automated sequence analysis on an ABI Prism 373 DNA sequencer (Applied Biosystems) was performed at the Biotech Facility at Northwestern University Medical School, Chicago, Ill. Primers were obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa). Sequence database searches as well as protein alignments were performed by using the BLAST algorithm (1). The nucleotide sequence was also analyzed for promoters (44), and the predicted protein was analyzed with the SignalP program for a signal sequence (41).

Gene cloning and *Legionella* mutant construction. To assist with ascribing function to *plaA*, several recombinant plasmids were derived from pAF1, a *plaA*-containing plasmid derived from our screen of the genomic library. First, a 2.3-kb *Bam*HI/*Stu*I fragment of pAF1 was subcloned into the *Bam*HI/*Eco*RV sites of pBluescript II KS(+) (Stratagene, La Jolla, Calif.), yielding pAF2. Next, pAF2 was restricted with *Bst*98I in order to delete nucleotides 1078 to 1393 of *plaA*, treated with Klenow fragment, and ligated with a kanamycin resistance gene (Km^r) cassette from pVK3 (60), resulting in pAF3. Furthermore, a PCR product that contained only *plaA* was amplified using primers plaa-d1 and plaa-m1 (5′-ATAAGGACCATTGCGCTG-3′) and cloned into the T-tailed *Eco*RV site of pGEM-Teasy (Promega, Madison, Wis.), resulting in pAF7. Subsequently, the *Sal*I and *Sph*I sites of the pGEM-Teasy backbone were used to subclone *plaA* into the corresponding sites of pMMB207 (40), yielding pAF8. Plasmids were isolated from *E. coli* by alkaline lysis using the Midiprep kit from Bio-Rad Laboratories (Hercules, Calif.).

To isolate an *L. pneumophila plaA* mutant, pAF3 and allelic exchange were used to introduce a Km^r insertion mutation into the chromosome of strain 130b (35). Plasmid pAF3 was introduced into *L. pneumophila* by natural transformation (53). Based upon the observations that *L. pneumophila* transformation is correlated with type IV pilus expression and that pili are more prominent at 30°C than at 37°C (35, 53), modifications to the original transformation method were made (K. Allard and N. P. Cianciotto, unpublished observations). In detail, 130b bacteria were inoculated (i.e., OD_{660} = 0.2) into 2 ml of BYE broth contained within a polypropylene plastic tube, and then 5 μg of plasmid DNA/ml was added. After growing the culture for approximately 18 h at 30°C with moderate shaking until mid-exponential phase, the bacteria were plated on BCYE agar supplemented with kanamycin. PCR and Southern blot analysis were used to examine Km^r legionellae for the presence of the *plaA* mutation (2, 48). For generation of strains for complementation experiments, pMMB207 and pAF8 were introduced into wild-type and mutant *L. pneumophila* by electroporation as described previously (45).

Enzymatic assay for lipolytic activities. Enzymatic activities were detected as described previously, with minor changes (2, 27, 28). In detail, 25 μl of different phospholipids or lipids were incubated with 25 μl of culture supernatant, cell lysate, or concentrated culture supernatant in a mixture containing 6 mM lipid substrate (3.4 mg of MPLPC/ml, 3.4 mg of 1-monopalmitoyllysophosphatidylglycerol [MPLPG]/ml, 2.2 mg of 1-monopalmitoylglycerol [1-MPG]/ml, 5 mg of 1,2-dipalmitoylphosphatidylglycerol [DPPG]/ml, 5 mg of 1,2-dipalmitoylphosphatidylcholine [DPPC]/ml), 3 mM NaN_3, 0.5% (vol/vol) Triton X-100, and 20 mM Tris-HCl (pH 7.2). When concentrated culture supernatants were assessed for glycerophospholipid-cholesterol acyltransferase (GCAT) activity, 6 mM (0.25 mg/ml) cholesterol was added. All lipids, including thin-layer chromatography (TLC) standards, were obtained from Sigma Chemical (St. Louis, Mo.) 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 (Vibracell; Sonics and Materials Inc., Danbury, Conn.) three times for 20 s at an intensity of 5. In order to test whether the lysophospholipase A activity was trypsin resistant, we added 10 μl from a 10-mg/ml stock of trypsin (Sigma Chemical) in 20 mM Tris-HCl (pH 7.2) to the reaction mixtures. The incubations with bacterial products were performed at 37°C with continuous agitation at 250 rpm for 2.5 h in the case of unconcentrated *L. pneumophila* supernatants and lysates, for 18 h in the case of concentrated *L. pneumophila* supernatants, and for 5 h in the case of *E. coli* supernatants. Free fatty acids (FFA) were determined by means of the NEFA-C kit (WAKO Chemicals, Neuss, Germany) according to the instructions of the manufacturer. The assay was modified for the use of microtiter plates, i.e., 2 to 10 μl of the reaction mixture was analyzed following the addition of 50 μl of reagent A and 100 μl of reagent B. Depending upon the nature of the experiment, BYE broth, concentrated BYE broth, or LB broth was incubated and treated like the cultures and subsequently used as a negative control.

Lipid extraction and TLC. For the detection of distinct polar and apolar lipids, reaction mixtures of lipids with concentrated culture supernatants, corresponding negative controls, or pieces from egg yolk agar were subjected to a lipid

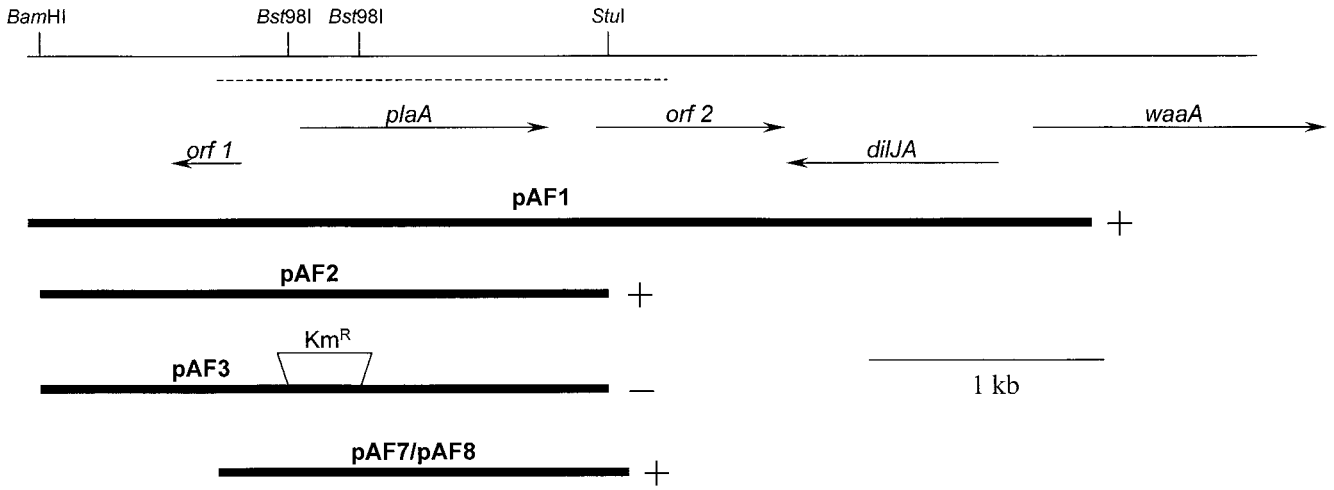

FIG. 2. The *plaA* locus in *L. pneumophila* and recombinant *E. coli*. The upper line represents a 5-kb region of the *L. pneumophila* chromosome that contains the lysophospholipase A gene (*plaA*), along with the location of relevant restriction enzyme sites. The dashed line represents the DNA region that we sequenced and have deposited in the GenBank database. The arrows below this line depict the relative location, size, and orientation of *plaA* and neighboring genes. The thick lines at the bottom of the figure represent the segments of *Legionella* DNA that were cloned into plasmid vectors. Plasmid pAF3 contained a Km^r gene cassette in place of the *Legionella* sequences that normally exist between the indicated *Bst*98I sites. The + and − symbols denote whether supernatants from the recombinant *E. coli* exhibited increased lysophospholipase A activity.

extraction (9, 27). 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 ratio of 65:25:4 (vol/vol/vol) (27, 55). A mixture of petroleum ether-diethylether-glacial acetic acid in a ratio of 90:10:1 (vol/vol/vol) was used for separation of apolar lipids, including cholesterol esters (37). For visualization, silica plates were then stained with naphthol blue black (Aldrich Chemical Company, Milwaukee, Wis.) (42).

Intracellular infection of U937 cells and *Hartmannella vermiformis* amoebae. U937, a human cell line that differentiates into macrophage-like cells upon treatment with phorbol esters, and *H. vermiformis* amoebae were used as hosts for in vitro infection by *L. pneumophila* (15, 16). The cell line and amoebae were maintained and infected as previously described (15, 16, 35). To assess intracellular growth of *L. pneumophila*, wells containing U937 cells or amoebae at a concentration of 10^6/ml and 10^5/ml, respectively, were infected with wild-type bacteria or isogenic mutants at a multiplicity of infection of 0.1. At various time points, the number of intracellular plus extracellular bacteria per well was determined by plating serial dilutions on BCYE agar (35). To measure the cytopathic effect of *L. pneumophila* strains on U937 cells, the ability of the infected monolayer to reduce alamar blue (Biosource International, Vacaville, Calif.) was determined (3, 52). Briefly, at various time points, the infected cells were thoroughly washed to eliminate the extracellular bacteria and then incubated with a mixture of medium and alamar blue at 10:1 (vol/vol) at 37°C for 3 h. After this time, the fluorescence (excitation, 540 nm; emission, 584 nm) was read in a Spectra Max Gemini fluorescence reader (Molecular Devices, Sunnyvale, Calif.).

Nucleotide sequence accession number. The *L. pneumophila* 130b *plaA* sequence has been deposited in the GenBank database at the National Center for Biotechnology Information under accession number AF510106.

RESULTS

Identification of an *L. pneumophila* lysophospholipase A gene. Recently, we determined the N-terminal sequence of a lysophospholipase A purified from culture supernatants of *L. pneumophila* strain 130b (28). The 17 N-terminal amino acids (i.e., TPLNNIVVFGDSLSDNG) were used in a BLAST search of the incomplete genome database of *L. pneumophila* Philadelphia strain 1 (http://genome3.cpmc.columbia.edu/~legion/int_blast.html). One incomplete ORF encoded residues that matched exactly with the N-terminal sequence. Using primers based on the sequence of the putative lysophospholipase gene, we were able to PCR amplify a 1,151-bp fragment from 130b genomic DNA. Then, using labeled PCR product and colony blot hybridization screens, five positive clones were found in our *L. pneumophila* 130b genomic library. Sequencing of the 4.3-kb insertion in one of the recombinant plasmids, pAF1, confirmed the existence of an intact *L. pneumophila* lysophospholipase A gene, which we designated as *plaA*, for PLA gene A (Fig. 2).

The 930-bp *plaA* sequence (GenBank accession no. AF510106) was predicted to encode a protein of 309 amino acids. The deduced PlaA sequence showed homology with numerous proteins, most of which are lipolytic enzymes. The closest overall homologies were with the GCAT of *Aeromonas salmonicida* (identity, 31%; similarity, 47%), phosphatidylcholine-sterol *O*-acyltransferase from *Aeromonas hydrophila* (identity, 31%; similarity, 47%), secreted effector J of *Salmonella enterica* serovar Typhimurium (identity, 29%; similarity, 47%), and the lecithinases of *Vibrio cholerae* and *Vibrio mimicus* (identity, 27%; similarity, 47 to 48%) (Fig. 3). These five enzymes and most of the PlaA homologs belong to a family of lipolytic proteins from prokaryotes as well as eukaryotes that contain a GDSL motif near their N termini (23, 57). Members of this group can be aligned along five blocks (I to V) of amino acid homology (57) (Fig. 3). We observed that this family of enzymes could be further classified into three subgroups, depending on the absence (group A) or presence of long N-terminal tails prior to block I (group B) or long C-terminal stretches after block V (group C). PlaA contains the GDSL motif close to its N terminus and possesses each of these blocks, suggesting that it is a member of this family (Fig. 3). *L. pneumophila* PlaA, like the GCAT protein of *A. hydrophila*, does not show any prolonged tails on either its N terminus or its C terminus and would therefore be a member of group A. After defining the three-dimensional structure of a GDSL-

	Organism	Accession No.	Function		Block I	
					******* *	
a)	*Legionella pneumophila*	AF510106	Lysophospholipase A	23	IVVFGD*S*LSDNG	41
b)	*Aeromonas hydrophila*	P10480	Acyltransferase (GCAT)	27	IVMFGDSLSDTG	37
c)	*Salmonella typhimurium*	AF294582	Secreted effector SseJ	144	LVFFGDSLSDSL	39
d)	*Acidiphilium sp.*	AB026254	Esterase	25	LYVFGDSLSDDG	36
e)	*Vibrio cholerae*	U50074	Lecithinase	148	VIAFGDSLSDTG	36
f)	*Vibrio harveyi*	AF293430	Hemolysin	147	VVALGDSLSDTG	36
g)	*Xylella fastidiosa*	D82761	Lipase/esterase	29	TIFFGDSLSDSG	43
h)	*Vibrio mimicus*	AF035162	Lecithinase	200	VIVFGDSLSDTG	36
i)	*Vibrio parahemolyticus*	Q99289	Hemolysin/PLA_2/LPLA	146	VVALGDSLSDTG	36
j)	*Vibrio vulnificus*	AF291424	Phospholipase	144	IVAFGDSLSDTG	36
k)	*Pseudomonas aeruginosa*	G83030	Hypothetical protein	31	IHAFGDSYSDNG	45
l)	*Xenorhabdus luminescens*	P40601	Triacylglycerol lipase	27	LYVFGDSLSDGG	34
m)	*Pseudomonas putida*	P40604	Hypothetical protein in *trpE-trpG* region	30	MIVFGDSLSDAG	48

	Block II		Block III		Block IV		Block V	
	* *****		** * **		** * ****		*** ***	
a)	SHLLDYAFG*G*AG	34	FVIWIGA-*N*NYL	26	KGAKHILVLNLPD	104	FF*D*LV*H*PT	25
b)	LTIANEAEGGPT	37	VILWVGA-NDYL	26	NGAKEILLFNLPD	128	FWDQVHPT	24
c)	KEMLNFAEGGST	31	AIFLLGA-NDYM	23	GGVNNVLVMGIPD	93	FNDLVHPT	22
d)	SSANDYAYGGAF	42	VTLWGGA-NNYF	37	LGARMLIVPNLPD	89	FWDNVHPT	342
e)	VPLYNWAVGGAA	34	FTLEFGL-NDFM	22	AGAKNLVLMTLPD	101	FWEVTHPT	21
f)	LPLYNWAVGGAA	34	FTLEFGL-NDFM	22	AGAKNLLLMTLPD	101	FWDVTHPT	23
g)	QTGNNYAAGGAH	35	YTLWGGG-NDLL	29	AGARYIVVATIPD	89	FADDIHPT	316
h)	VPIYNWAVGGAA	34	FTLEFGL-NDFM	22	ARAKNIVLLTLPD	101	FWEVTHPT	21
i)	LPLYNWAVGGAA	34	FTLEFGL-NDFM	22	AGAKNFMLMTLPD	101	FWNVTHPT	23
j)	LPLYNWAVGGAA	38	FGLEPTFMNYNR	18	AGAKNIMLMTLPD	101	FWGVTHPT	23
k)	AQLADHAVGGAK	39	HFIFVSA-NDFF	31	AGARRFLVVSSTD	88	FWDEWHPT	17
l)	KGGTNYAAGGAT	31	YVHWIGG-NDVD	29	AGAGLVIVPTVPD	155	FADDFHPT	313
m)	PDGNNWAVGGYT	45	YYLTGGG-NDFL	29	GGARYIMVWLLPD	94	FNDLVHPT	293

FIG. 3. Sequence alignment of *L. pneumophila* PlaA with members of the GDSL family. Sequences of the 12 closest matches to PlaA are aligned along the five conserved blocks of the GDSL family. An asterisk designates those positions where an amino acid is conserved in at least six of the homologs. The amino acids comprising the putative catalytic triad of PlaA are shown in italics, and the residues conserved in the SGNH family are underlined. The digits before and after blocks I to V indicate the number of amino acid residues present before and after the conserved regions.

containing rhamnogalacturonan acetylesterase, Molgaard et al. identified a catalytic triad that is composed of the first serine in block I and the aspartic acid and histidine in block V (39). These authors further showed that only four amino acids (i.e., S, G, N, and H) are completely conserved among the GDSL enzymes, and they argued for this group of hydrolases to be designated as the SGNH hydrolase family (39). With its possession of SGNH (Fig. 3), *L. pneumophila* PlaA fulfills the proposed requirements of an SGNH hydrolase family member. PlaA was predicted to have an 18-amino-acid signal sequence, supporting our belief that it is a secreted enzyme (28, 46). The amino acids that occur after the predicted signal sequence are in exact agreement with the N-terminal amino acid sequence of the lysophospholipase A purified from *L. pneumophila* supernatants (28).

Two uncharacterized genes (i.e., *orf 1* and *orf 2*), predicted to encode products with no significant homology to known proteins, flanked *plaA* (Fig. 2). Whereas the upstream *orf 1* was oriented in the opposite direction from *plaA*, the downstream *orf 2* was oriented in the same direction as *plaA*. We suspect that the *plaA* message is monocistronic, since *plaA* and *orf 2* are separated by 192 bp and since −10 and −35 promoter sequences exist within the intergenic region (GenBank accession no. AF510106). The *L. pneumophila* genomic database (http://genome3.cpmc.columbia.edu/~legion/int_blast.html) indicates that the *dilJA* gene, predicted to encode a chaperone, and the *waaA* gene, shown to encode a 3-deoxy-D-*manno*-oct-2-ulosonic acid transferase (10), lie downstream of *orf 2* (Fig. 2).

Enzymatic activities of *E. coli* clones containing *plaA*. In order to confirm that *plaA* encodes a lysophospholipase A, we sought to examine secreted enzymatic activities associated with a set of recombinant *E. coli* clones. Toward that end, a 2.3-kb fragment containing *plaA* was subcloned from pAF1 into pBluescript KS(+), resulting in pAF2 (Fig. 2). Furthermore, the subcloned *plaA* was interrupted by deletion of the region between its two *Bst*98I sites, yielding pAF3 (Fig. 2). Finally, a 1.7-kb PCR fragment containing *plaA*, but no other complete ORF, was cloned into pGEM-Teasy and pMMB207, yielding pAF7 and pAF8, respectively (Fig. 2). Culture supernatants of *E. coli* clones containing either pAF1, pAF2, pAF7, or pAF8 released significantly more FFA from MPLPC, the known substrate of the *L. pneumophila* lysophospholipase A (28), than *E. coli* containing the corresponding vector (Fig. 4A and data not shown). Moreover, the clone harboring pAF3 and its inactivated *plaA* did not liberate increased amounts of FFA from MPLPC (Fig. 4A). Since *L. pneumophila* supernatants do not

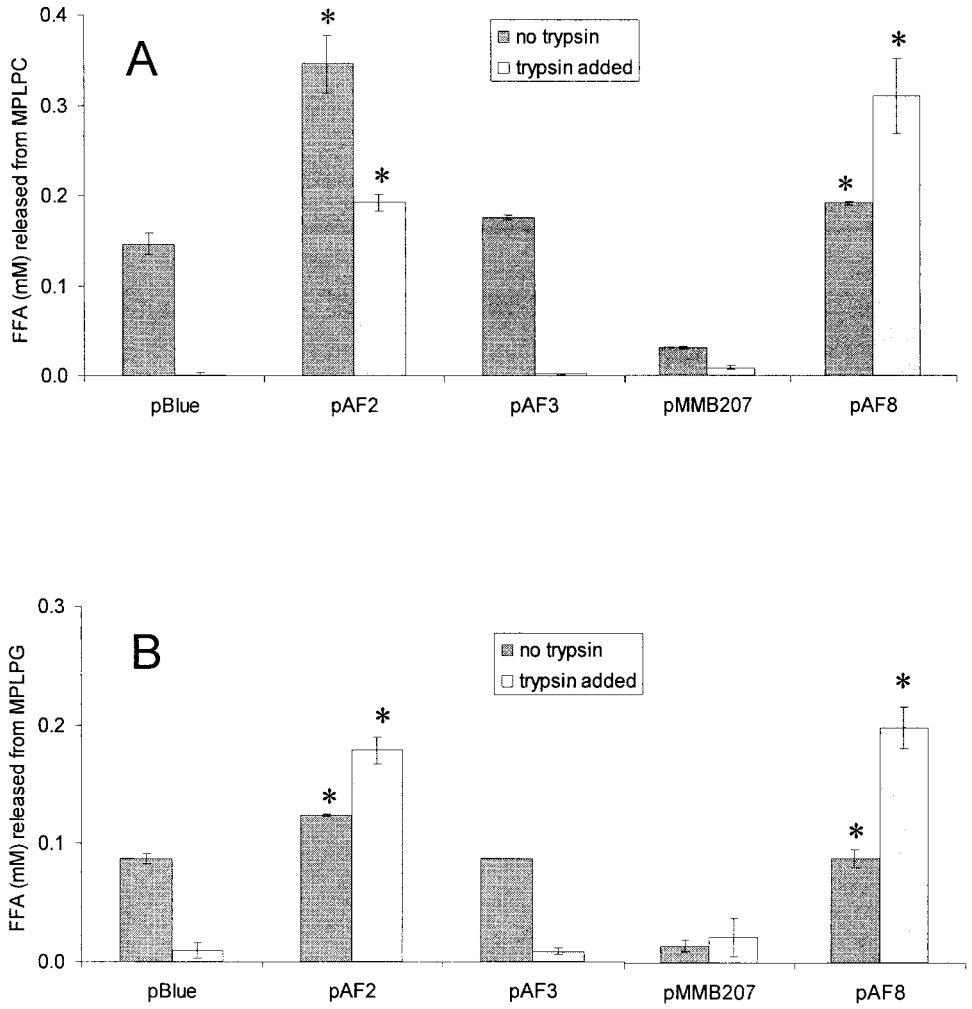

FIG. 4. Lysophospholipase A activity of *E. coli* containing *L. pneumophila plaA*. Culture supernatants of *E. coli* NovaBlue containing pBluescript KS(+) (pBlue) or its derivatives pAF2 or pAF3 as well as *E. coli* DH5α harboring pMMB207 or its derivative pAF8 were mixed with MPLPC (A) or MPLPG (B) in the presence or absence of trypsin and, after a 5-h 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 and the amount released by uninoculated LB broth. The results represent the means ± standard deviations of duplicate cultures and are representative of three independent experiments. Asterisks denote significant differences in lysophospholipase activity between *E. coli* containing *plaA* and the respective vector control ($P < 0.05$; Student's *t* test).

yield lysophosphatidylglycerol following exposure to lung surfactant (i.e., a source of phosphatidylglycerol) and since they release more FFA from DPPG than from DPPC (25, 26, 28), we hypothesized that lysophosphatidylglycerol might also be a substrate for PlaA. Therefore, we tested the supernatants from recombinant *E. coli* for the hydrolysis of MPLPG. As predicted, the *E. coli* clones containing pAF1, pAF2, pAF7, and pAF8, but not pAF3, were able to release more FFA from MPLPG (Fig. 4B and data not shown). Thus, PlaA confers activity towards both lysophosphatidylcholine and lysophosphatidylglycerol.

Whereas some lipolytic enzymes are susceptible to inactivation by common proteases (8), the activated form of the GCAT of *A. hydrophila*, a homolog of PlaA (see above), is stable towards further proteolytic cleavage (11, 32). Hence, we were interested to test the effect of the serine protease trypsin on the cloned PlaA activity as well as the background lysophospholipase A activity of *E. coli*. As shown in Fig. 4, lysophospholipase activity in supernatants from *E. coli* clones containing either empty vector or the insertionally inactivated *plaA* was eliminated by the addition of trypsin. In contrast, the PlaA activity associated with pAF2 and pAF8 persisted after protease treatment (Fig. 4). Thus, *L. pneumophila* PlaA is a trypsin-resistant lysophospholipase A.

None of the culture supernatants cleaved the phospholipids DPPG and DPPC, which contain both fatty acids and are substrates for the *L. pneumophila* PLA (28) (data not shown). Taken together, these data confirm that *L. pneumophila plaA* specifically encodes a lysophospholipase A.

Isolation of *L. pneumophila plaA* mutants. In order to determine the degree to which PlaA is responsible for the lysophospholipase A activity in *Legionella* supernatants, we constructed

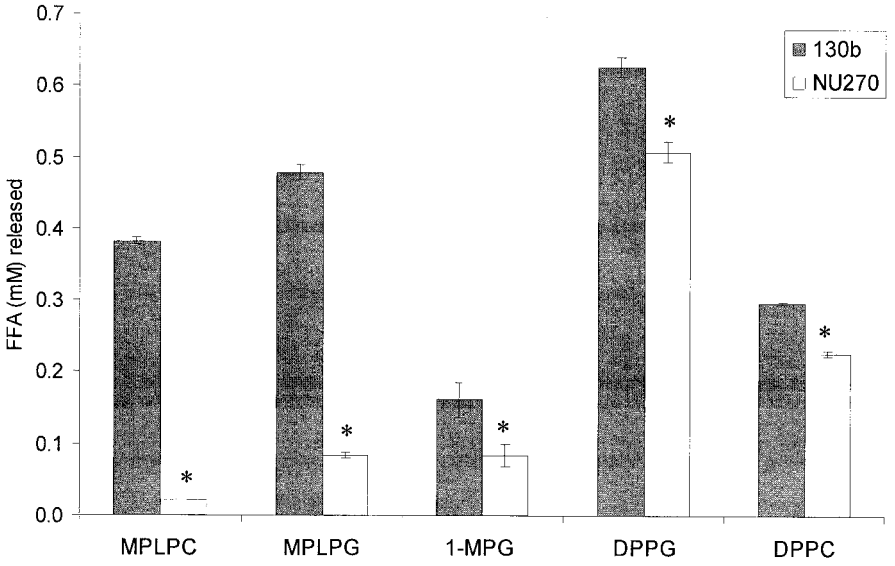

FIG. 5. Lipolytic activities of culture supernatants of wild-type and *plaA* mutant *L. pneumophila*. Culture supernatants from late log phase of BYE cultures of strains 130b and NU270 were incubated with MPLPC, MPLPG, 1-MPG, DPPG, or DPPC for 2.5 h at 37°C, and then the release of FFA was quantified. Data are expressed as differences between the amount of FFA released by the culture supernatant and the amount released by uninoculated BYE broth. The results represent the means ± standard deviations of triplicate cultures and are representative of two independent experiments. Asterisks denote significant differences in lipolytic activities between wild-type *L. pneumophila* and NU270 ($P < 0.01$ for MPLPC, MPLPG, DPPG, and DPPC hydrolysis, and $P < 0.05$ for hydrolysis of 1-MPG; Student's *t* test).

a set of *L. pneumophila plaA* mutants. More specifically, pAF3 (Fig. 2) and allelic exchange were used to introduce a Km^r cassette into the *plaA* gene of strain 130b. Two *plaA* mutants (i.e., NU270 and NU271) were obtained following two separate DNA transformations and allelic exchange selections. PCR and Southern blot analysis confirmed the mutations in *plaA* (data not shown). All of the following experiments, with the exception of the MPLPC sensitivity assays (see below), were performed with both NU270 and NU271 with comparable results, although for clarity only data for NU270 are presented. Thus, the phenotypes observed resulted directly or proximately from the mutation in *plaA* and not from spontaneous, second-site mutations.

To assess the importance of *plaA* for *L. pneumophila* extracellular growth, we compared, on three separate occasions, strains 130b and NU270 for their growth in BYE broth, the standard medium for culturing legionellae. As measured by the OD_{660} of the cultures, NU270 grew comparably to wild type throughout the logarithmic and stationary growth phases when incubated at 37°C with shaking (data not shown). Furthermore, the mutant grew normally on BCYE agar, the standard solid medium for culturing legionellae. Since *L. pneumophila* lysophospholipase A activity is dependent upon the *lsp* type II protein secretion system, and since wild-type and isogenic *lspDE* and *lspG Legionella* mutants have different colony morphologies (28, 46), we examined the colonial growth of NU270. After 4 days of growth on BCYE agar at 37°C and 10 days at room temperature, no differences in colony morphology were observed between the mutant and wild type (data not shown), indicating that the altered colony morphology of the secretion mutants is not due to a loss of PlaA. Thus, these data indicate that *plaA* is not required for normal extracellular growth in liquid or on solid bacteriological media.

Lipolytic activities of an *L. pneumophila plaA* mutant. Growth of bacteria, including *L. pneumophila*, on egg yolk agar is often used to estimate secretion of lipolytic factors (6, 7, 14). PLAs generate clearing, due to their ability to produce lysophospholipids. Lysophospholipases A alleviate that clearing. Therefore, an *L. pneumophila* mutant lacking a lysophospholipase A should generate more clearing than the wild type. Indeed, NU270 produced more clearing on egg yolk agar than did strain 130b (data not shown). When the agar medium surrounding the bacterial growth was examined by TLC, we observed an enrichment of lysophosphatidylcholine associated with the mutant (data not shown). The phenotype of NU270 observed on egg yolk agar was fully complemented by a plasmid copy of *plaA*, indicating that alterations in clearing are due to the loss of *plaA* (data not shown). Taken together, these observations suggest a decrease in secreted lysophospholipase A activity in the *L. pneumophila plaA* mutant.

To more carefully assess the *plaA* mutant with respect to lipid hydrolysis, we tested culture supernatants for their ability to release FFA from MPLPC and MPLPG. Whereas hydrolysis of MPLPC by NU270 was reduced to less than 10% of wild-type activity, release of FFA from MPLPG was reduced to about 20% of normal levels (Fig. 5). These data indicate that PlaA is the major lysophospholipase A of *L. pneumophila*. The residual activities towards MPLPC and MPLPG suggest the presence of an additional, secreted lysophospholipase(s) A. Since partially purified PlaA had some activity against nonphospholipids (28), we next tested the relative ability of the mutant to hydrolyze 1-MPG. The hydrolysis of 1-MPG was reduced approximately 50% in NU270, confirming that PlaA is active against phospholipids and nonphospholipids (Fig. 5). The residual activity is likely due to other lipolytic enzymes, such as the recently described LipA lipase (4). Culture super-

TABLE 1. Genetic complementation of a lipolytic defect in the *L. pneumophila plaA* mutant

Supernatant sample	FFA (mM) released from[a]:				
	MPLPC	MPLPG	1-MPG	DPPG	DPPC
130b(pMMB207)	0.437 ± 0.005	0.516 ± 0.018	0.169 ± 0.028	0.482 ± 0.011	0.201 ± 0.004
130b(pAF8)	3.055 ± 0.112†[b]	3.004 ± 0.138†	0.468 ± 0.027†	0.755 ± 0.138†	0.323 ± 0.013†
NU270(pMMB207)	0.020 ± 0.001	0.100 ± 0.011	0.081 ± 0.021	0.402 ± 0.011	0.161 ± 0.003
NU270(pAF8)	2.493 ± 0.062†	2.849 ± 0.216†	0.435 ± 0.029†	0.654 ± 0.009†	0.294 ± 0.005†

[a] Supernatants from late-log phase BYE cultures of strains 130b and NU270 containing pMMB207 or pAF8 were incubated with the indicated substrates for 2.5 h at 37°C, and subsequently the release of FFA was quantified. Data are expressed as differences between the amount of FFA released by the culture supernatant and the amount released by uninoculated BYE broth. The results represent the means ± standard deviations of triplicate cultures and are representative of two independent experiments.

[b] A † denotes significant differences in lipolytic activity between the wild type or *plaA* mutant harboring the vector pMMB207 and the respective strains with the *plaA*-containing vector pAF8 ($P < 0.05$; Student's *t* test).

natants from the *plaA* mutant also had a modestly reduced release of FFA from DPPG and DPPC, two PLA substrates (Fig. 5). Since the *plaA*-containing *E. coli* clones did not liberate FFA from DPPG or DPPC (data not shown), this observation is likely due to an inability of mutant supernatants to further cleave those lysophospholipids produced by the action of the *Legionella* PLA. The ability of NU270 to fully release FFA from MPLPC, MPLPG, 1-MPG, DPPG, and DPPC was restored after *trans* complementation with *plaA* on pAF8 (Table 1). The activities of both the complemented wild type and NU270 against MPLPC and MPLPG were six- to ninefold higher than wild-type levels, a result that is likely due to multiple copies of *plaA*.

To confirm that the *plaA* mutant had altered lipolytic activities, we used TLC to examine the pattern of phospholipid cleavage caused by concentrated culture supernatants. In agreement with our FFA release data, strain 130b supernatants hydrolyzed more MPLPC and MPLPG than did those of NU270 (Fig. 6 and data not shown). Furthermore, the NU270 samples contained more lysophosphatidylcholine or lysophosphatidylglycerol following incubation with DPPC or DPPG, respectively (Fig. 6 and data not shown). These experiments also confirmed the reduced lysophospholipase A and PLA activities of *L. pneumophila* type II secretion mutants (Fig. 6 and data not shown). Taken together, the TLC experiments confirmed the significant loss of secreted lysophospholipase A activity that is associated with the *plaA* mutation in NU270.

With the isolation of a 130b mutant that does not express the major secreted lysophospholipase A, we were in a position to more clearly assess the prevalence of cell-associated lysophospholipase A activity. Therefore, we tested wild-type and mutant cell lysates for activities towards PlaA substrates MPLPC, MPLPG, and 1-MPG as well as PLA substrates DPPG and DPPC. High levels of activity against MPLPC, MPLPG, and 1-MPG were found in both strains (Fig. 7), implying the presence of a cell-associated lysophospholipase(s) A and perhaps lipase activity. Hydrolysis of DPPG and DPPC was also observed (Fig. 7), suggesting the presence of some unprocessed forms of the secreted PLA and/or an additional PLA(s) that is strictly linked to the bacterial cell.

Susceptibility of *L. pneumophila* to cytotoxic lysophosphatidylcholine. The PlaA substrate MPLPC is a potentially cytotoxic lipid (61) which can be generated by the action of host and pathogen PLAs on the membranes of eukaryotes and prokaryotes as well as lung surfactant (25, 33, 36, 56). Indeed, one of the suggested roles of lysophospholipases is the maintenance of lysophospholipids, such as MPLPC, at safe levels (29). For this reason, we examined whether wild-type and *plaA* mutant *L. pneumophila* differed in their susceptibility to lysophosphatidylcholine. More specifically, we added 0.05, 0.1, and 0.2 mM MPLPC to mid-log-phase cultures and then assessed changes in the viability of the bacteria. Previous experiments had shown that *L. pneumophila* lysophospholipase A secretion

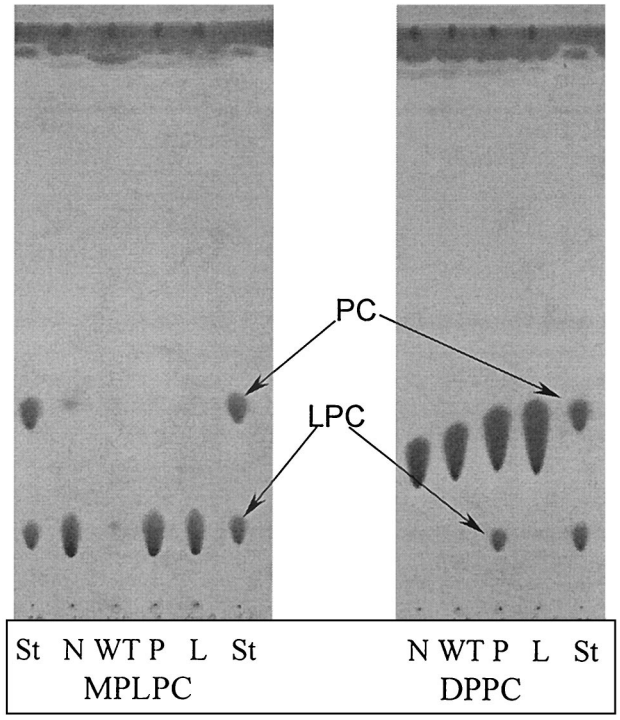

FIG. 6. TLC analysis of lipid hydrolysis by wild-type and mutant *L. pneumophila*. Tenfold-concentrated culture supernatants from late log phase of strains 130b (WT), NU270 (P), and an *lspDE* mutant (L) were incubated with MPLPC or DPPC for 18 h at 37°C. Subsequently, the lipids were extracted and separated by TLC. A mixture of concentrated BYE broth and the lipids was also incubated and served as a negative control (N). In the case of incubations with MPLPC (left panel), the samples were examined for the degradation of the lipid substrate and, in the cases of incubations with DPPC, for enrichment of lysophosphatidylcholine (LPC). For the qualitative identification of the lipid spots, lanes containing MPLPC and DPPC standards are marked St. The observations depicted here were made on at least two occasions.

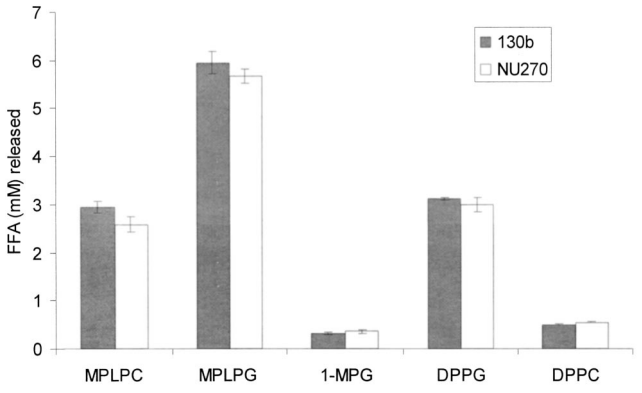

FIG. 7. Lipolytic activities of cell lysates of wild-type and *plaA* mutant *L. pneumophila*. Cell lysates from late log phase BYE cultures of strains 130b and NU270 were incubated with MPLPC, MPLPG, 1-MPG, DPPG, or DPPC for 2.5 h at 37°C. Subsequently, the release of FFA was quantified. Data are expressed as differences between the amount of FFA released by the culture supernatant and the amount released by uninoculated BYE broth that, like the cell samples, had been treated with lysozyme and Triton X-100. Results represent the means ± standard deviations of triplicate cultures and are representative of two independent experiments.

starts during mid-log phase and that wild-type cells grown in the presence of lung surfactant produce as much as 0.5 mM lysophosphatidylcholine (25, 26). Compared to an untreated control, wild-type bacteria showed reduced growth when exposed to 0.2 mM MPLPC (Fig. 8A). The viability difference between treated and untreated wild-type cultures was also confirmed when the cultures were plated for CFU determinations (Fig. 8B). These data show that lysophosphatidylcholine can be cytotoxic towards *L. pneumophila*. Mutant NU270 was only slightly more susceptible to lysophosphatidylcholine than the wild type (Fig. 8A and B), suggesting that PlaA is not critical for protection of *L. pneumophila* against MPLPC. Indeed, an *L. pneumophila lspDE* mutant was dramatically more sensitive to 0.2 mM MPLPC than was the wild type or the *plaA* mutant (Fig. 8A and B), indicating that other type II secreted factors can insure protection from toxic lysophosphatidylcholine. However, when *plaA* was introduced on a multicopy plasmid into the wild type or NU270, the bacteria were resistant to MPLPC (Fig. 8C), suggesting that PlaA may, under certain circumstances, play a role in detoxification of lysophosphatidylcholine.

Influence of the *L. pneumophila* zinc metalloprotease on secreted lipolytic activities. The PlaA-like GCAT of *A. hydrophila* is activated by a serine protease, which removes a small peptide between cysteine residues C_{225} and C_{281} (11, 32). This cleavage event occurs after the removal of the protein's 18-amino-acid signal peptide, and the resulting disulfide bridge formed between the cysteines renders the GCAT relatively resistant toward trypsin treatment (11, 17, 59). Since PlaA has two cysteines (i.e., C_{225} and C_{254}) in locations similar to those of the *Aeromonas* cysteines and is trypsin resistant, we were interested in whether the type II-secreted zinc metalloprotease of *L. pneumophila* is involved in PlaA activation. Therefore, we compared culture supernatants from strain 130b and its isogenic *proA* mutant AA200 for their ability to release FFA from different lipid substrates. Although the strains showed no difference in FFA liberation from MPLPC, the major substrate of PlaA, the protease mutant released less FFA from MPLPG, 1-MPG, DPPG, and DPPC (Fig. 9). These data indicate that the *L. pneumophila* metalloprotease has no role in PlaA processing but may promote activation of the *Legionella* PLA.

Identification of an *L. pneumophila* GCAT activity. Since PlaA shared homology and trypsin resistance with the GCAT from *Aeromonas*, we sought to determine if PlaA can transfer fatty acids from phospholipids to cholesterol. Since it was not known whether *L. pneumophila* possesses this activity, we first examined wild-type legionellae for GCAT activity. Toward that end, we incubated concentrated culture supernatants of strain 130b with cholesterol-lipid mixtures containing DPPG, DPPC, MPLPC, MPLPG, or 1-MPG and subsequently analyzed the lipid extracts for cholesterol esters by TLC. Most obvious, the wild type transferred fatty acids from DPPG to cholesterol, showing that *L. pneumophila* indeed confers GCAT activity (Fig. 10A). Transfer of fatty acids from DPPC, MPLPC, and MPLPG to cholesterol was less prominent (Fig. 10A), suggesting that phosphatidylglycerol is the preferred fatty acyl donor for *L. pneumophila* GCAT. Interestingly, cholesterol esters were also generated from mixtures of wild-type supernatants, cholesterol, and the nonphospholipid 1-MPG (Fig. 10A), indicating that *L. pneumophila* acyltransferase activity also accepts fatty acyl donors without an intramolecular phosphate. Next, to find out whether PlaA acts as a GCAT, we examined concentrated culture supernatants of NU270 for a loss of acyltransferase activity. However, cholesterol esters were observed in comparable quantities for the wild type and the *plaA* mutant (Fig. 10B and data not shown), suggesting that factors other than PlaA confer *L. pneumophila* GCAT activity. When concentrated culture supernatants of wild-type *L. pneumophila* were incubated with lipid-cholesterol mixtures, unknown apolar compounds were detected (Fig. 10). Interestingly, these compounds were absent in MPLPC-cholesterol incubations with the *plaA* mutant (Fig. 10B), suggesting that PlaA is involved in their generation. These compounds were not derivatives of cholesterol, since they were found when cholesterol was excluded from the reactions (data not shown).

Intracellular infection by an *L. pneumophila plaA* mutant. Since the type II protein secretion system of *L. pneumophila* promotes infection of macrophages and protozoa and since lysophospholipase A activity is dependent on type II secretion (46), we were interested in determining the role of *plaA* in intracellular infection. Toward that end, we first compared the ability of strains 130b and NU270 to infect monolayers of U937 cells, a standard model for *L. pneumophila* macrophage infection. Strains 130b and NU270 behaved similarly, showing a typical pattern of intracellular growth in which bacterial numbers increased from 100,000- to 1,000,000-fold by 72 h postinoculation (Fig. 11A) (2, 35). Furthermore, NU270 did not seem to have a defect in entry or attachment to the macrophages, since the numbers of bacteria recovered after the uptake period (0 h) were comparable for the wild type and the mutant (Fig. 11A). It is well known that *L. pneumophila* replication leads to death of the macrophage (45, 46). However, strains 130b and NU270 produced comparable effects on host cell viability (data not shown), indicating no major role for *plaA* in this in vitro model of cytopathogenicity. To determine the role of PlaA in amoeba infection, *H. vermiformis* cultures

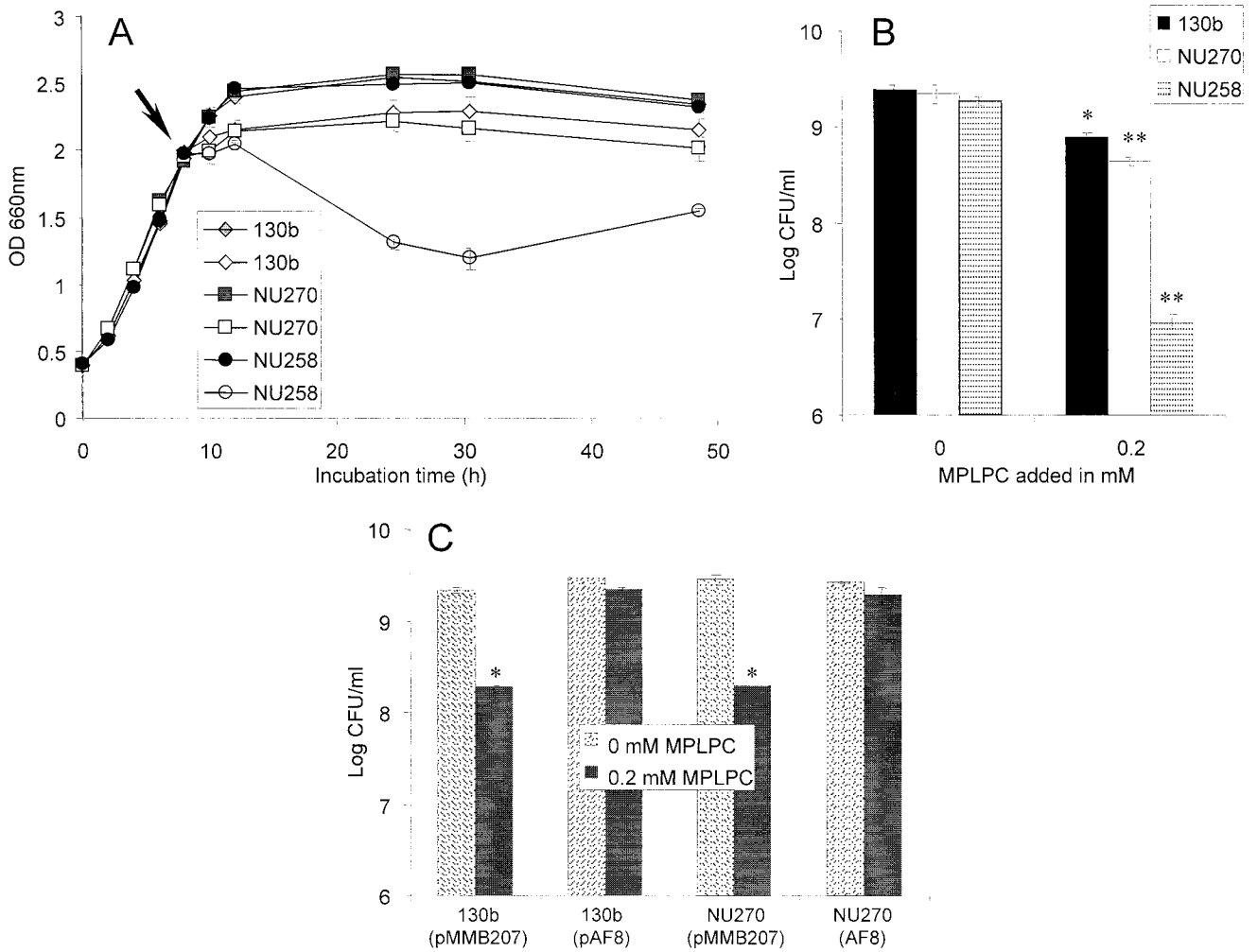

FIG. 8. Effect of MPLPC on the viability of wild-type and mutant *L. pneumophila*. Strains 130b, *plaA* mutant NU270, and *lspDE* mutant NU258 were inoculated into BYE broth at an OD_{660} of 0.2 to 0.3 and grown at 37°C with shaking. (A) When the cultures reached mid-log phase (arrow), 0.2 mM MPLPC (◇, □, ○) or medium control (◆, ■, ●) was added. Bacterial growth was monitored by recording the cultures' OD_{660}. (B) After 16 h of incubation with MPLPC, cultures were serially diluted and plated on BCYE agar for determination of CFU. Wild-type and *plaA* mutant *L. pneumophila* containing pMMB207 or pAF8 were treated as described above. (C) Bacteria were plated for determination of CFU after 4 h of incubation with 0.2 mM MPLPC. Data represent the means ± standard deviations of triplicate cultures and are representative of two independent experiments. One asterisk designates significant differences between *L. pneumophila* cultures untreated versus treated with MPLPC ($P < 0.05$; Student's *t* test). An additional asterisk denotes significant differences between the wild type and the *plaA* mutant or between the wild type and the *lspDE* mutant ($P < 0.05$; Student's *t* test).

were inoculated with strains 130b and NU270 and the number of bacteria was recorded at different times. As in the macrophage infection system, comparable numbers of the wild type and mutant were recovered from the amoeba cocultures (Fig. 11B). Taken together, these data indicate that *plaA* is not required for intracellular infection by *L. pneumophila*.

DISCUSSION

In this study, we identified the gene for the major *L. pneumophila* lysophospholipase A, an enzyme that cleaves fatty acids from lysophospholipids but not phospholipids containing both fatty acids. The substrates for PlaA are produced, at least in part, through the action of a secreted PLA (Fig. 1) recently identified (27). Since the *plaA* mutant showed residual (i.e., ca. 10%) lysophospholipase A activity in its culture supernatants, we conclude that *L. pneumophila* secretes lysophospholipases A in addition to PlaA. Furthermore, we found that *L. pneumophila* possesses cell-associated lysophospholipase A, as well as PLA, activities that are distinct from PlaA. In support of these data, we found two predicted homologs of PlaA in the unfinished *L. pneumophila* genome database (unpublished observations). Because the *plaA* mutant also had a reduced ability to release FFA from the nonphospholipid 1-MPG, we believe that PlaA also possesses lipase activity. This observation is in good agreement with the fact that PlaA-containing fractions obtained from *L. pneumophila* supernatants release FFA from both MPLPC and 1-MPG (28). The cleavage of both phospholipids and non-phosphate-containing lipids has also been described for staphylococcal (phospho)lipases (51).

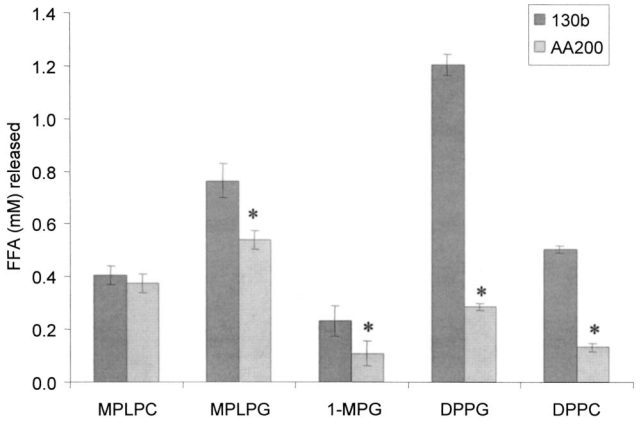

FIG. 9. Lipolytic activities of culture supernatants of wild-type and *proA* mutant *L. pneumophila*. Culture supernatants from log-phase cultures of strains 130b and *proA* mutant AA200 were incubated with MPLPC, MPLPG, 1-MPG, DPPG, or DPPC for 2.5 h at 37°C. Subsequently, the release of FFA was quantified. Data are expressed as differences between the amount of FFA released by the culture supernatant versus the amount released by uninoculated BYE broth. Results represent the means ± standard deviations of four cultures and are representative of two independent experiments. Asterisks denote significant differences in lipolytic activities between wild-type *L. pneumophila* and the *proA* mutant ($P < 0.05$; Student's *t* test).

Based upon its predicted amino acid sequence, *L. pneumophila* PlaA is a new member of the GDSL and SGNH hydrolase families (39, 57). The best-characterized lipolytic protein in the GDSL group is the *Aeromonas* GCAT, an enzyme that will also behave as a lysophospholipase or phospholipase, in the absence of an acceptor molecule (12). We observed that *L. pneumophila* supernatants do contain a GCAT activity. However, the *plaA* mutant was not lacking this secreted activity, suggesting that *L. pneumophila* PlaA, unlike its *Aeromonas* homolog, is not acting as a cholesterol acyltransferase. Since the *Legionella* GCAT activity, like that of *A. salmonicida* (13), transferred fatty acids from DPPG, it may be identical with the secreted, DPPG- and DPPC-cleaving PLA of *L. pneumophila* (26, 27, 28). Further investigations are necessary to elucidate the structural determinants for the *L. pneumophila* acyltransferase activity and to understand why only some GDSL proteins act as acyltransferases.

Since the predicted size of the translated *plaA* sequence is 34.5 kDa but purified PlaA migrates as a 28- or 29-kDa protein upon reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28), we suspect that PlaA is proteolytically modified. The first cleavage of PlaA is undoubtedly the removal of its 18-amino-acid signal sequence by leader peptidase, an event that is a prerequisite for type II secretion. Following upon the *Aeromonas* GCAT example (11), the second modification may represent a restriction of the protein between cysteines at positions 225 and 281, a process that would yield a mature

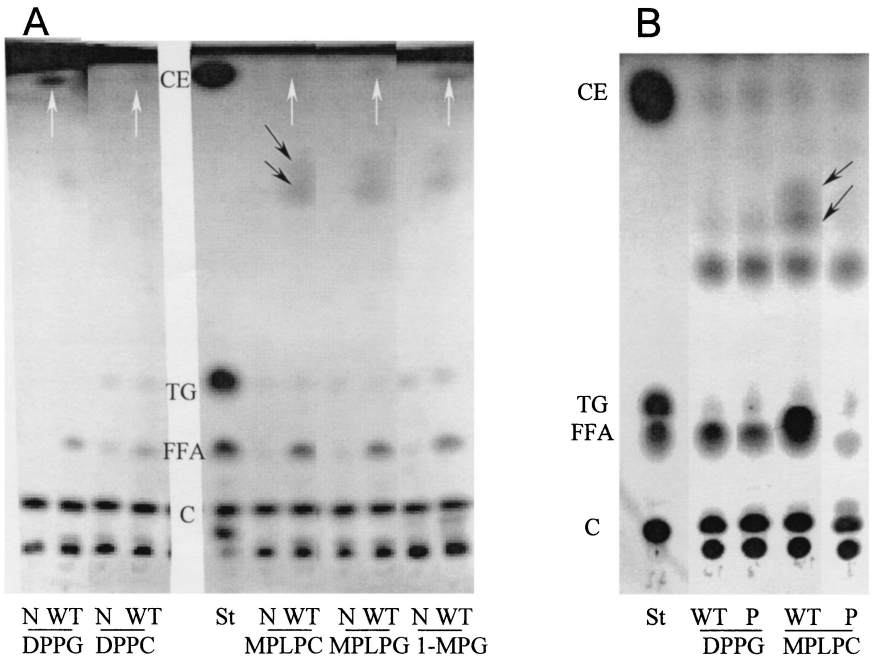

FIG. 10. GCAT activity of culture supernatants from wild-type and *plaA* mutant *L. pneumophila*. (A) Twenty-fold-concentrated culture supernatants of strains 130b (WT) incubated with mixtures of DPPG, DPPC, MPLPC, MPLPG, or 1-MPG with cholesterol. (B) Twenty-fold-concentrated culture supernatants of strains 130b (WT) and NU270 (P) incubated with mixtures of DPPG or MPLPC with cholesterol for 18 h at 37°C. Subsequently, the lipids were extracted and separated by TLC. A mixture of concentrated BYE broth and the lipids served as a negative control (N). The samples were examined for generation of cholesterol esters as a measure of GCAT activity. Lanes containing cholesterol palmitate (CE), tripalmitoylglycerol (TG), palmitic acid (FFA), and cholesterol (C) standards are marked St. The white arrows in panel A indicate cholesterol esters formed by *L. pneumophila* GCAT activity. The black arrows indicate the position of unknown polar compounds produced by *L. pneumophila* concentrated supernatants. Data are representative of two independent experiments.

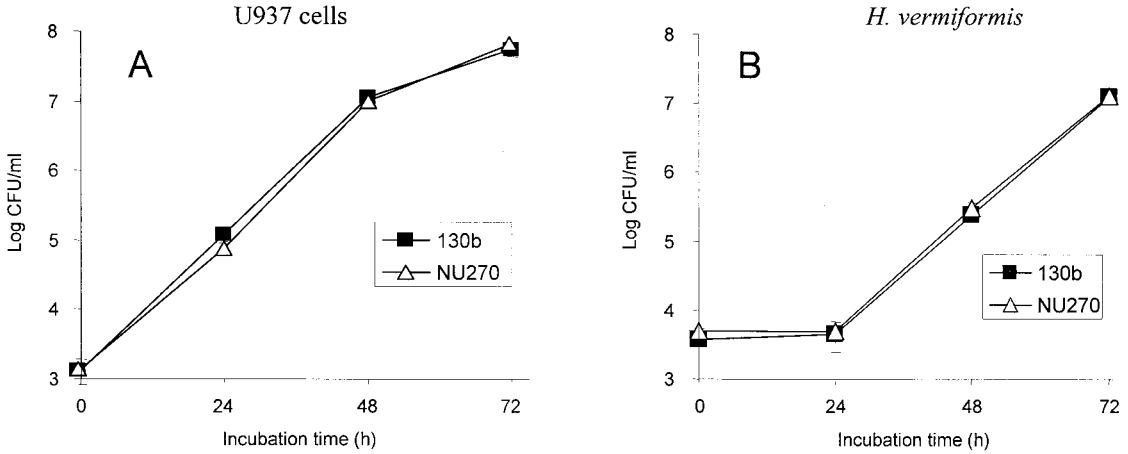

FIG. 11. Intracellular infection by wild-type and *plaA* mutant *L. pneumophila*. Strains 130b and NU270 were used to infect monolayers of U937 macrophages (A) or cultures of *H. vermiformis* amoebae (B) at a multiplicity of infection of 0.1. At 0, 24, 48, and 72 h postinoculation, the numbers of bacteria were quantitated by plating aliquots on BCYE agar. Results represent the means ± standard deviations of triplicate samples and are representative of two independent experiments.

protein of about 29 kDa. Because the *Legionella* metalloprotease mutant was normal for lysophospholipase A activity, the final stage of PlaA maturation and the relevant protease remain to be determined. Interestingly, though, the culture supernatants of the *proA* mutant showed reductions in PLA activity and, to a lesser extent, MPLPG-specific lysophospholipase A and lipase activities. These data suggest that some of the virulence attenuation observed with the protease mutant (38) might be due to a loss of lipolytic activities.

One of the suggested functions of lysophospholipases is protection from high levels of cytotoxic lysophosphatidylcholine (29, 61). Indeed, increased expression of *plaA* afforded *L. pneumophila* greater survival in the presence of MPLPC. However, since an *lsp* mutant, unlike the *plaA* mutant, was hypersensitive to MPLPC, we believe that other type II secreted factors are most critical for *L. pneumophila* resistance to lysophosphatidylcholine. The other secreted lysophospholipase A activity and the newly discovered cell-associated activity are candidate effectors of lysophospholipid detoxification. Interestingly, it has been shown that gram-negative bacteria are more resistant to lysophosphatidylcholine than gram-positive bacteria (18, 58). In support of these data, when we searched the finished and unfinished bacterial genome databases we only found evidence for GDSL protein homologs in gram-negative organisms (unpublished observations).

Recently, lysophospholipases A and members of the GDSL family have been associated with virulence and/or potentially pathogenic activities. For example, a lysophospholipase A activity of the fungus *Cryptococcus neoformans* was implicated in intracellular replication and in virulence in both mouse inhalational and rabbit meningitis models (19). Furthermore, a *pldA* mutant of *Campylobacter coli* that is reduced in lysophospholipase A and PLA activity is defective for lysis of erythrocytes (30). Finally, an *sseJ* mutant of *S. enterica* serovar Typhimurium is mildly attenuated for systemic virulence in mice, and the SseJ protein, a member of the GDSL family (see Fig. 3), appears to be involved in the formation of tubular extensions of the *Salmonella*-containing vacuole (47). On the other hand, GCAT loss in *A. salmonicida* produces no decrease in virulence in Atlantic salmon (59), and the absence of Lec phospholipase, another GDSL protein, does not diminish fluid accumulation by *V. cholerae* in rabbit ileal loops (24).

The *L. pneumophila plaA* mutant revealed no defect in intracellular multiplication within U937 cell macrophages and *H. vermiformis* amoebae, suggesting that PlaA is not critical for intracellular infection. However, it is possible that the other *Legionella* lysophospholipases A compensate for the lack of PlaA, necessitating a need to generate and examine mutants defective for multiple GDSL proteins. Furthermore, it is conceivable that PlaA plays a role in extracellular events and, thus, examination of *plaA* mutants in an animal model is of interest. Additional targets for future investigation involve identifying the type II exoenzymes that promote *L. pneumophila* intracellular infection and resistance to cytotoxic lysophosphatidylcholine, the enzyme(s) that is responsible for *Legionella* GCAT activity, and the function of the other lysophospholipase A activities and GDSL proteins that are encoded by *L. pneumophila*.

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