

# Phospholipase PlaB of *Legionella pneumophila* Represents a Novel Lipase Family

## PROTEIN RESIDUES ESSENTIAL FOR LIPOLYTIC ACTIVITY, SUBSTRATE SPECIFICITY, AND HEMOLYSIS\*

Received for publication, May 27, 2009, and in revised form, July 26, 2009. Published, JBC Papers in Press, July 29, 2009, DOI 10.1074/jbc.M109.026021

Jennifer Bender<sup>‡§</sup>, Kerstin Rydzewski<sup>§</sup>, Markus Broich<sup>‡</sup>, Eva Schunder<sup>§</sup>, Klaus Heuner<sup>§</sup>, and Antje Flieger<sup>‡§1</sup>

From the <sup>‡</sup>Division of Bacterial Infections, FG11, Robert Koch-Institut, Burgstrasse 37, Wernigerode 38855 and the

<sup>§</sup>Robert Koch-Institut, Project Group P26, Nordufer 20, 13353 Berlin, Germany

*Legionella pneumophila* possesses several phospholipases capable of host cell manipulation and lung damage. Recently, we discovered that the major cell-associated hemolytic phospholipase A (PlaB) shares no homology to described phospholipases and is dispensable for intracellular replication *in vitro*. Nevertheless, here we show that PlaB is the major lipolytic activity in *L. pneumophila* cell infections and that PlaB utilizes a typical catalytic triad of Ser-Asp-His for effective hydrolysis of phospholipid substrates. Crucial residues were found to be located within the N-terminal half of the protein, and amino acids embedding these active sites were unique for PlaB and homologs. We further showed that catalytic activity toward phosphatidylcholine but not phosphatidylglycerol is directly linked to hemolytic potential of PlaB. Although the function of the prolonged PlaB C terminus remains to be elucidated, it is essential for lipolysis, since the removal of 15 amino acids already abolishes enzyme activity. Additionally, we determined that PlaB preferentially hydrolyzes long-chain fatty acid substrates containing 12 or more carbon atoms. Since phospholipases play an important role as bacterial virulence factors, we examined cell-associated enzymatic activities among *L. pneumophila* clinical isolates and non-*pneumophila* species. All tested clinical isolates showed comparable activities, whereas of the non-*pneumophila* species, only *Legionella gormanii* and *Legionella spiritensis* possessed lipolytic activities similar to those of *L. pneumophila* and comprised *plaB*-like genes. Interestingly, phosphatidylcholine-specific phospholipase A activity and hemolytic potential were more pronounced in *L. pneumophila*. Therefore, hydrolysis of the eukaryotic membrane constituent phosphatidylcholine triggered by PlaB could be an important virulence tool for *Legionella* pathogenicity.

Bacterial phospholipases are involved in many disease-promoting processes ranging from production of bioactive molecules influencing cellular signal transduction pathways to formation of membrane pores and depletion of essential components from lipid layers (1–3). They often induce massive

membrane destruction, as shown for *Pseudomonas aeruginosa* type III secreted cytotoxin ExoU, a phospholipase A (PLA)<sup>2</sup>/lysophospholipase A (LPLA), or *Clostridium perfringens*  $\alpha$ -toxin, a phospholipase C (4–8). We recently identified PlaB, the major cell-associated PLA/LPLA of *Legionella pneumophila*, to be hemolytic and thereby a membrane-destroying enzyme (9). Accordingly, activity of PlaB fits into the picture of Legionnaires' disease, the severe pneumonia caused by *L. pneumophila* bacteria, showing loss of a functional epithelial cell layer in the lung as well as degradation of lung macrophages in infected individuals (10, 11). Now it is indeed clear that PlaB contributes to pathogenesis in a guinea pig infection model (1),<sup>3</sup> although it is dispensable for intracellular replication in macrophage and amoeba host cells (1, 9).<sup>3</sup> This clearly underlines the importance of PlaB as a virulence factor of *L. pneumophila*; however, its mechanism of action has not yet been studied in detail.

PlaB was initially detected by screening an *L. pneumophila* genomic library in *Escherichia coli*, thus yielding a clone with remarkable hemolytic activity on human red blood agar (9). Subsequently, *plaB* insertion mutagenesis revealed that the gene encodes the most prominent cell-associated PLA/LPLA activity, being ~100-fold more active than secreted phospholipolytic activities of *L. pneumophila*. Interestingly, PlaB has no significant protein homology to any established lipase/phospholipase at all. Further, considering the putative domains responsible for catalytic activity, it does not align significantly to any defined class of lipolytic enzymes. As proposed by Arpigny and Jaeger (12), lipases can be divided into eight families characterized by conserved amino acid motifs. Members of those families in general exhibit a conserved pentapeptide of GX SXG surrounding the catalytically important serine residue except of proteins belonging to GDSL hydrolases, which harbor the catalytic active serine within a differing GDSL motif (13). Further, lipases or phospholipases usually constitute a catalytic triad of serine, aspartate, and histidine; however, combinations of Ser-Glu-His or catalytic dyads have been reported as well (12,

\* This work was supported by Deutsche Forschungsgemeinschaft (Bonn, Germany) Grants HE 2845/4-1 and FL 359/4-1 (to K.H. and A.F., respectively).

<sup>1</sup> To whom correspondence should be addressed: Robert Koch-Institut, Division of Bacterial Infections, FG11, Burgstrasse 37, 38855 Wernigerode, Germany. Tel.: 49-30-18754-2522; Fax: 49-30-18754-4207; E-mail: fliegera@rki.de.

<sup>2</sup> The abbreviations used are: PLA, phospholipase; LPLA, lysophospholipase; DPPG, dipalmitoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; MPLPG, 1-monopalmitoylphosphatidylglycerol; MPLPC, 1-monopalmitoylphosphatidylcholine; PC, phosphatidylcholine; 1-MPG, 1-monopalmitoylphosphatidylglycerol.

<sup>3</sup> E. Schunder, P. Adam, F. Higa, K. A. Remer, U. Lorenz, J. Bender, A. Flieger, M. Steinert, J. Hacker, and K. Heuner, submitted for publication.

## Phospholipase *PlaB* of *L. pneumophila*

14, 15). The latter one is present within a recently discovered group of bacterial lipolytic enzymes, the patatin-like proteins (16, 17). Although variability in amino acid homology between phospholipases is quite elevated, we propose *PlaB* and homologs to represent a novel division of lipolytic proteins.

To understand the biochemical nature of *L. pneumophila* *PlaB*, in this study we (a) determined expression of *PlaB* during host cell infection, (b) identified the catalytically important residues and further protein domains crucial for lipid hydrolysis, (c) analyzed the contribution of the catalytic and other important sites to hemolytic activity, and (d) investigated lipid substrate specificity with respect to fatty acid chain length. We additionally addressed the questions of whether cell-associated phospholipase activity is found in various clinical isolates and whether *PlaB* is unique to *L. pneumophila* or also present within non-*pneumophila* species.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains and Growth Conditions**—*L. pneumophila*, environmental and clinical isolates, and different *E. coli* strains used in this study are listed in Table 1 and were grown as described previously (9, 18).

**Complementation of the *plaB* Deletion Mutant**—Amplification of *L. pneumophila* Corby *plaB* (*lpc*\_1029) was carried out by using primers *plaB*\_SalIF and *plaB*\_EagIr (oligonucleotides were purchased from TIB MOLBIOL (Berlin) or Eurofins MWG Operon (Ebersberg)). The resulting fragment was cloned into *Sal*I and *Eag*I (New England Biolabs) restricted pBCKS vector to yield pJB04. This additional complementation vector was constructed because the *plaB* gene within pKH192 harbors an amino acid conversion, although not influencing lipolytic activities.

**Site-directed Mutagenesis**—Mutagenesis of important amino acids was carried out by utilizing the combined chain reaction method, as described elsewhere (19). Minor modifications included performance of PCR with 20 ng of each primer and 20 ng of pJB04 plasmid DNA (Table 1), a 0.25 mM concentration of each dNTP, 2.5 units of Pfu polymerase (Fermentas), and 3 units of Ampligase (Epicenter). Mutagenesis primers were phosphorylated in advance by T4 polynucleotide kinase (New England Biolabs). Plasmid pJB04, carrying wild-type *plaB*, served as template for single amino acid exchanges, whereas pJB06, which harbors already *plaB* S85A, was used for creating loss of function double mutants S85A/D203N and S85A/H251N. Amplification of the designated fragments was performed by using *plaB*\_SalIF and *plaB*\_EagIr primers and the appropriate mutagenesis primer (Table 2). Restriction endonucleases *Sal*I and *Eag*I were used for site-specific integration of the desired fragments into vector pBCKS. Mutants were verified by sequencing both DNA strands, and resulting plasmids were electroporated into *L. pneumophila* *plaB* mutant strain. Table 1 gives a summary of all generated plasmids and respective *plaB* mutants.

**Construction of C-terminally Truncated Versions of *PlaB***—For construction of full-length glutathione *S*-transferase-*PlaB* fusion protein, pGEX-5X-1 vector backbone was linearized by *Eco*RI and *Eag*I restriction enzymes and ligated to the appropriate digested *plaB* fragment, amplified by using gene-specific

primers *plaB*\_EagIr and *plaB*\_Ecof2 (Table 2). Truncated versions were cloned into vector pGEX-6P-1. Genes for *PlaB*-(1–459) and *PlaB*-(1–388) were amplified by *PlaB*\_truncC1\_P1 and *PlaB*\_truncC2\_P2, respectively, in combination with *plaB*\_TOPf2. Both fragments were inserted over *Sma*I restriction sites into vector pGEX-6P-1. The truncated version *PlaB*-(1–307) was amplified by means of *plaB*\_Ecof2 and *plaB*\_trunc3Eag primers and ligated to pGEX-6P-1 via *Eco*RI and *Eag*I restriction sites. All clones were verified by sequencing before electroporation into protein expression host *E. coli* BL21.

**Cloning of *Legionella spiritensis* and *Legionella gormanii* *plaB***—The *plaB* gene from *L. spiritensis* was amplified from genomic DNA using primers *plaB*\_pBADf and *plaB*\_c1-r. Subsequently, the product was cloned into pGEMT-EZ, resulting in vector pKR8 before primers *PlaB*spir\_BamHI and *PlaB*spir\_NotI were used to precisely ligate the amplicon into digested pBCKS backbone vector, yielding pJB37 (Tables 1 and 2). *plaB* gene sequences from strains *L. spiritensis* and partially for *L. gormanii* were determined from pKR8 and pKR10, respectively. The latter vector resulted from an amplification product generated from *L. gormanii* genomic DNA by using primers *plaB*\_pBADf and *plaB*\_g1\_r and cloned into pGEMT-EZ.

**Preparation of Culture Supernatants and Cell Lysates and Determination of Lipolytic Activities**—Samples were obtained from cultures grown to an  $A_{660}$  of 2. For assessment of lipolytic activities, via release of fatty acids, bacterial cell lysates and supernatants were prepared and incubated with deduced lipids as described previously (9). Briefly, incubation of bacterial cell lysates or supernatants with different lipids (dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), 1-monopalmitoyllysophosphatidylcholine (MPLPC), 1-monopalmitoyllysophosphatidylglycerol (MPLPG), 1-monopalmitoylglycerol, 1-monolauroyllysophosphatidylcholine, or 1-monooctanoyllysophosphatidylcholine) was performed at 37 °C with continuous agitation at 100 rpm for overnight incubations and at 150 rpm for various shorter time points. Final concentration of each lipid was set to 6.7 mM. Free fatty acids as a marker of lipid hydrolysis by PLA/LPLA/lipase were determined by means of the 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. All lipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) or Sigma.

**Expression of *PlaB***—*E. coli* BL21 cells harboring the designated vectors were grown overnight prior to inoculation into fresh media. At an  $A_{660}$  of 0.6–0.8, protein expression was induced by using isopropyl 1-thio- $\beta$ -D-galactopyranoside to a final concentration of 1 mM. Bacterial cells were harvested at an  $A_{660}$  of 2 and centrifuged for 5 min at 5,000  $\times$  g, and pellets were stored overnight at –20 °C. Cell lysates were obtained according to our previously published protocol and subsequently used for determination of lipolytic activity (9).

**Hemolysis Assay**—For detection of hemolytic activity, *E. coli* strains were grown for 1 day on LB agar plates. Bacteria were

**TABLE 1**  
Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<b>Strains</b>		
<i>L. pneumophila</i> strains		
Corby	serogroup 1, clinical isolate	(39)
Corby (pBCKS)	carrying control vector pBCKS	this study
130b	serogroup 1, clinical isolate	ATCC BAA-74
Corby <i>plaB</i> 1	<i>plaB::km</i> insertion mutant (Km <sup>r</sup> )	(9)
Corby <i>plaB</i> 1 (pBCKS)	<i>plaB::km</i> insertion mutant (Km <sup>r</sup> ), carrying control vector pBCKS	this study
Corby <i>plaB</i> 1 (pKH192, pJB04 or derivatives)	complementing strain carrying pBCKS- <i>plaB</i> or mutagenized <i>plaB</i> variants	(9); this study
L02-705	serogroup 1, clinical isolate	Dr. Lück
L03-638	serogroup 1, clinical isolate	Dr. Lück
L04-41	serogroup 3, clinical isolate	Dr. Lück
L05-410	serogroup 3, clinical isolate	Dr. Lück
L06-281	serogroup 1, clinical isolate	Dr. Lück
L07-2	serogroup 1, clinical isolate	Dr. Lück
L08-147	serogroup 1, clinical isolate	Dr. Lück
L08-336	serogroup 1, clinical isolate	Dr. Lück
W08-231	serogroup 2, clinical isolate	Dr. Lück
water conduit isolate #257	environmental isolate	Robert Koch-Institut
Non- <i>pneumophila</i> strains		
<i>L. anisa</i>	environmental isolate	ATCC 35292; Dr. Neumeister
<i>L. feeleii</i>	serogroup 1, environmental isolate	ATCC 35072; Dr. Neumeister
<i>L. gormanii</i>	environmental isolate	ATCC 33297; Dr. Neumeister
<i>L. jordanis</i>	environmental isolate	ATCC 33623; Dr. Neumeister
<i>L. longbeachae</i>	serogroup 1, clinical isolate	ATCC 33462; Dr. Neumeister
<i>L. micdadei</i>	clinical isolate	ATCC 33218; Dr. Neumeister
<i>L. micdadei</i>	environmental isolate	CDC F976; Barry Fields
<i>L. oakridgensis</i>	environmental isolate	ATCC 33761; Dr. Neumeister
<i>L. parisiensis</i>	environmental isolate	ATCC 35299; Dr. Neumeister
<i>L. sainthelensis</i>	environmental isolate	ATCC 35248; Dr. Neumeister
<i>L. spiritensis</i>	environmental isolate	ATCC 35249; Dr. Neumeister
<i>L. steigerwaltii</i>	environmental isolate	ATCC 35302; Dr. Neumeister
<i>E. coli</i> strains		
DH5 $\alpha$ (pBCKS)	<i>E. coli</i> DH5 $\alpha$ harboring pBCKS vector	this study
DH5 $\alpha$ (pJB04 or derivatives)	<i>E. coli</i> DH5 $\alpha$ harboring pJB04 or derivatives	this study
BL21 (pGEX or derivatives)	<i>E. coli</i> BL21 harboring pGEX or derivatives	this study
<b>Plasmids</b>		
pBCKS	control vector	Stratagene
pKH192	expression vector for <i>L. pneumophila</i> Corby <i>plaB</i>	(9)
pGEX-5X-1/-6P-1	expression vectors to generate N-terminal translational fusions to GST	GE healthcare
pJB04	pBCKS- <i>plaB</i> ; wild type <i>L. pneumophila</i> Corby	this study
pJB06	pBCKS- <i>plaB</i> ; S85A site-directed mutant	this study
pJB07	pBCKS- <i>plaB</i> ; S129A site-directed mutant	this study
pJB08	pBCKS- <i>plaB</i> ; S200A site-directed mutant	this study
pJB09	pBCKS- <i>plaB</i> ; S229A site-directed mutant	this study
pJB10	pBCKS- <i>plaB</i> ; S250A site-directed mutant	this study
pJB11	pBCKS- <i>plaB</i> ; D75N site-directed mutant	this study
pJB12	pBCKS- <i>plaB</i> ; D203N site-directed mutant	this study
pJB13	pBCKS- <i>plaB</i> ; H270N site-directed mutant	this study
pJB18	pGEX-5X-1 with <i>plaB</i> full length construct	this study
pJB19	pBCKS- <i>plaB</i> ; T83G site-directed mutant	this study
pJB20	pBCKS- <i>plaB</i> ; D167N site-directed mutant	this study
pJB21	pBCKS- <i>plaB</i> ; D342N site-directed mutant	this study
pJB22	pBCKS- <i>plaB</i> ; D381N site-directed mutant	this study
pJB23	pBCKS- <i>plaB</i> ; H7N site-directed mutant	this study
pJB24	pBCKS- <i>plaB</i> ; H339N site-directed mutant	this study
pJB25	pBCKS- <i>plaB</i> ; H433N site-directed mutant	this study
pJB26	pBCKS- <i>plaB</i> ; H251N site-directed mutant	this study
pJB28	pBCKS- <i>plaB</i> ; S85A/D203N site-directed mutant	this study
pJB29	pBCKS- <i>plaB</i> ; S85A/H251N site-directed mutant	this study
pJB31	pGEX-6P-1 with <i>plaB</i> truncation encoding amino acids 1 to 459	this study
pJB32	pGEX-6P-1 with <i>plaB</i> truncation encoding amino acids 1 to 388	this study
pJB33	pGEX-6P-1 with <i>plaB</i> truncation encoding amino acids 1 to 307	this study
pJB36	pBCKS- <i>plaB</i> ; T83V site-directed mutant	this study
pJB37	pBCKS- <i>plaB</i> from <i>L. spiritensis</i>	this study
pKR8	pGEMT-EZ- <i>plaB</i> from <i>L. spiritensis</i>	this study
pKR10	pGEMT-EZ- <i>plaB</i> from <i>L. gormanii</i> , partial sequence	this study

inoculated into PBS medium and set to a final  $A_{660}$  of 0.3. As described by Kirby *et al.* (20), human red blood cells were mixed with an equal volume of bacterial suspension, centrifuged at  $16,000 \times g$  for 2 min before incubation was carried out at 37 °C. At the desired time points, the suspension was vortexed and repelleted by centrifugation, and release of hemoglobin into the supernatant was determined at an optical density of 415 nm.

*Determination of Lipolytic Activities during Intracellular Infection of Host Cells by L. pneumophila*— $10^6$ /ml U937 macrophages were infected with *L. pneumophila* Corby (9, 21, 22). For detection of enzymatic activities, samples were taken from co-incubations as well as from uninfected U937 cells and divided into culture supernatant and cell pellet. Culture supernatant was furthermore passed through a 0.2- $\mu$ m filter, and cell



**TABLE 2**  
Primers used for site-directed mutagenesis, cloning, and sequence analysis

Oligonucleotide	Sequence (5'-3')	Application
plaB_H7N	-gTTATCTTCgTCAATggCTggAgT-	site-directed mutagenesis resulting in pJB23
plaB_D75N	-gATAAgCTACgAAATgggCgAgCgTT-	site-directed mutagenesis resulting in pJB11
plaB_T83G	-TTgCTTgTATCggTCACTCTAAC-	site-directed mutagenesis resulting in pJB19
plaB_T83V	-TTgCTTgTATCgTTCACCTACCg-	site-directed mutagenesis resulting in pJB36
plaB_S85A	-gTATCACTCACgCTACCggTgggCC-	site-directed mutagenesis resulting in pJB06
plaB_S129A	-CAGCTTggCAAAGCCgCCTAg-	site-directed mutagenesis resulting in pJB07
plaB_D167N	-gAAAgCTggCTTAAATATgATTgC-	site-directed mutagenesis resulting in pJB20
plaB_S200A	-ACAggAgAAgCTggATCCgATg-	site-directed mutagenesis resulting in pJB08
plaB_D203N	-gAATCTggATCCAAIggggTggTA-	site-directed mutagenesis resulting in pJB12
plaB_S229A	-gATAACggTgAAgCTCTTgTTgTCg-	site-directed mutagenesis resulting in pJB09
plaB_S250A	-CTACCTggACTTgCACATCCg-	site-directed mutagenesis resulting in pJB10
plaB_H251N	-CTACCTggACTTCAAATTCg-	site-directed mutagenesis resulting in pJB26
plaB_H270N	-AATgCAgCCACTAACCCACg-	site-directed mutagenesis resulting in pJB13
plaB_H339N	-gACCgCggCAATAACCTTAT-	site-directed mutagenesis resulting in pJB24
plaB_D342N	-CACCTTATCAAITATgATCTTTACC-	site-directed mutagenesis resulting in pJB21
plaB_D381N	-CTgACTTATTTCTTAACTACgAT-	site-directed mutagenesis resulting in pJB22
plaB_H433N	-CACAAAATACTTAAATCCgAATgAAA-	site-directed mutagenesis resulting in pJB25
plaB_Sallf	-gATgTCgACAAgCTTgAggATTgAg-	cloning of site-directed mutagenized <i>plaB</i>
plaB_Eaglr	-gACggCCgAATgTggCTAgATgAC-	cloning of site-directed mutagenized <i>plaB</i>
pBC_a1_f	-ggTTTTCCCAgTCACgA-	sequence analysis
pBC_b1_r	-CgCgCAATTAACCCTCAC-	sequence analysis
plaB_b1_r	-CCAgTAAgTACAAAggAATAg-	sequence analysis
plaB_c1_r	-TgCTATATCAAATgTAAgAg-	cloning of pKR8
plaB_d1_f	-gCATTgAACCGggAAAgTgTg-	sequence analysis
plaB_f1_f	-gCgATgCCTgCAAATgTAAgAg-	sequence analysis
plaB_g1_r	-gATTgCCgCggTCATCAA-	sequence analysis; cloning of pKR10
plaB_pBADf	-AggAgCgTTATgATTgTTATC-	cloning of pKR8/pKR10
plaB_Ecof2	-CCggAATTCAGgAgCgTTACCATgATTgTT-	cloning of pJB18/pJB33
plaB_TOPf2	-CACCAgCgTTATgATTgTTATCT-	cloning of pJB31/pJB32
plaB_truncC_P1	-TCAAAGTTgTTgCTgATACgAAA-	cloning of pJB31
plaB_truncC_P2	-TCATCCACCTTCCATAATATCgTAGTC-	cloning of pJB32
plaB_trunc3Eag	-gACggCCgTCAATgCTCATTTTTCTgAgTC-	cloning of pJB33
plaBspir_BamHI	-gAggATCCATgATTgTTATCTTCCCTACAT-	cloning of pJB37
plaBspir_NotI	-gTTAgCggCCgCTCAATCTACCgTTTTTCC-	cloning of pJB37

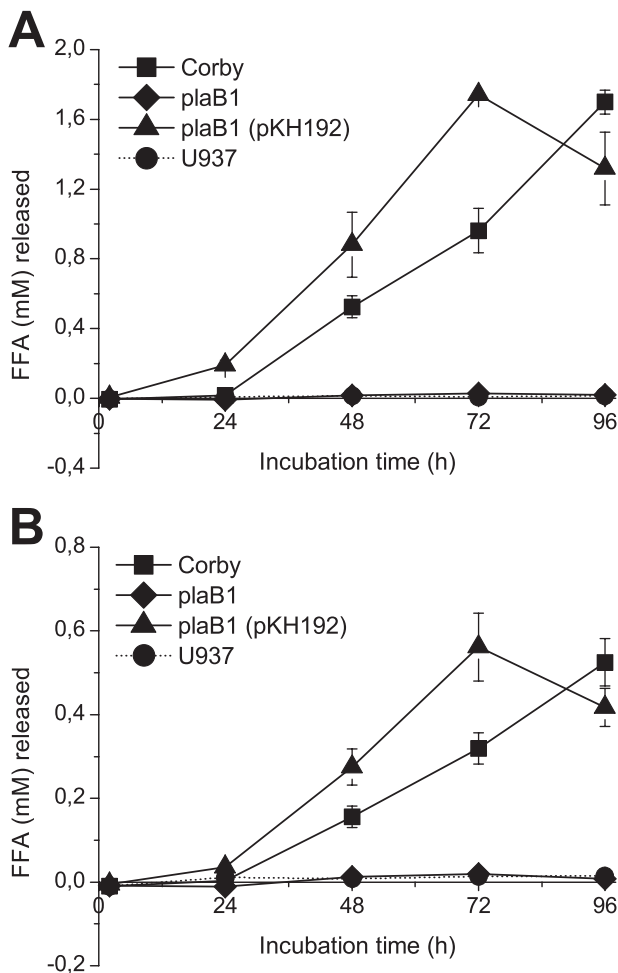
pellet was treated for 10 min by ultrasonication, stored at  $-20^{\circ}\text{C}$  for several days, thawed, and then resuspended in 20 mM Tris-HCl, pH 7.5 ( $25^{\circ}\text{C}$ ), to original culture volume of the U937 infections. Culture supernatants and cell lysates were then incubated for 3 h with different lipid substrates, and enzymatic activities were determined. Hydrolysis in the presence of 6.25 mM EDTA accounted for bacterial PLA and LPLA activities.

**RESULTS**

*PlaB Is Expressed during Host Cell Infections*—Although *PlaB* was shown to be dispensable for intracellular replication (9), we were interested whether *PlaB*-dependent lipolytic activities are expressed during the infection process. To address this question, U937 macrophages were infected with wild-type *L. pneumophila* Corby, *plaB* mutant *plaB1*, and the complementing strain *plaB1* (pKH192). Both replication of bacteria and PLA (via hydrolysis of DPPG)/LPLA (via hydrolysis of monopalmitoyllysophosphatidylcholine (MPLPC)) activities associated with cells and present in the culture supernatant were monitored. Although bacterial replication was comparable for all three strains (data not shown), wild-type infection comprised high PLA/LPLA activities, whereas the *plaB* mutant infection or uninfected U937 cells did not exhibit these activities (Fig. 1, A and B). A similar pattern was observed for cell culture supernatants (data not shown). The introduction of a functional copy of *plaB* into the *plaB1* mutant complemented demonstrated activity defects (Fig. 1, A and B). Expression of *plaB* was additionally confirmed by reverse transcription-PCR analysis (not

shown). Comparable data were obtained both for infections of U937 macrophages and *Acanthamoeba castellanii* amoebae with *L. pneumophila* strain 130b, *plaB* mutant, and the complementing strain (data not shown). These data demonstrate that *L. pneumophila* *PlaB* is indeed expressed and presumably the most prominent PLA/LPLA of the pathogen during intracellular infection of eukaryotic host cells.

*PlaB Utilizes a Typical Catalytic Triad for Effective Hydrolysis*—Lipase or phospholipase families typically contain a catalytic triad of Ser, Asp, and His, mostly arranged within conserved amino acid blocks. Assuming that *PlaB* exhibits a catalytic triad as well, we determined conserved and reasonable residues of *PlaB* and homologs to be used for site-directed mutagenesis. Interestingly, none of the potential catalytically active site serines is located within the typical pentapeptide GX SXG of true lipases or patatin-like proteins (12, 16); however, five serines were found to be part of similar amino acid motifs, such as Ser-85 (THSTG), Ser-129 (GKSRL), Ser-200 (GESGS), Ser-229 (GESLV), and Ser-250 (GLSHS). On the basis of this reasoning, serines were replaced by alanine, whereas reasonable aspartates and histidines were replaced by asparagine residues (Table 1). Resulting genes were expressed in a *L. pneumophila* *plaB*-deficient background, and cell lysates were subsequently tested for their ability to release fatty acids from various lipid substrates. In particular, dipalmitoylphosphatidylcholine (DPPC) and DPPG were used to check for PLA activity, and MPLPC and MPLPG were used to examine LPLA activity.



**FIGURE 1. Phospholipase A and lysophospholipase A activities upon intracellular infection of macrophages by *L. pneumophila* wild-type, *plaB* mutant and complementing strain.** Wild-type Corby, mutant *plaB1*, and complementing strain *plaB1* (pKH192) were used to infect monolayers of U937 cells, and PLA activity toward DPPG (A) and LPLA activity toward MPLPC (B) were determined for cell lysates at various time points postinoculation and after an additional 3 h of lipid hydrolysis. As a control, PLA and LPLA activities were monitored from uninfected U937 cells (U937).

All of those activities are usually comprised by the PlaB enzyme. We thereby could identify Ser-85, Asp-203, and His-251 to form the catalytic triad, since corresponding mutants were reduced in >99% of activity against all tested substrates (Fig. 2A).

Further, mutant S129A was severely reduced in hydrolysis of choline-containing phospholipids, such as both DPPC and MPLPC, but not remarkably against substrates containing glycerol esterified to the phosphate residue instead (Fig. 2A). Moreover, we found that H270N mutants retained only ~10% of their original hydrolytic properties and found His-7 to be particularly linked to substrate specificity regarding choline-containing lipids as well, since the mutant was about 10-fold less active against DPPC when compared with wild-type PlaB (Fig. 2A). Other mutants (S200A, S229A, S250A, D75N, D167N, D342N, D381N, H339N, and H433N) retained their ability to hydrolyze deduced substrates (data not shown). Reduction of enzymatic activity of described PlaB mutants was not due to second site mutations or decreased expression levels, as deter-

mined by sequencing and reverse transcription-PCR analysis (data not shown).

Together, amino acids crucial for PlaB enzymatic activity constitute a typical lipase Ser-Asp-His triad, arranged within the N-terminal part of the protein, thus leaving an extension of about 174 amino acids at the C-terminal domain, a function which still requires further investigation (Fig. 2B).

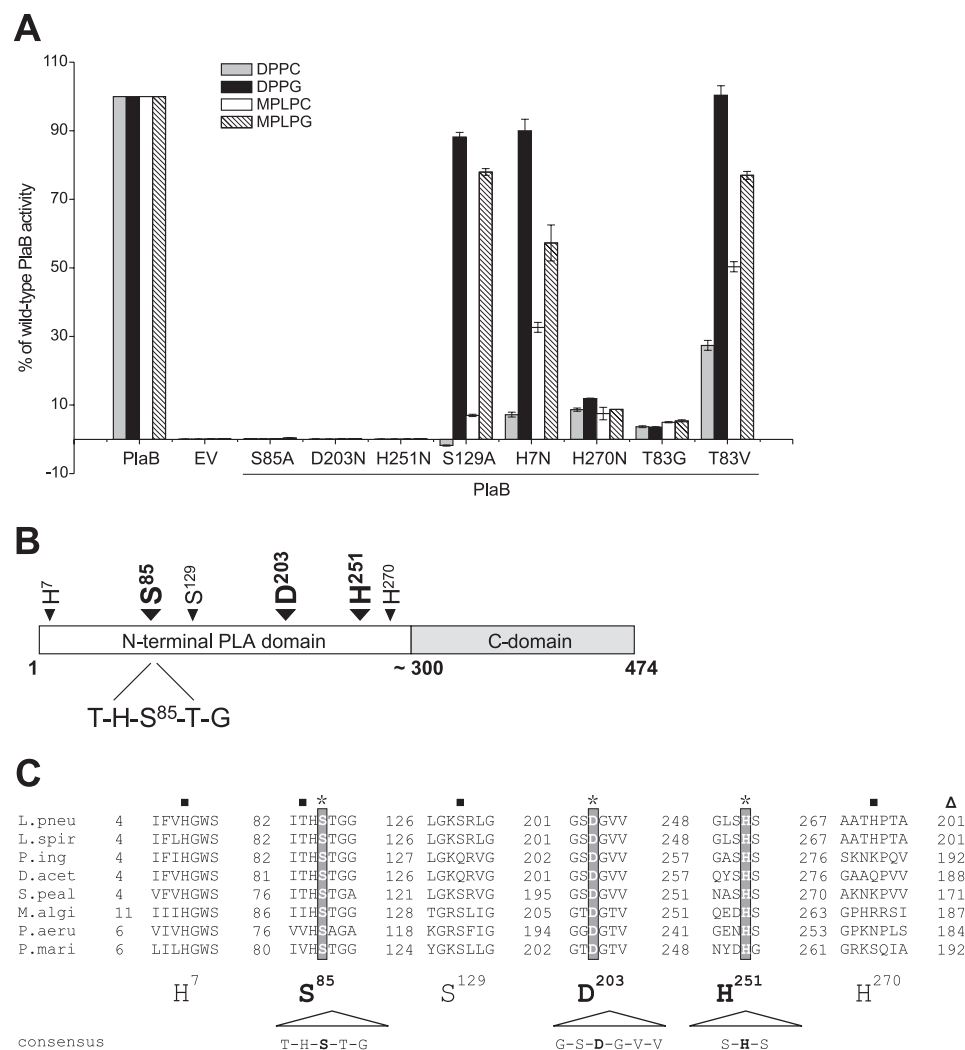
*The Crucial Active Sites Are Embedded into a New Amino Acid Environment*—Amino acid sequence alignment to related proteins (ClustalW2; EMBL-EBI) revealed that the designated residues composing the catalytic triad and embedding amino acid motifs are well conserved throughout PlaB homologs (Fig. 2C), underlining their necessity for enzymatic activity. Interestingly, the crucial Ser-85 in PlaB is surrounded by similar amino acids, as is the case for most lipases; however, a threonine substitutes for the usually comprised glycine residue within the GX SXG motif. Omitting Thr in the immediate vicinity of the active site Ser through Gly substitution strongly reduces PlaB activity by 95%, revealing its particular importance for PlaB enzyme activity (Fig. 2A). Additional replacement of Thr-83 by Val, a residue found in a PlaB homolog of *P. aeruginosa* (Fig. 2C) underlined this observation, since mutant T83V was reduced in DPPC/MPLPC hydrolysis to 27 or 50% of wild-type activity, respectively (Fig. 2A).

Ultimately, PlaB utilizes a well known catalytic triad, although conserved motifs embedding the crucial catalytic amino acids, designated THSTG, GSDGVV, and SHS (Fig. 2C), are unique among described phospholipases. PlaB thus represents the first described member of a new class of lipolytic families.

*Phosphatidylcholine (PC)-specific Catalytic Activity of PlaB Is Responsible for *plaB*-dependent Hemolysis*—PlaB has been shown to contribute to hemolytic activity of *L. pneumophila* (9). Although it seems reasonable to assume that extensive phospholipase activity correlates with cytolytic activity, we aimed to clarify whether the identified catalytic domains are indeed essential for hemolysis. Corresponding PlaB site-directed mutant genes were expressed in non-hemolytic *E. coli* DH5 $\alpha$ . Disrupting catalytically important amino acids, such as Ser-85 or Ser-85/Asp-203, a double knock-out without residual lipolytic activity, diminished hemolytic potential of *plaB* to the level of the empty vector-containing strain (Fig. 3). Mutant H433N did not show altered lipolytic quantities and profiles and therefore lysed human erythrocytes to the same extent as *E. coli* DH5 $\alpha$  cells containing wild-type *plaB*. Interestingly, when we expressed mutant Ser-129, which was shown to hydrolyze DPPC/MPLPC less efficiently, hemolysis was comparable with vector control level (Fig. 3). Thus, PC-hydrolyzing activity of PlaB was directly responsible for its hemolytic effects.

*The C Terminus of PlaB Is Important for Catalytic Activity*—As demonstrated in Fig. 2B, PlaB catalytically important residues are present within the N-terminal region; therefore, the protein exhibits an extended C terminus of additional 174 amino acids, a function that remains to be elucidated. To this end, we constructed three truncated versions of the protein, ranging from 15 to 167 amino acids less than wild-type PlaB. Deletion of only 15 or more amino acids completely abolished enzymatic activities of PlaB, whereas protein expression levels

## Phospholipase PlaB of *L. pneumophila*



**FIGURE 2. Enzymatic activities of PlaB and its site-directed mutant derivatives and distribution of crucial amino acids within the PlaB protein of *L. pneumophila* and homologs.** A, mutagenesis of several amino acids severely influences PlaB activity. Values are expressed as percentages of wild-type PlaB activity. B, PlaB can be divided into an N-terminal region (amino acids 1 to ~300) containing the catalytic triad of Ser-85/Asp-203/His-251 and further amino acids important for hydrolytic activity and a C-terminal domain with unknown function (amino acids ~300–474). C, sequence alignment of *L. pneumophila* PlaB to potential phospholipases or hypothetical proteins of several bacterial species with an expect value of <0.01. Amino acids comprising the catalytic triad are marked with an asterisk and are highlighted by a gray background; further amino acids important for *L. pneumophila* PlaB activity are labeled with black squares (■). The last digits indicate the number of amino acids representing the residual C-terminal domain and are marked with a triangle. The consensus sequence is based on the amino acids most frequently found at this position. *L. pneu.*, *L. pneumophila*; *L. spir.*, *L. spiritensis*; *P. ing.*, *Psychromonas ingrahamii*; *D. acet.*, *Desulfuromonas acetoxidans*; *S. peal.*, *Shewanella pealeana*; *M. algi*, *Marinobacter algicola*; *P. aeru.*, *P. aeruginosa* PA7; *P. mari.*, *Persephonella marina*. EV, empty vector pBCKS.

of the truncated enzymes were comparable with the full-length construct (data not shown). Thus, catalytic activity of PlaB depends on both the N-terminal sequence harboring the crucial amino acids and the C-terminal region.

**PlaB Mainly Hydrolyzes Long-chain Fatty Acid Substrates—**To further examine biochemical properties, we analyzed acyl chain length selectivity of wild-type PlaB enzyme. To this end, cell lysates of *L. pneumophila* Corby wild-type, the *plaB* mutant, and complementing strain were exposed to comparable concentrations of lysophosphatidylcholines varying in chain length from C8 to C16, and the release of fatty acids was quantified. We demonstrate here that PlaB almost solely hydrolyzed long fatty acid chained substrates, such as MPLPC (C16) and 1-monolauryllysophosphatidylcholine (C12) (Fig. 4). 1-Mono-

octanoyllysophosphatidylcholine (C8) was not affected by PlaB-dependent lipolysis under our experimental conditions (Fig. 4). This observation was verified by using *p*-nitrophenyl-based substrates esterified to carboxylic acids from C4, C12, and C16 in chain length (data not shown). Thus, PlaB acts mostly against long-chain fatty acid substrates with preferences for lipids containing fatty acyl residues larger than eight carbon atoms.

**Distribution of Cell-associated Lipolytic Activities among *L. pneumophila* Clinical Isolates—**We demonstrated above that PlaB is a potent hemolytic agent and is expressed during intracellular replication, which probably contributes to extensive lung destruction during Legionnaires' disease. Thus, we investigated the distribution of cell-associated PLA/LPLA activity within nine clinical isolates. We observed comparable hydrolytic activities for various *L. pneumophila* strains, including different serogroups (Fig. 5A). Investigation of putative *plaB* expression by reverse transcription-PCR analysis revealed that these isolates indeed possess and actively transcribe *plaB*-like genes (not shown). This suggests that PlaB is widely spread among *L. pneumophila* bacteria isolated from various patients. Interestingly, an environmental *L. pneumophila* strain (Lpn 257), obtained from a water conduit, showed lipolytic potential comparable with that of the clinical isolates tested (Fig. 5A).

***L. gormanii* and *L. spiritensis* Possess Reduced Potential for Phosphatidylcholine Hydrolysis—***L. pneumophila* has been most often associated with Legionella infections, and therefore researchers seek to identify virulence factors that are present only in pathogenic but not in less pathogenic species (23, 24). Although we previously determined *plaB* genetically to be restricted to *L. pneumophila* (9), we now wanted to verify whether non-*pneumophila* strains possess cell-associated PLA/LPLA activities. To this end, we tested *L. pneumophila* 130b and 12 non-*pneumophila* (Table 1) cell lysates for fatty acid release from phospholipids, lysophospholipids, and a lipase substrate, 1-MPG. Only *L. gormanii* and *L. spiritensis* possessed a spectrum and quantity of PLA/LPLA/lipase activities comparable with that of *L. pneumophila* 130b (Fig. 5B). However, both *L. gormanii* and *L. spiritensis* showed a



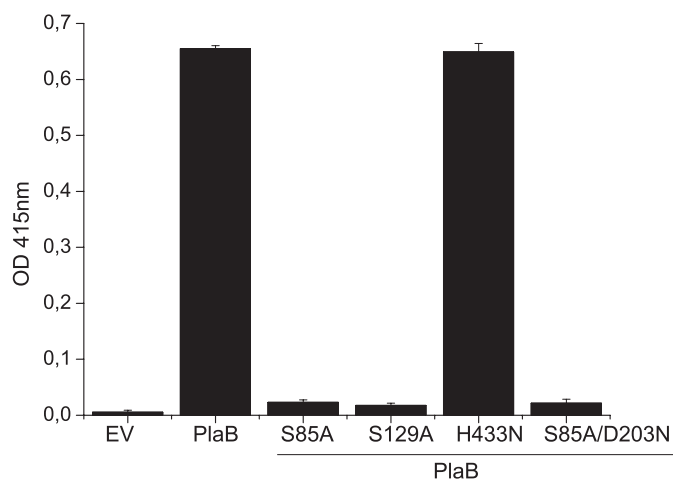


FIGURE 3. Contact-dependent hemolysis of human red blood cells upon incubation of *E. coli* strains expressing mutated variants of PlaB. Bacteria, obtained from 1-day-old agar plates, were set to an equal  $A_{660}$  before added to human erythrocytes. Cell lysis was determined by measuring absorbance after 18 h of incubation at 37 °C. EV, empty vector pBCKS.

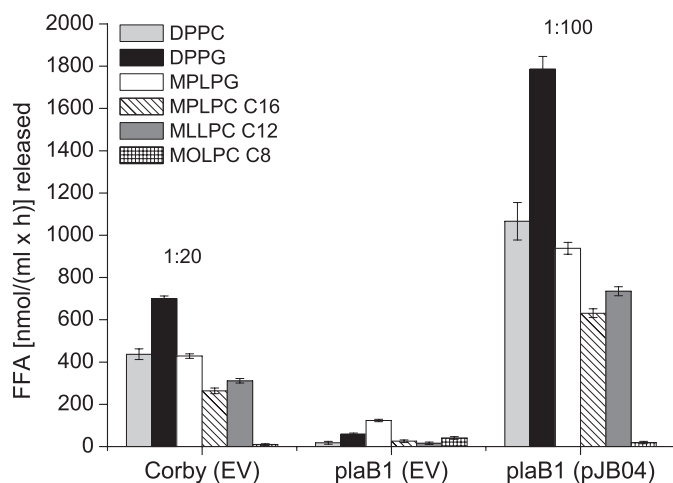


FIGURE 4. Acyl chain length selectivity of wild-type *L. pneumophila* PlaB. Cell lysates of *Legionella pneumophila* strain Corby wild-type (empty vector pBCKS), plaB1 (empty vector pBCKS) mutant, or the complementing strain plaB1 (pJB04), diluted prior to incubation as indicated, were exposed to various lipids ranging from 8 (1-monoctanoyllysophosphatidylcholine) to 12 (1-monolauroyllysophosphatidylcholine) to 16 (MPLPC) carbon atoms in chain length, and the release of fatty acids was quantified. As an internal control, DPPG, DPPC, and MPLPG were carried along under equal conditions. EV, empty vector pBCKS.

lower PLA activity toward DPPC than *L. pneumophila* strains (Fig. 5, A and B). Additionally, by increasing the incubation time, *Legionella anisa*, *Legionella jordanis*, *Legionella parisiensis*, and *Legionella sainthelensis* very predominantly hydrolyzed phospho- and lysophospholipids with an attached glycerol (DPPG and MPLPG) instead of choline (DPPC and MPLPC) (data not shown).

*L. gormanii* and *L. spiritensis* Possess Genes Encoding PlaB Homologs—To address the question of *plaB*-like gene presence in *L. gormanii* and in *L. spiritensis*, a pool of primers were used for PCR with *L. gormanii* and *L. spiritensis* genomic DNA. For *L. spiritensis* amplification, products of a size comparable with that of *L. pneumophila* DNA were obtained and subsequently sequenced. The resulting *L. spiritensis* *plaB* gene (GenBank™ accession number EF408871) showed 86% identity, and its pro-

tein product showed 88% protein identity and 94% similarity to *L. pneumophila* Corby *plaB* or PlaB, respectively. Some of the primers yielded PCR products from *L. gormanii* DNA. The resulting translated partial sequence also showed 86% of amino acid identity and 95% similarity to the *L. pneumophila* Corby PlaB sequence (amino acids 16–256; data not shown). As demonstrated for *L. spiritensis* (Fig. 2C), *L. gormanii* as well comprises the crucial amino acids representing the catalytic active triad of serine, aspartate, and histidine, as well as important Ser-129 responsible for substrate specificity.

Furthermore, we cloned *L. spiritensis* *plaB* into vector pBCKS and compared lipolytic and hemolytic potential of transformed *E. coli* DH5 $\alpha$  to *L. pneumophila* Corby *plaB*. Although no major differences in DPPG hydrolysis were observed, *L. spiritensis* was about 2.5-fold less able to hydrolyze DPPC than *L. pneumophila* Corby PlaB protein (Fig. 5C) ( $p < 0.05$ ). Furthermore, lysis of human red blood cells agreed with this observation, since *L. spiritensis* *plaB* expressing DH5 $\alpha$  cells were 2.2-fold reduced in hemolytic potential against human erythrocytes (Fig. 5D). These results emphasize again that only *L. pneumophila*-derived PlaB is most efficiently able to hydrolyze PC, the most abundant lipid in the outer leaflet of eukaryotic membranes.

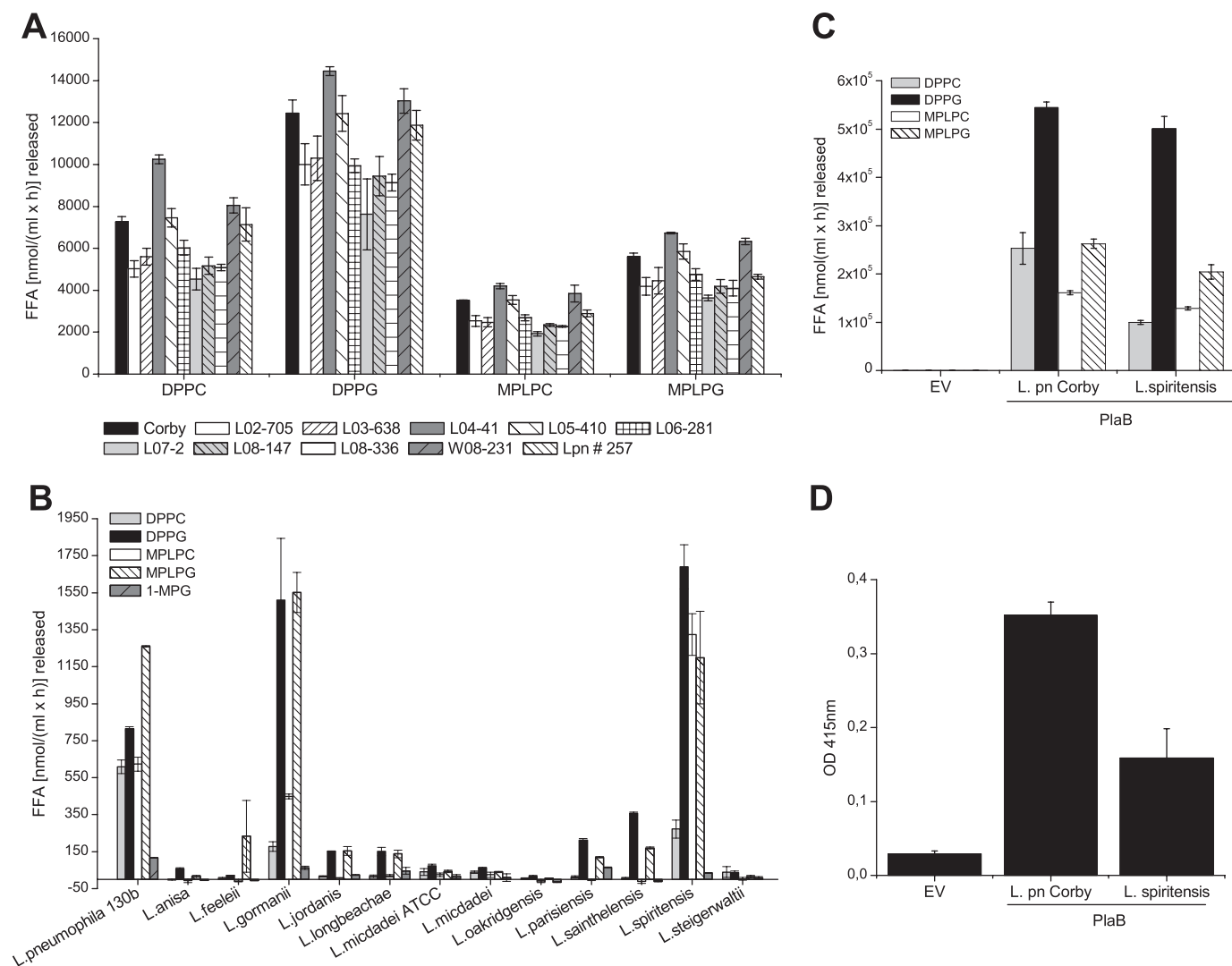
## DISCUSSION

*L. pneumophila* possesses a variety of phospholipid-hydrolyzing enzyme activities both of a secreted, such as PlaA and PlaC, and cell-associated nature, such as PlaB (9, 17, 25–28). *Legionella* phospholipases A may contribute to bacterial virulence in several ways. Those activities destroy lung surfactant phospholipids of mainly PC and phosphatidylglycerol thereby endangering lung function by increasing breathing effort. On the other hand, as shown for PlaB, they contribute to cell destruction by means of cytolytic/hemolytic properties and additionally by releasing toxic lysophospholipids, thus perturbing membrane integrity (9, 26, 29, 30).

As demonstrated recently, the cell-associated PLA activity of *L. pneumophila* is about 100-fold more active than the total of secreted PLA activity present in the culture supernatant (9). Since an *L. pneumophila* *plaB* knock-out mutant basically loses all of its cell-associated lipolytic activity, PlaB is a very potent and most importantly the major phospholipase A of the lung pathogen. PlaB is not essential for intracellular replication under laboratory conditions; nevertheless it represents the most prominently expressed PLA/LPLA activity throughout bacterial host cell infections. Although macrophage or amoeba cell infection rates were not influenced by the presence or absence of PlaB, guinea pig infection experiments show its importance for *Legionella* replication and dissemination *in vivo* (1).<sup>3</sup>

PlaB does not comprise hydrophobic stretches to form  $\beta$ -barrel structures; nevertheless, it could resemble *E. coli* or *Helicobacter pylori* outer membrane phospholipase A function, to allow bacterial adaptation through membrane alteration during the infection process (31, 32). However, PlaB does not show protein homology to outer membrane phospholipase A enzymes or to any other phospholipase described so far. We therefore analyzed the role of several

## Phospholipase PlaB of *L. pneumophila*



**FIGURE 5. Cell-associated PLA activity within clinical isolates of *L. pneumophila* and PlaB-like enzyme properties of non-pneumophila Legionellae.** A, cell lysates of various clinical isolates (Table 1) were incubated with PLA (DPPG and DPPC) and LPLA (MPLPG and MPLPC) substrates. Nine clinical isolates were compared with *L. pneumophila* Corby and with the water conduit strain *Legionella* 257. B, determination of released fatty acids by *L. pneumophila* 130b and 12 different non-pneumophila strains. Cells were grown to an  $A_{660}$  of 1.5, pelleted, lysed, and added to different lipids, followed by quantification of released fatty acids. C, cell lysates of *E. coli* DH5 $\alpha$  expressing either *L. pneumophila* or *L. spiritensis* plaB were incubated with different lipid substrates, and released fatty acids were quantified. DPPC hydrolysis of *L. spiritensis* PlaB was significantly decreased ( $p < 0.05$ ) compared with *L. pneumophila* Corby PlaB. D, lysis of human red blood cells by *E. coli* strains as mentioned in C was measured at 415 nm after the bacteria-cell suspension was incubated for 4 h at 37 °C. EV, empty vector pBCKS.

putative active sites and clarified that PlaB and homologs exhibit a “classical” catalytic triad of Ser, Asp, and His (Ser-85, Asp-203, His-251). Lipases mostly share a common  $\alpha/\beta$ -hydrolase folding pattern, at which conserved motifs surround the nucleophilic residue (Ser, Asp, or Cys), the catalytic acid (Asp or Glu) and histidine (33). However, PlaB and homologs differ from previously published enzymes, since they do not exhibit a GX SXG or a GDSL motif, characteristically displayed by eukaryotic and prokaryotic lipases and esterases as well (12, 34). Instead, PlaB constitutes the designated motif THSTG, surrounding the catalytically active serine. Reconstitution of the lipase-based amino acid sequence by changing Thr-83 to Gly severely affected hydrolysis. Interestingly, replacing Thr-83 by Val, as found in PlaB homologs of several *P. aeruginosa* strains, only affected substrate specificity regarding choline-containing phospholipids. Thus, to our knowledge, this is the first

description of an amino acid other than glycine to be of major importance in the immediate vicinity of the active site Ser residue, which probably favors special steric conditions for effective hydrolysis of deduced substrates. This distinguishes PlaB from other lipases, such as Vst of *Vibrio harveyi*, since the usually invariant glycine was shown to be indispensable for catalytic activity (35).

We further determined His-7 and Ser-129 to be involved in substrate specificity of the protein. Alignment to PlaB homologs displays His-7 to be as conserved as residues constituting the catalytic triad. Adjacent to a central glycine, which most likely represents a part of the oxyanion hole, His-7 probably facilitates hydrolysis of deduced substrates through stabilization of the tetrahedral intermediate (34). Regarding conservation of Ser-129, PlaB-like proteins of *Psychromonas* or *Desulfuromonas* exhibit a Gln residue at the designated position. Both amino acids, His-7 and Ser-129,



selectively contribute to hydrolysis of phospholipids harboring choline side chains. Similar results were obtained by replacing Thr-83 by Val. Apparently, PC is not the main substrate used by the PlaB enzyme, because minor modifications beyond the catalytic triad severely decrease corresponding hydrolysis. Instead, *Legionella* might additionally benefit from PlaB action by using its force to destruct this most abundant lipid within the outer leaflet of eukaryotic membranes (36). Further, lung surfactant is mainly composed of PC, therefore representing a perfect target for PlaB to deplete this important monolayer in the lung and contribute to the loss of lung function as observed in Legionnaires' disease (29). This assumption was underlined by the observation that hemolytic properties of PlaB almost solely depend on its ability to hydrolyze PC.

Another potent phospholipase and virulence factor, ExoU (exotoxin U) of *P. aeruginosa*, was shown to be dispensable for bacterial infection, since some clinical isolates lack the *exoU* encoding gene (37). Nevertheless, increased virulence was directly associated with secretion of the enzyme (38). As demonstrated within this study, different clinical isolates of *L. pneumophila* serogroup 1–3 were tested positive for cell-associated PLA activity. Taking into account host cell and guinea pig infection studies (Fig. 1) (1),<sup>3</sup> the presence of PlaB-like activity within all clinical and environmental isolates tested encourages our assumption of PlaB being valuable for pathogenic *L. pneumophila*.

Regarding distribution of PlaB throughout Legionellae other than *L. pneumophila*, we determined by means of biochemical activity assays that two of the 12 tested non-*pneumophila* strains, *L. gormanii* and *L. spiritensis*, showed cell-associated PLA and LPLA activities in quantities comparable with *L. pneumophila*. We additionally found that the *plaB* genes and deduced proteins of *L. gormanii* or *L. spiritensis* share a high degree of similarity to *L. pneumophila plaB* or PlaB, respectively. This shows that *plaB* paralogs are present in certain other *Legionella* species and may contribute to bacterial virulence as described for *L. pneumophila* PlaB. Indeed *L. gormanii* is a known but rare agent of Legionnaires' disease; however, this has not yet been demonstrated for *L. spiritensis* (23, 24). As already mentioned, *L. pneumophila* cell-associated PLA is strongly directed against phosphatidylglycerol, which is also the case for *L. gormanii*, *L. spiritensis*, and other non-*pneumophila* species showing cell-associated lipolytic activities. Of all tested *Legionella* species, *L. pneumophila* interestingly hydrolyzed the highest percentage of PC. This was verified when the *plaB* genes of *L. pneumophila* and *L. spiritensis* were expressed in *E. coli* DH5 $\alpha$  cells. Since *L. pneumophila* is responsible for more than 90% of Legionnaires' disease cases (24), specificity for cleavage of typical eukaryotic phospholipids, like PC, could be a potent tool for bacterial pathogenicity.

To summarize, we have shown here that cell-associated *L. pneumophila* PlaB is the major PLA and LPLA of the lung pathogen expressed during host cell infection. The highly active enzyme exhibits a "classical" catalytic triad of serine hydrolases, although special motifs surrounding the active sites distinguish PlaB and homologs from previously discov-

ered phospholipases. Thus, PlaB represents the first described member of a new subfamily of lipolytic enzymes. Although it is not restricted to the most prominent pathogenic species, *L. pneumophila*, it probably confers additional colonization advantages through hydrolysis of certain phospholipids, such as PC, the most abundant lipid within the outer leaflet of eukaryotic membranes.

---

*Acknowledgments*—We are grateful to Dave Matthews for critically reading the manuscript and to Ferda Ersöz for excellent technical laboratory support. We further thank Birgid Neumeister (Tübingen University, Germany), Christian Lück (German Reference Laboratory for Legionella, Dresden, Germany), and Barry Fields (Centers for Disease Control and Prevention, Atlanta, GA) for providing non-*pneumophila* strains and clinical isolates, respectively.

---

## REFERENCES

- Bender, J., and Flieger, A. (2009) *Microbiology of Hydrocarbons, Oils, Lipids, and Derived Compounds*, Springer-Verlag GmbH, Heidelberg, Germany, in press
- Istivan, T. S., and Coloe, P. J. (2006) *Microbiology* **152**, 1263–1274
- Schmiel, D. H., and Miller, V. L. (1999) *Microbes Infect.* **1**, 1103–1112
- Jepson, M., and Titball, R. (2000) *Microbes Infect.* **2**, 1277–1284
- Phillips, R. M., Six, D. A., Dennis, E. A., and Ghosh, P. (2003) *J. Biol. Chem.* **278**, 41326–41332
- Sato, H., Frank, D. W., Hillard, C. J., Feix, J. B., Pankhaniya, R. R., Moriyama, K., Finck-Barbançon, V., Buchaklian, A., Lei, M., Long, R. M., Wiener-Kronish, J., and Sawa, T. (2003) *EMBO J.* **22**, 2959–2969
- Sato, H., and Frank, D. W. (2004) *Mol. Microbiol.* **53**, 1279–1290
- Titball, R. W., Naylor, C. E., and Basak, A. K. (1999) *Anaerobe* **5**, 51–64
- Flieger, A., Ryzdzewski, K., Banerji, S., Broich, M., and Heuner, K. (2004) *Infect. Immun.* **72**, 2648–2658
- Winn, W. C., Jr., and Myerowitz, R. L. (1981) *Hum. Pathol.* **12**, 401–422
- Blackmon, J. A., Chandler, F. W., Cherry, W. B., England, A. C., 3rd, Feeley, J. C., Hicklin, M. D., McKinney, R. M., and Wilkinson, H. W. (1981) *Am. J. Pathol.* **103**, 429–465
- Arpigny, J. L., and Jaeger, K. E. (1999) *Biochem. J.* **343**, 177–183
- Upton, C., and Buckley, J. T. (1995) *Trends Biochem. Sci.* **20**, 178–179
- Rydel, T. J., Williams, J. M., Krieger, E., Moshiri, F., Stallings, W. C., Brown, S. M., Pershing, J. C., Purcell, J. P., and Alibhai, M. F. (2003) *Biochemistry* **42**, 6696–6708
- Vernet, T., Ziomek, E., Recktenwald, A., Schrag, J. D., de Montigny, C., Tessier, D. C., Thomas, D. Y., and Cygler, M. (1993) *J. Biol. Chem.* **268**, 26212–26219
- Banerji, S., and Flieger, A. (2004) *Microbiology* **150**, 522–525
- Banerji, S., Aurass, P., and Flieger, A. (2008) *Int. J. Med. Microbiol.* **298**, 169–181
- Edelstein, P. H. (1981) *J. Clin. Microbiol.* **14**, 298–303
- Bi, W., and Stambrook, P. J. (1998) *Anal. Biochem.* **256**, 137–140
- Kirby, J. E., Vogel, J. P., Andrews, H. L., and Isberg, R. R. (1998) *Mol. Microbiol.* **27**, 323–336
- Cianciotto, N. P., and Fields, B. S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5188–5191
- Liles, M. R., Edelstein, P. H., and Cianciotto, N. P. (1999) *Mol. Microbiol.* **31**, 959–970
- Fang, G. D., Yu, V. L., and Vickers, R. M. (1989) *Medicine* **68**, 116–132
- Muder, R. R., and Yu, V. L. (2002) *Clin. Infect. Dis.* **35**, 990–998
- Banerji, S., Bewersdorff, M., Hermes, B., Cianciotto, N. P., and Flieger, A. (2005) *Infect. Immun.* **73**, 2899–2909
- Flieger, A., Gong, S., Faigle, M., Deeg, M., Bartmann, P., and Neumeister, B. (2000) *J. Bacteriol.* **182**, 1321–1327
- Flieger, A., Gong, S., Faigle, M., Stevanovic, S., Cianciotto, N. P., and Neumeister, B. (2001) *J. Bacteriol.* **183**, 2121–2124
- Flieger, A., Neumeister, B., and Cianciotto, N. P. (2002) *Infect. Immun.* **70**, 6094–6106

## Phospholipase *PlaB* of *L. pneumophila*

29. Flieger, A., Gongab, S., Faigle, M., Mayer, H. A., Kehrer, U., Mussotter, J., Bartmann, P., and Neumeister, B. (2000) *FEMS Microbiol. Lett.* **188**, 129–133
30. Weltzien, H. U. (1979) *Biochim. Biophys. Acta* **559**, 259–287
31. Snijder, H. J., and Dijkstra, B. W. (2000) *Biochim. Biophys. Acta* **1488**, 91–101
32. Tannaes, T., Dekker, N., Bukholm, G., Bijlsma, J. J., and Appelmek, B. J. (2001) *Infect. Immun.* **69**, 7334–7340
33. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., and Schrag, J. (1992) *Protein Eng.* **5**, 197–211
34. Jaeger, K. E., Ransac, S., Koch, H. B., Ferrato, F., and Dijkstra, B. W. (1993) *FEBS Lett.* **332**, 143–149
35. Teo, J. W., Zhang, L. H., and Poh, C. L. (2003) *Gene* **312**, 181–188
36. Devaux, P. F., and Morris, R. (2004) *Traffic* **5**, 241–246
37. Hirakata, Y., Finlay, B. B., Simpson, D. A., Kohno, S., Kamihira, S., and Speert, D. P. (2000) *J. Infect. Dis.* **181**, 765–769
38. Schulert, G. S., Feltman, H., Rabin, S. D., Martin, C. G., Battle, S. E., Rello, J., and Hauser, A. R. (2003) *J. Infect. Dis.* **188**, 1695–1706
39. Jepras, R. I., Fitzgeorge, R. B., and Baskerville, A. (1985) *J. Hyg. (Lond.)* **95**, 29–38