***Legionella pneumophila* genes that encode lipase and phospholipase C activities**

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***Legionella pneumophila*, the agent of Legionnaires' disease, is an intracellular parasite of aquatic protozoans and human macrophages. The type II protein secretion system of the Gram-negative *Legionella* organism promotes intracellular infection. A lipase activity and a *p*-nitrophenylphosphorylcholine (pNPPC) hydrolytic activity are two of the factors that are diminished in *L. pneumophila* type II secretion mutants. The *Legionella* lipase activity was found to include free fatty acid release from di- and triacylglycerol substrates, in addition to the previously reported cleavage of monoacylglycerol. In a number of other bacterial systems, the release of *p*-nitrophenol from pNPPC is due to a phospholipase C. In an attempt to identify exoproteins that potentiate intracellular infection, three genes were identified and mutated in *L. pneumophila* strain 130b that were predicted to encode either a secreted lipase or a phospholipase C. The first two genes, which were designated *lipA* and *lipB*, encoded proteins containing the lipase consensus sequence [LIV]-X-[LIVFY]-[LIVMST]-G-[HYWV]-S-X-G-[GSTAC]. Mutations in *lipA* in particular reduced supernatant activity against mono- and triacylglycerols. However, loss of *lipA* and/or *lipB* did not impair the ability of *L. pneumophila* to infect *Hartmannella* amoebae or U937 cell macrophages. The third *L. pneumophila* gene, which was denoted *plcA*, encoded a protein that was highly homologous with a phospholipase C from *Pseudomonas fluorescens*. Inactivation of *plcA* diminished secreted pNPPC hydrolase activity but did not influence *Legionella* infection of host cells. Taken together, these data indicate that *L. pneumophila* has multiple lipases and possibly several phospholipase C enzymes but that *LipA*, *LipB* and *PlcA* are not among those exoproteins required for optimal intracellular infection.**

Keywords: Legionnaires' disease, type II secretion, lipolytic enzymes, macrophages, intracellular bacteria

INTRODUCTION

Legionella pneumophila is the primary aetiological agent of Legionnaires' disease, a potentially fatal pneumonia that especially afflicts immunocompromised individuals (Swanson & Hammer, 2000; Winn, 1988). An inhabitant of fresh water environments, *L. pneumophila* generally enters the lower respiratory tract following the in-

halation of aerosols generated by air conditioners, cooling towers and other devices (Atlas, 1999). Within its aquatic habitats, the *Legionella* bacterium survives as an intracellular parasite of amoebae and ciliates (Fields, 1996; Harb *et al.*, 2000; Swanson & Hammer, 2000). Within the mammalian lung, the microbe flourishes as a parasite of alveolar macrophages (Horwitz, 1992; Roy, 1999; Shuman *et al.*, 1998; Swanson & Hammer, 2000; Vogel & Isberg, 1999). Thus, investigations into the molecular basis of intracellular infection are critical for understanding the natural history and pathogenesis of legionellosis (Cianciotto *et al.*, 1989a; Cianciotto, 2001; Hacker *et al.*, 1993).

Mutational analysis of the *L. pneumophila* *lsp* genes has

Abbreviations: 1,2-DG, 1,2-dipalmitoylglycerol; FFA, free fatty acid; 1-MG, 1-monopalmitoylglycerol; PLC, phospholipase C; PNP, *p*-nitrophenol; pNPPC, *p*-nitrophenylphosphorylcholine.

The GenBank accession numbers for the *L. pneumophila* *lipA*, *lipB* and *plcA* sequences are AF454863, AF454864 and AF454865, respectively.

determined that the *Legionella* type II protein secretion system is required for intracellular infection (Hales & Shuman, 1999; Polesky *et al.*, 2001; Rossier & Cianciotto, 2001). Although necessary for optimal infection of human macrophages, the *L. pneumophila* type II system appears to be most critical for growth within protozoa (Rossier & Cianciotto, 2001). One of six protein secretion systems that can occur in Gram-negative bacteria, type II secretion is a two-step process (Koster *et al.*, 2000; Lee & Schneewind, 2001; Sandkvist, 2001a; Thanassi & Hultgren, 2000). In the first step, the proteins destined for export are transported across the inner membrane and into the periplasmic space via the general secretory (Sec) pathway, and then, in the second step, the proteins translocate the outer membrane by interacting with the multiprotein type II secretion apparatus (Nunn, 1999; Russel, 1998; Sandkvist, 2001a). Type II secretion is dependent upon the PilD prepilin peptidase, an inner membrane enzyme that processes, among other things, pseudopilins which help form the type II apparatus (Liles *et al.*, 1998; Nunn, 1999). In *L. pneumophila*, the type II- and PilD-dependent enzymic activities include a zinc metalloprotease, phospholipase A, lysophospholipase A, lipase, *p*-nitrophenylphosphorylcholine (pNPPC) hydrolase, and tartrate-sensitive and tartrate-resistant acid phosphatase (Aragon *et al.*, 2000, 2001; Flieger *et al.*, 2001; Hales & Shuman, 1999; Liles *et al.*, 1999; Rossier & Cianciotto, 2001). Since the *L. pneumophila* system is the only type II secretion pathway that has been implicated in intracellular infection (Lee & Schneewind, 2001; Rossier & Cianciotto, 2001; Sandkvist, 2001b), identifying the biological significance of each of the *Legionella* type II exoenzymes is an important goal. Thus far, mutational analysis has determined that the *L. pneumophila* metalloprotease and tartrate-sensitive acid phosphatase are not required for intracellular infection (Aragon *et al.*, 2001; Moffat *et al.*, 1994; Szeto & Shuman, 1990).

For three reasons, we chose the lipase and pNPPC hydrolase activities for further study. First, bacterial lipases have been implicated in a variety of pathogenic processes, including the inhibition of phagocyte function (Gribbon *et al.*, 1993; Jaeger *et al.*, 1994; Konig *et al.*, 1996; Pratt *et al.*, 2000; Rollof *et al.*, 1988). Second, pNPPC hydrolysis has been associated with the production of phospholipase C (PLC) enzymes in *L. pneumophila* and others, and in some of those other bacteria, the PLC is implicated in virulence (Baine, 1988; Dowling *et al.*, 1992; Filloux *et al.*, 1987; Jepson *et al.*, 1999; Merino *et al.*, 1999; Schmiel & Miller, 1999; Songer, 1997; Strom *et al.*, 1991; Swanson & Hammer, 2000; Terada *et al.*, 1999; Titball, 1998; Weingart & Hooke, 1999). Third, lipase- and PLC-like activities are conserved among clinical isolates of *L. pneumophila* as well as other pathogenic *Legionella* species (Baine, 1985; Muller, 1981; Nolte *et al.*, 1982; Thorpe & Miller, 1981). Here, we demonstrate that *L. pneumophila* supernatants have activity against mono, di- and triacylglycerols, and that these lipase activities require at least two genes (*lipA*, *lipB*), whose predicted products share

structural characteristics with known lipases. Furthermore, we identify a *L. pneumophila* gene (*plcA*), whose predicted product catalyses pNPPC hydrolysis and is homologous with a *Pseudomonas fluorescens* PLC. Mutants defective for *lipA*, *lipB* or *plcA* were examined for their relative ability to infect protozoan and macrophage hosts.

METHODS

Bacteria and media. *L. pneumophila* serogroup 1 strain 130b (ATCC BAA74), a virulent clinical isolate, was described previously (Engleberg *et al.*, 1984). Strains NU243 and NU258, direct derivatives of 130b, contain kanamycin-resistance gene insertions in the *Legionella pilD* and *lspDE* genes, respectively (Liles *et al.*, 1999; Rossier & Cianciotto, 2001). Legionellae were routinely cultured on buffered charcoal yeast extract (BCYE) agar for 3 days at 37 °C (Edelstein, 1981). To determine the levels of secreted enzymes and compare the growth rates between strains, bacteria were cultured in buffered yeast extract (BYE) broth (Aragon *et al.*, 2000, 2001). Bacterial growth was assessed by measuring the OD₆₆₀. *Escherichia coli* DH5 α , the host of recombinant plasmids, was grown in Luria-Bertani broth or agar (Aragon *et al.*, 2001).

Lipase and phospholipase assays. To test for the presence of secreted enzymes, supernatants from *L. pneumophila* BYE cultures were prepared as before (Aragon *et al.*, 2000). Briefly, supernatants were obtained by centrifugation followed by filtration, and then, in some cases, concentrated 100-fold by passage through a YM10 ultrafiltration cell (Millipore). For the detection of lipase activity, samples were tested for their ability to release free fatty acid (FFA) from 1-monopalmitoylglycerol (1-MG), 1,2-dipalmitoylglycerol (1,2-DG), tributyrin, tricaprilyn, tripalmitin, triolein or lard oil (Aragon *et al.*, 2000). Thus, supernatants were incubated for up to 1 h at 37 °C in 20 mM Tris/HCl (pH 7.2) containing 3 mM sodium azide, 0.5% Triton X-100 and either 2 mg 1-MG ml⁻¹, 1.6 mg 1,2-DG or tripalmitin ml⁻¹, or 0.5% tributyrin, tricaprilyn, triolein or lard oil. After this incubation, FFA levels were determined by the NEFA-C-Kit obtained from Wako Chemicals (Aragon *et al.*, 2000; Flieger *et al.*, 2001; Hoffmann *et al.*, 1986; Rossier & Cianciotto, 2001). The release of FFA was also confirmed by TLC, as previously described (Aragon *et al.*, 2000). Esterase-lipase activity was assayed by monitoring the ability of supernatant samples to release *p*-nitrophenol (pNP) from *p*-nitrophenyl caprylate and *p*-nitrophenyl palmitate (Anguita *et al.*, 1993; Aragon *et al.*, 2000; El Khattabi *et al.*, 1999; Filloux *et al.*, 1987; Jaeger *et al.*, 1999; Rossier & Cianciotto, 2001; Thorpe & Miller, 1981). As before, 100 μ l sample was added to 1 ml buffer (i.e. 100 mM Tris/HCl, pH 8, 0.2% Triton X-100) containing 1 mM substrate, and the increase in absorbance at 410 nm was measured after incubation at 37 °C. For the present study, we confirmed that the wild-type reaction was optimal at pH 7.5–8.0, with no activity detectable below pH 6.5, and was not inhibited by the addition of 50 mM EDTA, a chelator of divalent cations such as calcium (data not shown). One unit of enzyme activity was defined as that which yields 1 nmol pNP in 1 min. PLC activity was assayed as the ability of samples to release pNP from pNPPC (Baine, 1988; Filloux *et al.*, 1987; Kurioka & Matsuda, 1976; Strom *et al.*, 1991). Briefly, 100 μ l supernatant was added to 1 ml 50 mM HEPES (pH 7.5) buffer containing 5 mM CaCl₂, 5 mM MnCl₂, 3 mM sodium azide, 0.5% Triton X-100 and 2.5 mM pNPPC, and then after overnight incubation at 37 °C, the amount of pNP was recorded as above. The PLC of *Bacillus cereus* served as a control in the pNPPC

hydrolysis tests, with one unit of enzyme activity being defined as that which yields 1 nmol pNP in 1 min. Unless noted otherwise, all enzyme substrates and standards were obtained from Sigma. To minimize experimental variation, the supernatants, whether used for lipase or phospholipase assays, were always derived from cultures that had grown to a similar OD₆₆₀.

PCR and sequencing analysis. *L. pneumophila* DNA was extracted as previously described (Aragon *et al.*, 2001). Based on data from the *L. pneumophila* Philadelphia I genome (<http://genome3.cpmc.columbia.edu/~legion/>), three pairs of DNA primers were designed for the amplification of genes from 130b genomic DNA. The pair consisting of 5'-CAACAG-GCTACCGCTAACTT-3' and 5'-CAAGCCGTGATGGTA-TGTCT-3' amplified a 2.8 kb fragment containing *lipA*. Primers 5'-GCATGAAGTGGATGTGGTGT-3' and 5'-CTC-TCCTGAAGAAGATGTCG-3' generated a 1.7 kb fragment containing *lipB*. Primers 5'-CAGGACAGCATCACCATC-TT-3' and 5'-GACTTCTGTCACTGGTCTTG-3' yielded a 3.1 kb *plcA*-containing fragment. Sequencing reactions were performed using at least two different PCR amplicons per gene, a series of custom primers and the BigDye terminator cycle-sequencing mix from PE Applied Biosystems. Automated sequence analysis was performed on an ABI Prism 373 DNA sequencer (Applied Biosystems) at the Biotech Facility at Northwestern University Medical School, Chicago, IL. Primers were obtained from Integrated DNA Technologies. Sequence database searches were performed using programs based on the BLAST algorithm (Altschul *et al.*, 1997). The predicted protein was analysed with SignalP program (Nielsen *et al.*, 1997) and Psort (Nakai & Kanehisa, 1991) for a signal sequence, and for protein motifs with the PROSITE database (Hofmann *et al.*, 1999). Protein alignments were done using the CLUSTAL method (Higgins & Sharp, 1988).

Gene cloning and *Legionella* mutant constructions. To facilitate mutant construction, the PCR fragments were ligated into the pGem-T Easy vector (Promega), yielding pVA14 for the cloned *lipA*, pVA15 for the cloned *lipB*, and pVA16 for the cloned *plcA*. The *lipA* mutants were generated by allelic exchange, a process that proceeded in three steps. First, a kanamycin-resistance gene cassette, obtained from pVK3 (Viswanathan *et al.*, 2000), was cloned into the *Bgl*III site of pVA14, yielding pVA14-1. Then, a *Bst*ZI fragment containing the insertional inactivated *lipA* was released from pVA14-1 and ligated into *Not*I-digested pBOC20, producing pVA14-2. The pBOC20 vector facilitates allelic exchange in *Legionella* by virtue of its counterselectable *sacB* gene (Cianciotto *et al.*, 1988; O'Connell *et al.*, 1995). Following electroporation of pVA14-2 into competent 130b cells (Cianciotto & Fields, 1992), mutants were selected based upon their kanamycin and sucrose resistance. The construction of the *lipB* mutant followed a similar progression. After a gentamicin-resistance gene cassette, obtained from pBBR1MCS-5 (Kovach *et al.*, 1995), was inserted into the *Xcm*I site within pVA15, the insertional inactivated *lipB* was transferred, on a *Not*I-*Sal*I fragment into *Not*I/*Sal*I-digested pBOC20. Following electroporation or transformation of the resulting pVA15-2 into strain 130b (Cianciotto & Fields, 1992; Stone & Abu Kwaik, 1999), mutants were obtained based upon their resistance to gentamicin and sucrose. To obtain a single strain deficient in both *lipA* and *lipB*, pVA15-2 was also introduced into one of the *lipA* mutants, and the resulting allelic exchange event yielded a kanamycin-, gentamicin-, sucrose-resistant double mutant. The *plcA* mutant was obtained as follows. pVA16 was digested with *Eco*RV and then religated, producing pVA16-1, which contains a 310 bp deletion in the cloned *plcA*.

Next, the kanamycin resistance cassette from pVK3 was inserted into *ΔplcA*, and then the mutated, tagged gene was transferred, on a *Bst*ZI fragment, into *Not*I-digested pBOC20. Following electroporation of the resulting pVA16-3 into strain 130b, mutants were obtained based upon their resistance to kanamycin and sucrose. Verification of all of the mutant genotypes was carried out by PCR and Southern hybridization (Robey *et al.*, 2001).

Intracellular infection of U937 cells and *Hartmannella* amoebae. U937, a human cell line that differentiates into macrophage-like cells after treatment with phorbol esters, served as a host for *in vitro* infection by *L. pneumophila* (Cianciotto *et al.*, 1989b). The cell line was infected as previously described (Aragon *et al.*, 2000, 2001; Liles *et al.*, 1999; Rossier & Cianciotto, 2001). To quantitate intracellular growth, monolayers containing 10⁶ macrophages were inoculated with approximately 10⁵ c.f.u., incubated for 0, 24, 48 or 72 h and then lysed. Serial dilutions of the lysates were plated on BCYE agar, supplemented with kanamycin or gentamicin for the mutants, to determine the numbers of bacteria per monolayer. To establish the cytopathic effect of *L. pneumophila* on U937 cells, the viability of infected monolayers was tested by their ability to reduce alamar blue (Aragon *et al.*, 2001). To examine the ability of legionellae to grow within a protozoan host, *Hartmannella vermiformis* was infected as previously indicated (Aragon *et al.*, 2000, 2001; Cianciotto & Fields, 1992; Liles *et al.*, 1999). Thus, approximately 10⁵ c.f.u. were added to wells containing 10⁵ amoebae and then, at 0, 24, 48 or 72 h post-inoculation, the numbers of bacteria within the co-culture were determined by plating serial dilutions on BCYE agar.

RESULTS AND DISCUSSION

Clarification of lipase secretion by *L. pneumophila*

We previously demonstrated that unconcentrated supernatants from cultures of *L. pneumophila* strain 130b have the ability to release both pNP from *p*-nitrophenyl caprylate and *p*-nitrophenyl palmitate and FFA from 1-MG (Aragon *et al.*, 2000). Since these supernatants were unable to release FFA from 1,2-DG and since other supernatants that had been concentrated 40-fold did not release resorufin from 1,2-O-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester, we reasoned that the carboxylesterase activity of *L. pneumophila* supernatants includes, perhaps exclusively, a monoacylglycerol lipase (Aragon *et al.*, 2000; Jaeger *et al.*, 1999). We began the present study by determining whether *Legionella* culture supernatants could, if concentrated, release FFA from 1,2-DG and triacylglycerols. In addition to cleaving 1-MG, strain 130b supernatants that had been concentrated 100-fold released FFA from 1,2-DG and triacprilin (Table 1). They also cleaved tripalmitin, triolein and lard oil, but not tributyrin (data not shown). Previously, we observed that FFA release from 1-MG was diminished in *L. pneumophila pilD* and *lsp* mutants (Aragon *et al.*, 2000; Rossier & Cianciotto, 2001). Mutations in *pilD* and *lspDE* also reduced the ability of strain 130b to secrete the enzyme(s) that is active against di- and triacylglycerol substrates (Table 1). Taken together, these data indicate that *L. pneumophila* supernatants contain a variety of lipolytic activities that are dependent upon the type II protein secretion system.

Table 1. FFA release from mono-, di- and triacylglycerol substrates

Late-exponential phase concentrated supernatants of wild-type 130b, *pilD* mutant NU243, *lspDE* mutant NU258, *lipA* mutant NU262 and *lipB* mutant NU265 were tested for their ability to release FFA from 1-MG, 1,2-DG and tricaprylin. The values presented are the mean $\pm$ SD from three cultures. The results obtained were observed on at least one other occasion.

Strain	FFA release from lipid substrates (mM)*		
	1-MG	1,2-DG	Tricaprylin
Wild-type	2.700 $\pm$ 0.180	0.118 $\pm$ 0.002	0.351 $\pm$ 0.005
<i>pilD</i> mutant	0.009 $\pm$ 0.040*	0.030 $\pm$ 0.014*	0.144 $\pm$ 0.004*
<i>lspDE</i> mutant	0.069 $\pm$ 0.014*	0.040 $\pm$ 0.028*	0.137 $\pm$ 0.012*
<i>lipA</i> mutant	0.550 $\pm$ 0.114*	0.092 $\pm$ 0.013	0.089 $\pm$ 0.002*
<i>lipB</i> mutant	2.630 $\pm$ 0.000	0.122 $\pm$ 0.016	0.235 $\pm$ 0.002*

* Mutant values that are significantly less than 130b controls ($P < 0.05$, Student's *t* test).

Given what is known about other bacteria (Jaeger *et al.*, 1994, 1999), these activities may result from the action of multiple lipase enzymes or a single exoenzyme that acts upon mono-, di- and triacylglycerol substrates.

Identification of two *L. pneumophila* lipase genes

As a first step toward understanding the molecular basis of *Legionella* lipase activity, the *L. pneumophila* genome database was examined for genes that might encode lipases. Although the database is still incomplete and is reflective of the Philadelphia-1 strain of *L. pneumophila*, we have found it an accurate predictor of genes present in our strain 130b (Aragon *et al.*, 2001; Robey *et al.*, 2001; Rossier & Cianciotto, 2001). The sequence [LIV]-X-[LIVFY]-[LIVMST]-G-[HYWV]-S-X-G-[GSTAC] is known to be conserved among prokaryotic and eukaryotic lipases (PROSITE accession no. PS00120). By utilizing the sequences LXLGHXSXGS and KLXFVGH-SXGS to perform a BLAST search of the *Legionella* database, we identified two unlinked ORFs that were predicted to encode proteins containing both the lipase consensus sequence and a signal sequence, a situation that would be compatible with them being type II secreted lipases. Each of the ORFs was amplified by PCR from genomic 130b DNA and sequenced in its entirety. The first ORF, identified by BLAST searches using either variant of the consensus sequence, is predicted to encode a 282 aa protein, which has the alpha/beta hydrolase fold, a catalytic domain that is found in a variety of enzymes (Ollis *et al.*, 1992). We tentatively designated this ORF *lipA*, for lipase gene A (GenBank accession no. AF454863). The second ORF, only identified by the BLAST search that used KLXFVGH-SXGS, encodes a 254 aa protein and was tentatively denoted *lipB*, for lipase gene B (GenBank accession no. AF454864). An alignment of the lipase consensus sequences in LipA, LipB and some of the known lipases is shown in Fig. 1. LipA also aligns with and shares approximately 35% identity and 54% similarity with hypothetical hydrolases from *P. aeruginosa* and *Vibrio*

Lipase Consensus Sequence		L	I	V	-X-	L	I	V	F	Y	-	L	I	V	M	S	T	-G-	H	Y	W	V	-S-	X-	G-	G	S	T	A	C
<i>Lpn</i> LipA	K	L	H	L	L	G	H	S	M	G	A	-	102																	
<i>Lpn</i> LipB	K	I	H	F	V	T	H	S	L	G	G	-	128																	
<i>Bacillus</i>	K	V	D	I	V	A	H	S	M	G	G	-	111																	
<i>Burkholderia</i>	K	V	N	L	V	G	H	S	Q	G	G	-	134																	
<i>Streptococcus</i>	K	L	N	V	A	G	H	S	I	G	G	-	156																	
<i>Rattus</i>	N	V	H	L	I	G	H	S	L	G	A	-	187																	

Fig. 1. Sequence alignment of *L. pneumophila* LipA, LipB and known lipases. The PROSITE consensus sequence for lipases with a serine-containing active site appears at the top of the figure. Below the *L. pneumophila* strain 130b LipA and LipB sequences are the relevant amino acids from lipases of *Bacillus subtilis*, *Burkholderia cepacia*, *Streptococcus* sp. and *Rattus norvegicus*, which have GenBank accession numbers 523934, A39133, AAK81864 and A46696, respectively. Those residues that are completely conserved in this group of proteins are in bold. The amino acid residue immediately preceding the start of the consensus sequence has also been included, since it invariably was a lysine in the bacterial enzymes.

cholerae (GenBank accession nos NP250313 and NP231620). A known protein that shares significant overall homology (31% identity and 47% similarity) with LipA was Kraken from *Drosophila melanogaster* (GenBank accession no. AJ000516). In contrast to the BLAST results for LipA, the only protein that exhibited extensive stretches of homology with LipB (i.e., 30% identity and 49% similarity) was a hypothetical membrane protein from *Chlamydia trachomatis* (GenBank accession no. NC_000117). Sequence analysis further suggested that *lipA* and *lipB* are monocistronic, with *lipA* positioned downstream of the *L. pneumophila* zinc-metalloprotease gene (data not shown) (Moffat *et al.*, 1994).

To test whether LipA and LipB are associated with *Legionella* lipase activity, we used allelic exchange to

Table 2. Release of pNP from *p*-nitrophenyl caprylate and *p*-nitrophenyl palmitate

Wild-type 130b, *lipA* mutant NU262 and *lipB* mutant NU265 were grown in BYE broth, and then, at late-exponential phase, unconcentrated culture supernatants were tested for their ability to release pNP from *p*-nitrophenyl caprylate and *p*-nitrophenyl palmitate. The values presented are the mean $\pm$ SD from three cultures.

Strain	pNP released from substrate (U)	
	<i>p</i> -nitrophenyl caprylate	<i>p</i> -nitrophenyl palmitate
Wild-type	6.517 $\pm$ 0.294	2.188 $\pm$ 0.132
<i>lipA</i> mutant	1.804 $\pm$ 1.152*	0.371 $\pm$ 0.310*
<i>lipB</i> mutant	6.771 $\pm$ 0.871	4.854 $\pm$ 0.463

* Differences between 130b and NU262 activity were significant ($P < 0.05$, Student's *t* test) and were observed in an additional experiment.

isolate a series of 130b mutants containing an antibiotic resistance gene inserted into either *lipA* or *lipB*. For the *lipA* mutants, the insertion was placed 165 bp from the start of the 846 bp ORF, and for the *lipB* mutants, the insertion mutation was positioned 376 bp from the beginning of the 762 bp gene. Three independent *lipA* mutants, designated NU262, NU263 and NU264, were obtained. Two independent *lipB* defective strains were derived and named NU265 and NU266. All of the mutants grew in BYE broth similarly to wild-type 130b (data not shown), indicating that *lipA* and *lipB* are not generally required for extracellular replication. All further experiments described here were also performed with multiple *lipA* and *lipB* mutants with similar results. Thus, the phenotypes observed for the mutants result directly or proximately from the mutation in the *lipA* or *lipB* gene and not from spontaneous second-site mutations. For clarity, only data for NU262 and NU265 are presented in this paper.

Concentrated culture supernatants of NU262 had a reduced ability to release FFA from 1-MG and triacylglycerol (Table 1), as well as tripalmitin, triolein and lard oil (data not shown), further indicating that *lipA* encodes a secreted lipolytic enzyme. Although not defective for the cleavage of the monoacylglycerol substrate, NU265 was modestly impaired for FFA release from the triacylglycerol substrate (Table 1), suggesting that *lipB* also encodes a *Legionella* lipase-like enzyme. Neither the *lipA* nor the *lipB* mutant was significantly defective for FFA release from 1,2-DG (Table 1), suggesting that lipase(s) other than LipA and LipB are largely responsible for the cleavage of diacylglycerol substrates. The ability to release pNP from *p*-nitrophenyl caprylate and *p*-nitrophenyl palmitate has often been associated with lipase activities, including those of *L. pneumophila* (Aragon *et al.*, 2000; Thorpe & Miller, 1981). NU262 culture supernatants were lacking in their ability to cleave *p*-nitrophenyl caprylate and *p*-nitrophenyl palmitate, whereas the *lipB* mutant appeared to be unaltered for these activities (Table 2). Taken together, the relationship between LipA and LipB sequences and those of known lipases and the altered lipolytic activity

Fig. 2. Intracellular infection of *H. vermiformis* by wild-type and mutant *L. pneumophila*. (a) Infection profile for strain 130b (◆) versus *lipA* mutant NU262 (□). (b) Infection profile for strain 130b (◆) versus *lipB* mutant NU265 (△), *lipA lipB* double mutant NU267 (●), *plcA* mutant NU268 (□) and *lsdDE* mutant NU258 (■). Amoebae were infected at a m.o.i. of 1, and then the numbers of bacteria in each well were quantified at 0, 24, 48 and 72 h by plating aliquots on BCYE agar. Results represent the means $\pm$ SD of triplicate wells and are representative of two independent experiments.

of the mutants indicate that *lipA* and *lipB* are lipase genes of *L. pneumophila*. Since the *lipA* mutant was defective in assays that utilized unconcentrated super-

Fig. 3. Intracellular infection of U937 cells by wild-type and mutant *L. pneumophila*. Infection profile of strain 130b (●) versus *lipA* mutant NU262 (□) (a), *lipB* mutant NU265 (△) (b), *lipA lipB* double mutant NU267 (▽) (c), and *plcA* mutant NU268 (◇) (d). Macrophages were infected at a m.o.i. of 0.1. At 0, 24, 48 and 72 h post-inoculation, the monolayer was lysed and the total numbers of c.f.u. within the wells were determined. Each datum point represents the mean and standard deviation for three monolayers. Results are representative of two independent experiments. When culture supernatants obtained without the purposeful lysis of the monolayers were assayed for bacterial numbers, comparable numbers of c.f.u. of wild-type and mutant bacteria were recovered (data not shown), indicating that the *lipA*, *lipB* and *plcA* mutants are not defective for either intracellular growth or host cell lysis.

natants, we strongly suspect that LipA is a *bona fide* type II exoenzyme. In contrast, because the *lipB* mutant was only impaired in an assay that utilized concentrated supernatants, LipB is either an exoenzyme that is exported in low amounts or is a periplasmic enzyme that is released into supernatants upon cell lysis.

Intracellular infection by *L. pneumophila* lipase mutants

To determine whether *lipA* and *lipB* promote intracellular infection by *L. pneumophila*, we examined the ability of NU262 and NU265 to grow within *H. vermiformis* and U937 cell macrophages. Upon co-culture with the *Hartmannella* amoebae, the mutants grew comparably to wild-type, indicating that neither

lipA nor *lipB* is required for intracellular infection of protozoan hosts (Fig. 2). When the macrophage cell line was infected, NU262 and NU265 behaved, for the most part, similarly to wild-type (Fig. 3a, b). The only aberration was a modestly reduced recovery of the *lipA* mutant between 48 and 72 h incubation (Fig. 3a). Since that mutant also exhibited reduced survivability upon incubation in the RPMI tissue culture medium (data not shown), we suspect that this reduced recovery was not due to diminished intracellular growth but rather the death of bacteria following their release from the macrophages. As an alternative way of identifying an intracellular infectivity defect (Cianciotto *et al.*, 1989b; Robey *et al.*, 2001), we examined the ability of the mutants to kill the U937 cell monolayer. However, neither NU262 nor NU265 showed an impaired cyto-

pathic effect (data not shown). Even though the analysis of lipolytic activities in the mutants' supernatants suggested that *lipA* and *lipB* have different substrate spectra, we explored the possibility that the two genes have overlapping function in the intracellular environment, such that the loss of *lipA* is compensated for by *lipB* and *vice versa*. Thus, we constructed a 130b mutant, designated strain NU267, that was lacking both *lipA* and *lipB*; i.e., a gentamicin-resistance gene cassette was inserted into the *lipB* gene of the kanamycin-resistant *lipA* mutant. Strain NU267 grew in BYE broth as well as did wild-type, but its culture supernatants had, as expected, a reduced activity against lipase substrates (data not shown). However, the double lipase mutant did not display a significant defect in intracellular infection (Figs 2b and 3c). Taken together, these data indicate that the *lipA* and *lipB* genes are not required for intracellular infection by *L. pneumophila*. They also signal that the reduced intracellular infectivity of type II secretion mutants (see Fig. 2b, for example) is not due to the loss of LipA or LipB. Our observations do not, however, rule out a possible role for other *Legionella* lipases in infection of macrophages and/or protozoa. It should also be acknowledged that the apparent absence of a critical role for LipA and LipB in intracellular infection does not mean that these factors have no role in *L. pneumophila* virulence. Indeed, like the type II secretion dependent metalloprotease (Moffat *et al.*, 1994), LipA and LipB may be most relevant in the extracellular spaces of the lung.

Identification and mutation of a *L. pneumophila* PLC-like gene

Biochemical analysis of culture supernatants has yielded mixed results concerning the existence of *L. pneumophila* PLC, and it has been argued that the observed hydrolysis of pNPPC might be due to a phosphatase (Baine, 1988; Flieger *et al.*, 2000). Yet, a recently obtained acid phosphatase mutant is not defective for pNPPC hydrolysis (Aragon *et al.*, 2001). Thus, we felt that the type of genetic approach applied to *lipA* and *lipB* might be helpful in addressing the existence and relevance of a *L. pneumophila* PLC. Examination of the Philadelphia-I genome database suggested the presence of a *L. pneumophila* ORF that is related to a *Pseudomonas* PLC gene. Cloning of the ORF from strain 130b and subsequent complete sequence analysis confirmed that *L. pneumophila* contains a gene (GenBank accession no. AF454865) whose predicted 48 kDa product aligns with and is 43% identical and 62% similar to a recently described PLC that is present in *P. fluorescens* (GenBank accession no. AJ30443) (Preuss *et al.*, 2001). Since the *Legionella* protein was also predicted to contain a signal sequence, we viewed it as a potential type II exoenzyme. When the gene was inactivated by allelic exchange, the resulting mutant (NU268) grew normally in BYE broth (data not shown). Importantly, on five separate occasions, the mutant's unconcentrated culture supernatants displayed a reduced ability to release pNP from pNPPC. For example, in one ex-

periment, the wild type samples ($n=3$) had 2.947 ± 0.254 U activity, whereas the mutant samples ($n=3$) had 1.508 ± 0.096 ($P < 0.001$, Student's *t*-test). In that same experiment, the *pilD* and *lspDE* mutants had 0.174 ± 0.153 and 0.021 ± 0.019 U activity. Taken together, these data indicate that mutant NU268 has diminished PLC activity. Since *L. pneumophila* secretes both a phospholipase A (Flieger *et al.*, 2000) as well as multiple lipases (see above), we were unable to assay more specifically for PLC activity by monitoring diacylglycerol release from phospholipid substrates. On the basis of our current structural and functional data, we designated this new gene as *L. pneumophila plcA*, for phospholipase C gene A.

To determine the relevance of *plcA* for *Legionella* intracellular growth, we examined the relative ability of NU268 to multiply within *Hartmannella* co-cultures and U937 cell monolayers. The *plcA* mutant did not show evidence of a replication defect in either amoebae (Fig. 2b) or macrophages (Fig. 3d). Furthermore, it exhibited a cytopathic effect in U937 cells that was comparable to that of its wild-type parent (data not shown). Taken together, these data indicate that *plcA*, though associated with a PLC-like activity, is not required for *L. pneumophila* intracellular infection. This observation does not, however, rule out a possible role for *Legionella* phospholipase C enzymes in infection of macrophages and/or protozoa. Indeed, recent examination of the *L. pneumophila* genomic database suggests the presence of additional PLC genes (data not shown); a situation that is compatible with the residual pNPPC hydrolysis exhibited by the *plcA* mutant. Thus, our further exploration into the question of *Legionella* PLC enzymes will initially be targeted toward the characterization of other candidate phospholipase C genes.

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