Legionella pneumophila Type II Protein Secretion Promotes Virulence in the A/J Mouse Model of Legionnaires' Disease Pneumonia

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Legionella pneumophila, the gram-negative agent of Legionnaires' disease, possesses type IV pili and a type II protein secretion (Lsp) system, both of which are dependent upon the PilD prepilin peptidase. By analyzing multiple *pilD* mutants and various types of Lsp mutants as well as performing *trans*-complementation of these mutants, we have confirmed that PilD and type II secretion genes are required for *L. pneumophila* infection of both amoebae and human macrophages. Based upon a complete analysis of *lspDE*, *lspF*, and *lspG* mutants, we found that the type II system controls the secretion of protease, RNase, lipase, phospholipase A, phospholipase C, lysophospholipase A, and tartrate-sensitive and tartrate-resistant acid phosphatase activities and influences the appearance of colonies. Examination of the developing *L. pneumophila* genome database indicated that the organism has two other loci (*lspC* and *lspLM*) that are predicted to promote secretion and thus a set of genes that is comparable to the type II secretion genes in other gram-negative bacteria. In contrast to *lsp* mutants, *L. pneumophila* pilus mutants lacking either the PilQ secretin, the PspA pseudopilin, or pilin were not defective for colonial growth, secreted activities, or intracellular replication. *L. pneumophila dot/icm* mutants were also not impaired for type II-dependent exoenzymes. Upon intratracheal inoculation into A/J mice, *lspDE*, *lspF*, and *pilD* mutants, but not pilus mutants, exhibited a reduced ability to grow in the lung, as measured by competition assays. The *lspF* mutant was also defective in an in vivo kinetic assay. Examination of infected mouse sera revealed that type II secreted proteins are expressed in vivo. Thus, the *L. pneumophila* Lsp system is a virulence factor and the only type II secretion system linked to intracellular infection.

The gram-negative bacterium *Legionella pneumophila* is the agent of Legionnaires' disease, a pneumonia which especially affects immunocompromised individuals (28, 89). An inhabitant of freshwater environments, *L. pneumophila* naturally replicates within protozoan hosts and in biofilms (27, 89). Following inhalation of contaminated aerosols, the bacterium reaches the human respiratory tract. Bacterial multiplication in alveolar macrophages is concomitant with cell death and damage to the lung tissue (89, 100).

In gram-negative bacteria, the PilD prepilin peptidase is necessary for the cleavage and methylation of pilins and pseudopilins that assemble into type IV pili (Tfp) (55, 68, 69, 87). In addition, PilD processes other pseudopilins that are necessary for the biogenesis of a functional type II protein secretion system (8, 13, 55, 71, 86). Accordingly, our previous mutational analysis determined that *pilD* is required for *L. pneumophila* piliation and protein secretion (4, 52). In *L. pneumophila*, Tfp promote attachment to host cells and are involved in competence for DNA transformation (84, 85). The *L. pneumophila* proteins believed to be secreted via the type II system include a zinc metalloprotease, acid phosphatases, lipases, phospholipases C (PLC), a PLA, and a lysophospholipase A (LPLA) (3–5, 39, 52, 75). In addition to *pilD*, the loci known to be involved in *L. pneumophila* type II protein secretion are *lspDE*, which specifies the outer membrane secretin LspD and the ATPase LspE; *lspF*, which encodes the inner membrane protein LspF; and *lspGHIJK*, which encodes the pseudopilins

LspGHIJK (39, 75). Previous studies suggested that *L. pneumophila pilD*, though not required for extracellular replication, is essential for optimal infection of aquatic protozoa and human macrophages (52, 75). Whereas a type IV pilin (*pilE_L*) mutant exhibits intracellular growth identical to wild-type strains (75, 84), mutants lacking either *lspDE*, *lspG*, or *lspK* are critically impaired for replication in *Hartmannella vermiformis* and *Acanthamoeba castellanii* amoebae (39, 70, 75). The type II secretion mutants are also defective for growth in human macrophages (70, 75), but an absence of complementation analysis has prevented a definitive conclusion regarding the role of the Lsp system in *L. pneumophila* infection of mammalian cells. Furthermore, the importance of type II secretion as well as Tfp for *L. pneumophila* in vivo growth and virulence has remained unknown.

In this study, we constructed and analyzed a large panel of mutants and complemented derivatives in order to confirm the relative roles of PilD, the type II secretion system, and type IV piliation in *L. pneumophila* extracellular growth, colony formation, protein secretion, natural transformation, and in vitro intracellular infection. Furthermore, to assess the in vivo significance of *L. pneumophila* type II secretion and pilus biogenesis, we tested the mutants in the A/J mouse model of legionellosis. The results of these studies affirm, among other things, that the type II secretion system promotes the ability of *L. pneumophila* to infect both protozoan and macrophage hosts and to grow in the mammalian lung. In fact, the data from the animal studies indicate that type II secretion has a greater role in Legionnaires' disease than would have been predicted from the results of in vitro experiments.

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TABLE 1. Wild-type *Legionella* strains used in this study

Species and strain[a]	Serogroup	Source	Reference
L. pneumophila			
BAA-74	1	Clinical	25
33217	1	Clinical	15
33154	2	Clinical	58
33155	3	Clinical	58
33156	4	Clinical	58
33216	5	Clinical	36
33215	6	Clinical	59
33823	7	Clinical	10
35096	8	Clinical	12
43736	13	Clinical	54
43703	14	Clinical	9
L. cherrii 35252		Environmental	14
L. feeleii 35072	2	Clinical	92
L. gormanii 33297		Environmental	64
L. longbeachae 33462	1	Clinical	57
L. micdadei			
Stanford-R	1	Clinical	74
33218		Clinical	40
L. parisiensis 35299	1	Environmental	14
L. spiritensis 35249		Environmental	14

[a] Except for one *L. micdadei* isolate, the strain designations refer to ATCC numbers.

and N. P. Cianciotto, Abstr. Gen. Meet. Am. Soc. Microbiol. 2002, abstr. B-207, p. 67, 2002.])

MATERIALS AND METHODS

Bacterial strains and media. *L. pneumophila* serogroup 1 strain 130b (ATCC BAA-74), which served as the wild-type strain in this study, and its derivatives NU243, NU258, NU259, BS100, and AA200, which contain stable insertions of a kanamycin resistance (Km^r) gene in *pilD*, *lspDE*, *lspG*, *pilE$_L$*, and *proA*, respectively, were described previously (25, 52, 62, 75, 84). Table 1 lists additional strains of *L. pneumophila* and of *Legionella* spp. used in this study. Legionellae were cultured at 37°C in buffered yeast extract (BYE) broth or on buffered charcoal yeast extract (BCYE) agar (22). Growth in liquid medium was assessed by measuring the optical density of the culture at 660 nm (OD$_{660}$). *Escherichia coli* strains NovaBlue (Novagen, Madison, Wis.) and DH5α (Bethesda Research Laboratories), hosts for recombinant plasmids, were grown at 37°C on LB agar (6). The following antibiotics were added to the media at the indicated final concentrations (μg per ml): ampicillin, 100; chloramphenicol, 6 for *L. pneumophila* and 30 for *E. coli*; gentamicin, 2.5; and kanamycin, 25 for *L. pneumophila* and 50 for *E. coli*.

DNA isolation, PCR, and sequence analysis. Genomic DNA was isolated from *L. pneumophila* as described previously (24). Based on data from the *L. pneumophila* Philadelphia-I genome database (http://genome3.cpmc.columbia.edu/~legion/), four pairs of primers were designed for amplifying genes from 130b DNA. Primers F3 and R34 (52) yielded a 1,246-bp fragment containing *pilD*, OR7lspF (5′-CCTCCAGGATAGTCCGCGTA), and OR2lspH (5′-CTTGTTG TTGAGCCAGGCTT) yielded a 1,972-bp fragment containing *lspF* and *lspG*, OR33pilQ (5′-CAACCTGACGGTCAGATTAG) and OR34pilQ (5′-GCCAG CACGCTCTTCAATTA) yielded a 2,857-bp fragment encoding *pilQ*, and OR29pspA (5′-CATGACGATGTATCTCGGCT) and OR30pspA (5′-GGTAA CCGTCTCACACTACT) yielded an 1,830-bp fragment containing *pspA*. Sequencing reactions were performed using two different PCR amplicons, a series of custom primers, and the BigDye terminator cycle sequencing mix (PE Applied Biosystems, Foster City, Calif.). Automated sequence analysis was done on an ABI Prism 373 DNA sequencer (Applied Biosystems) at the Biotech Facility at Northwestern University. Primers were obtained from Integrated DNA Tech-

nologies (Coralville, Iowa). Database searches were performed using programs based on the BLAST algorithm (2), and protein sequences were analyzed for motifs using the PROSITE database (26).

Gene cloning and generation of mutants. To facilitate construction of *L. pneumophila* mutants, the PCR fragments encoding *pilD*, *lspF*, and *pilQ* were ligated into pGem-T Easy (Promega, Madison, Wis.), yielding pGD1, pGlspF, and pGQ211, respectively, and the PCR product encoding *pspA* was cloned into pGem-T (Promega), giving pGpspA. A gentamicin resistance (Gm^r) gene was isolated from pX1918GT after *Hin*cII and *Pvu*II digestion (82) and was ligated into the single *Nar*I site of the cloned *pilD* on pGD1, to give pGD::Gm. Plasmid pGlspF was digested with *Msc*I, which cuts in the middle of *lspF*, and subsequently ligated to a Km^r gene isolated from pMB2190 upon *Hin*cII digestion (38), to produce pGlspF::Km. Digestion of pGQ211 with *Mfe*I and *Cla*I led to the deletion of a 1.5-kb section of *pilQ* that, following Klenow treatment, was replaced by either the Km^r gene to generate pGQ::Km or the Gm^r gene to yield pGQ::Gm. Plasmid pGpspA was digested with *Mfe*I and *Ssp*I so as to remove a 0.4-kb fragment from *pspA* that, following Klenow treatment, was replaced by the Km^r gene producing pGpspA:::Km or by the Gm^r gene, yielding pGpspA::Gm. Finally, using our modification to the original *L. pneumophila* transformation protocol (30, 85), *pilD*, *lspF*, *pilQ*, and *pspA* mutants were obtained by natural transformation of strain 130b with pGD::Gm, pGlspF::Km, pGQ::Km, pGQ::Gm, pGpspA:::Km, and pGpspA::Gm. Briefly, bacteria were inoculated at an OD$_{660}$ of 0.3 into 2 ml of BYE broth in a 15-ml polypropylene tube, and then 5 μg of plasmid DNA/ml was added. After the culture was grown for approximately 20 h at 30°C with moderate shaking until mid- to late exponential phase, bacteria were plated on BCYE agar supplemented with kanamycin or gentamicin. Verification of all mutant genotypes was carried out by PCR and Southern hybridization (77). To construct an *lspDE pilQ* double mutant, the Km^r *lspDE* mutant NU258 (75) was transformed with plasmid pGQ::Gm, and mutants were selected on BCYE agar supplemented with kanamycin and gentamicin.

Complementation analysis. Although we and others have previously used pMMB207 (63) for *trans*-complementation of *L. pneumophila* mutants (56, 73, 96), the conjugal components of the vector can inhibit *L. pneumophila* growth within macrophages (83). Therefore, in order to facilitate present and future complementation studies, pMMB207 was digested with *Age*I and then religated, yielding pMMB2002, which has a 401-bp deletion in the *mobA* gene (80, 88). After plasmids were electroporated into competent 130b bacteria (52), the stability of pMMB2002 versus pMMB207 was determined by plating serial dilutions of transformants that had been grown without antibiotic selection on plain BCYE agar and BCYE agar supplemented with chloramphenicol. After growth in BYE broth or on BCYE agar to stationary phase, 85 to 100% and 51 to 70% of bacteria retained pMMB2002 and pMMB207, respectively. In addition, whereas 130b(pMMB207) had a 20- to 70-fold decrease in CFU recovery from infected U937 cells relative to 130b, bacteria containing pMMB2002 exhibited intracellular growth that was identical to that of wild type. Thus, to facilitate complementation of the *pilD* mutants, a 1.2-kb fragment containing only *pilD* was amplified by PCR using primers F3 and R34 (52) and then cloned under the control of the *tac* promoter in pMMB2002, yielding pMD1. To assess complementation of an *lspF* mutant, pMF1 was constructed; i.e., a 1.3-kb fragment containing only *lspF* was obtained from pGlspF using *Sst*I and *Ssp*I and then ligated into pMMB2002 that had been digested with *Sst*I and *Sma*I.

Analysis of *L. pneumophila* secreted enzymatic activities. *L. pneumophila* protease and lipolytic activities were observed on casein (1%) agar and egg yolk (5%) agar, respectively (4, 30, 93). Filter-sterilized supernatants from cultures in BYE broth in late exponential phase (4) were tested for tartrate-sensitive and tartrate-resistant acid phosphatases as determined by the release of *p*-nitrophenol (*p*-NP) from *p*-NP phosphate in 200 mM sodium acetate (pH 5.5) in the absence or presence of 5 mM tartrate (3). PLC activity was monitored by the release of *p*-NP from *p*-NP phosphorylcholine (4). Monoacylglycerol lipase, PLA, and LPLA activities were measured by the ability of supernatants to release free fatty acids from 1-monopalmitoyl glycerol, phosphatidylcholine, and lyso-phosphatidylcholine, respectively (4, 29, 75). Lipolytic activities were also determined by *p*-NP palmitate and *p*-NP caprylate hydrolysis (4). RNase activity was assayed using a protocol adapted from Kar et al. (45). Briefly, 40 μl of culture supernatants was incubated with 400 μl of assay buffer (50 mM Tris, pH 8) containing Baker's yeast type III RNA (10 mg/ml; Sigma Chemical Co., St. Louis, Mo.). At the beginning of the reaction, half of the sample was transferred to a fresh tube, chilled, and precipitated with 2 volumes of ice-cold 10% trichloroacetic acid for 25 min. The remainder was incubated at 37°C for 40 min, at which point the reactions were chilled and precipitated with trichloroacetic acid as described above. Following centrifugation (12 min at 18,000 × *g*) of the precipitated samples, the absorbance of the supernatants at 260 nm, reflective of

released soluble nucleotides, was determined. Bovine pancreas RNase A (Sigma) was used as a positive control.

Intracellular infection by *L. pneumophila*. To examine the ability of *L. pneumophila* to grow within a protozoan host, *H. vermiformis* was infected as previously described (3, 4, 18, 52). Thus, ca. 10^4 CFU was added to wells containing 10^5 amoebae, and then at 0, 24, 48, 72, or 96 h postinoculation, the numbers of bacteria per coculture were determined by plating serial dilutions on BCYE agar, supplemented with the appropriate antibiotics. To quantitate intracellular growth in human macrophages, U937 cells were infected as previously described (3, 4, 52, 75). Briefly, monolayers containing 10^6 macrophages were inoculated with approximately 10^5 CFU, incubated for 2 h to allow bacterial entry and then washed three times with media to remove unincorporated bacteria. After 0, 24, or 48 h of incubation, serial dilutions of the lysed monolayers were plated on BCYE agar, supplemented with antibiotics when appropriate, to determine the numbers of bacteria per monolayer.

Pulmonary infection of A/J mice with *L. pneumophila*. Pulmonary infection of A/J mice with *L. pneumophila* is a standard model for Legionnaires' disease (1, 16, 17, 19, 74). Female, 6- to 8-week-old mice (Jackson Laboratory, Bar Harbor, Maine) were anesthetized and then inoculated intratracheally with 25 μl of a bacterial suspension. To assess the virulence of bacterial strains, standard competition assays (31, 50, 60) were performed, as has been described previously (73, 74). Mice were inoculated with 10^5 CFU of a ca. 1:1 ratio of wild-type and mutant bacteria. At various time points, infected mice ($n = 4$ to 5) were sacrificed, and lungs were disrupted into 5 to 10 ml of phosphate-buffered saline using a homogenizer Pro 200 equipped with a generator (7 mm by 150 mm; Pro Scientific Inc., Monroe, Conn.). Host cell lysis was achieved by incubation of the tissue sample with 1% saponin for 15 min at 37°C, followed by vortexing. The numbers of viable bacteria and the ratio of wild type to mutant were estimated by plating 10-fold serial dilutions on both standard and antibiotic-supplemented BCYE. To determine the relative ability of strains to replicate and survive in mice lungs, mice ($n = 4$ or 5) were infected separately with 10^6 CFU of wild type or mutant, and at various hours postinoculation, the bacterial CFU in the lungs were determined by plating on BCYE agar.

Immunoblot analysis of *L. pneumophila* culture supernatants. Filter-sterilized supernatants from BYE cultures in late exponential phase were precipitated with 10% trichloroacetic acid on ice for 30 min. Following centrifugation for 20 min at 4°C, pellets were washed with 70% ethanol, air dried, and resuspended in sample buffer at 1/100 of the supernatant volume (51). Following separation by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) (51), proteins were transferred onto a nitrocellulose membrane (94) and reacted with a 1:100 dilution of mouse sera 3 weeks after inoculation with 10^6 CFU of strain 130b (above). After incubation with a 1:1,000 dilution of anti-mouse immunoglobulin G peroxidase-conjugated antibody (Chemicon International, Temecula, Calif.), immunodetection was performed using Enhanced Chemiluminescence Western blotting (Amersham Biosciences) and a charge-coupled device camera (Chemi Imager 5500; Alpha Innotech, San Leandro, Calif.).

Detection of *lsp* gene sequences in *Legionella* spp. and serogroups. Southern blot hybridization was carried out using *Eco*RI-restricted genomic DNA from strains representing several *L. pneumophila* serogroups and several *Legionella* spp. (Table 1). The digoxigenin labeling and detection system (Roche Molecular Biochemicals, Indianapolis, Ind.) was used according to the manufacturer's instructions. Probes were prepared by PCR incorporation using 130b DNA as a template and primers OR70lspC (5′-GTTTAACTGGGATTGGCAAC) and OR71lspC (5′-CAGGCAGCCATCATAGGAAT) for detection of *lspC*, OR5lspD (5′-TTGATTCTGTCTGGTCGAGC) and OR6lspD (5′-ATCAAGG ACTACTACGGAGG) for detection of *lspD*, OR7lspF or OR2lspH for detection of *lspFG*, and OR68lspL (5′-GGATTATGCGGCTTCTGGTT) and OR69lspM (5′-AGGTCAGGCTTATAGTCTTG) for detection of *lspLM*.

Nucleotide sequence accession number. The nucleotide sequence of *L. pneumophila pilQ* is deposited in GenBank under accession number AY320053.

RESULTS

Clarification of the role of *pilD* in *L. pneumophila* intracellular infection. In our previous studies of *L. pneumophila*, the prepilin peptidase mutant NU243 displayed a dramatic reduction in intracellular growth in both amoebae and macrophages (4, 52, 75). Introduction of pMRL13, a derivative of pBBR1MCS expressing *pilD*, into NU243 restored intracellular growth of the *pilD* mutant in *H. vermiformis* to wild-type

levels (52). In U937 cells, growth of NU243 was only partially complemented with pMRL13, but since pBBR1MCS derivatives are poorly maintained in strain 130b, we attributed the partial complementation to vector instability (52). With the development of pMMB2002, a vector that is stable in *L. pneumophila* (see Materials and Methods), we reinvestigated complementation of NU243 in order to definitively assess the importance of *pilD* for intracellular infection. As expected, NU243 carrying *pilD* cloned into pMMB2002 (i.e., pMD1) displayed full protease and lipolytic activities on casein agar and egg yolk agar, respectively (data not shown), confirming the role of PilD in *L. pneumophila* protein secretion. Upon infection of amoebae and macrophages, 130b(pMD1) exhibited the same replication profile as 130b(pMMB2002), demonstrating that expression of multiple copies of *pilD* does not alter the intracellular growth of wild-type *L. pneumophila* (Fig. 1). Expression from pMD1 allowed NU243 to effectively replicate in *H. vermiformis*, with the complementation of the *pilD* mutation complete after 72 h of coculture (Fig. 1A). NU243(pMD1) also exhibited robust intracellular replication in U937 macrophages, in contrast to NU243(pMMB2002) (Fig. 1B). However, NU243(pMD1)-infected cells always yielded significantly less CFU than did monolayers infected with 130b(pMD1) (Fig. 1B). Since >75% of 130b and NU243 retained pMMB2002 and pMD1 throughout the experiment (data not shown), such partial complementation could not be explained by loss of plasmid alone and suggested that some aspects of the NU243 phenotype are not due to the loss of PilD. Indeed, examination of 130b(pMMB2002), NU243 (pMMB2002), and NU243(pMD1) indicated that the grayer color but not the overall morphology of NU243 colonies (52) is associated with the *pilD* mutation (data not shown).

To explore further the possibility that NU243 has a second site mutation that affects infectivity and colonial growth, we used allelic exchange to isolate new *pilD* mutants. Three mutants were independently derived from 130b and designated NU272, NU273, and NU274. Each contained a Gm^r cassette inserted into the same site in *pilD* as the Km^r gene in NU243. Since the three new mutants behaved similarly in all experiments, findings will only be presented for NU272. Like NU243, the new mutants grew normally in BYE broth, were unable to secrete type II enzymatic activities (Table 2), and lacked protease and lipolytic activities on casein and egg yolk agar, a phenotype complemented by *pilD*-containing pMD1 (data not shown). They also had a severe growth defect in *H. vermiformis* that pMD1 could fully complement (Fig. 1A). However, unlike NU243, the new mutants had a modest growth defect in U937 cells but one that was complemented fully by pMD1 (Fig. 1B) and was comparable to that of the type II secretion (*lspG*) mutant NU259 (Fig. 1C). Finally, the new mutants' colonies differed from that of wild type and NU243 but were identical to that of the type II secretion mutants (75), and when pMD1 was introduced into NU272, normal colony morphology was restored (data not shown).

In sum, the observation of multiple *pilD* mutants and their complemented derivatives permits four conclusions. First, *L. pneumophila pilD* is required for the secretion of multiple enzymatic activities. Second, it promotes *L. pneumophila* intracellular growth in both amoebae and U937 cells, with its importance being most evident in protozoan hosts. Third, in
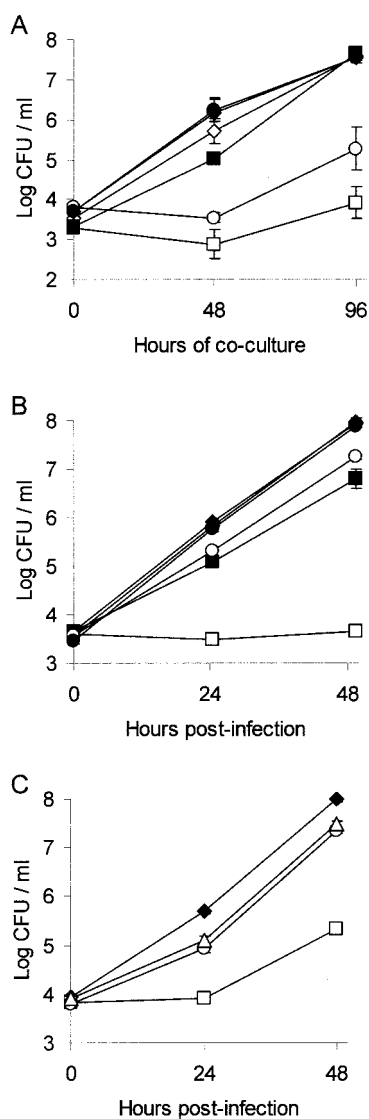
FIG. 1. Intracellular infection of *H. vermiformis* amoebae and U937 cell macrophages by *L. pneumophila pilD* mutants and their complemented derivatives. (A) Wells containing *H. vermiformis* were inoculated at a multiplicity of infection (MOI) of 0.1 with strains 130b(pMMB2002) (◇), 130b(pMD1) (◆), NU243(pMMB2002) (□), NU243(pMD1) (■), NU272(pMMB2002) (○), and NU272 (pMD1) (●), and then the numbers of bacteria in each well were quantified at various times postinoculation by plating on BCYE agar. Results are the means and standard deviations (error bars) of four wells and are representative of two independent experiments. In a third experiment, NU243(pMD1) and NU272(pMD1) also showed full complementation at 72 h postinoculation. (B) U937 cells were infected at an MOI of 0.1 with the same strains indicated above. At various times, the monolayers were lysed, and the total number of CFU in each well was determined. Results are the means and standard deviations (error bars) of triplicate wells and are representative of two independent experiments. The differences in recovery between 130b(pMD1) and both NU243(pMD1) and NU272(pMMB2002) were significant at 24 and 48 h (Student's *t* test, *P* < 0.005). (C) Macrophages were infected at an MOI of 0.1 with wild-type 130b (◆), Km^r mutant NU243 (□), Gm^r *pilD* mutant NU272 (○), and *lspG* mutant NU259 (△), and then at various times, the total number of CFU in each well was determined. Results are the means and standard deviations (error bars) of triplicate wells and are representative of three independent experiments. At 24 and 48 h postinoculation, significant differences in recovery were obtained between 130b and NU243, 130b and NU259, 130b and NU272, and NU243 and NU272 (Student's *t* test, *P* < 0.001).

addition to its *pilD* mutation, NU243 has a secondary site mutation that also affects colony morphology and intracellular growth in macrophages. Fourth, since the three new *pilD* mutants behaved identically to *lsp* mutants, type II secretion appears to be the main subset of the PilD-dependent activities that facilitate intracellular infection.

Clarification of the role of *lsp* genes in *L. pneumophila* protein secretion and intracellular infection. Given the equivalent behavior of the new *pilD* mutants and our previously isolated *lsp* mutants in macrophage (Fig. 1C) and protozoan (data not shown) infection, we next focused attention on further defining the structure and function of the *L. pneumophila* type II secretion system. Previously, the *lspDE* and *lspGHIJK* loci were shown to be involved in protein secretion (39, 75). As a next step toward identifying genes that promote *Legionella* type II secretion, we used allelic exchange to insert a Km^r cassette into *lspF* of strain 130b, generating mutants NU275 and NU276. The *lspF* gene maps 103 bp upstream of the *lspG* pseudopilin gene and is believed to encode an inner membrane component of the secretion apparatus (39, 75). NU275 and NU276 grew in BYE broth similarly to wild type (data not shown), indicating that *lspF*, like the previously studied *lsp* genes, is not generally required for extracellular replication. The color and morphology of the *lspF* mutants' colonies were identical to those of the *lspDE* and *lspG* mutants (data not shown). The *lspF* mutants were defective in the secretion of those protease, lipase, PLC, and LPLA activities previously linked to *pilD*, *lspDE*, and *lspG* (Table 2) (4, 75). In addition to a tartrate-sensitive acid phosphatase, there is a tartrate-resistant acid phosphatase in wild-type supernatants that is lacking in *pilD*-negative NU243 and NU272 (Table 2) (3). Thus, we analyzed the culture supernatants of *lsp* mutants for acid phosphatase activity in the presence and absence of tartrate. Enzyme activity was always diminished for *lspDE*, *lspF*, and *lspG* mutants (Table 2), suggesting that both of the acid phosphatase activities in *L. pneumophila* supernatants are dependent upon type II secretion. Prior examination of *pilD* mutant NU243 revealed a reduction in RNase activity on clear agar matrices impregnated with RNA (4). In order to quantitatively determine the role of *lsp* genes in RNase secretion, we incubated supernatants with RNA and then measured the release of nucleotides by monitoring increases in absorbance at 260 nm (Table 3). Wild-type supernatants caused an increase in absorbance in the presence but not in the absence of added RNA, confirming that *L. pneumophila* secretes an RNase. Activity in *lspDE* and *lspF* mutant supernatants was reduced by sixfold (Table 3). The introduction of *lspF*-containing pMF1 into NU275 restored all supernatant activities to wild-type levels (Tables 2 and 3), confirming that the loss of *lspF* is responsible for the reduced secretion by that strain. Thus, we can now conclude that, in *L. pneumophila*, the secretion of protease, acid phosphatase, lipase, PLA, PLC, LPLA, and RNase activities is dependent upon a type II secretion pathway.

The *L. pneumophila* zinc metalloprotease ProA appears to be one of the most abundant proteins secreted by the type II pathway (39, 52, 75), and it has been previously observed that the amount of phospholipase A and monoacylglycerol lipase activities in supernatants is reduced in the absence of the protease (30). Therefore, we tested whether other type II-secreted activities are influenced by the metalloprotease by

TABLE 2. Secreted activities of *L. pneumophila* strains

Strain	Genotype	Protease[a]	Activity (%) of supernatant[b]							
			Acid phosphatase	Tartrate-resistant acid phosphatase	PLC	PLA	LPLA	Lipase	*p*-NP caprylate hydrolase	*p*-NP palmitate hydrolase
130b	WT	+	100 ± 8	100 ± 4	100 ± 10	100 ± 7	100 ± 4	100 ± 4	100 ± 4	100 ± 4
NU243	*pilD*[c]	−	21 ± 1[d]	23 ± 2[d]	7 ± 4[d]	18 ± 1[d]	23 ± 1[d]	37 ± 1[d]	16 ± 1[d]	6 ± 1[d]
NU272	*pilD*	−	19 ± 0[d]	32 ± 0[d]	17 ± 7[d]	ND	ND	38 ± 2[d]	15 ± 2[d]	19 ± 6[d]
NU258	*lspDE*	−	16 ± 1[d]	33 ± 4[d]	9 ± 3[d]	20 ± 0[d]	23 ± 2[d]	43 ± 4[d]	15 ± 1[d]	11 ± 2[d]
NU259	*lspG*	−	20 ± 2[d]	27 ± 2[d]	31 ± 7[d]	ND	ND	ND	ND	ND
NU275	*lspF*	−	17 ± 3[d]	25 ± 1[d]	23 ± 14[d]	ND	45 ± 14[d]	34 ± 2[d]	13 ± 1[d]	27 ± 13[d]
130b(pMMB2002)		+	101 ± 5	102 ± 5	110 ± 11	ND	109 ± 11	93 ± 5	92 ± 9	86 ± 7
130b(pMF)		+	103 ± 9	92 ± 3	117 ± 14	ND	83 ± 13	108 ± 19	99 ± 4	101 ± 17
NU275(pMMB2002)		−	16 ± 1[d]	27 ± 0[d]	16 ± 8[d]	13 ± 0[d]	49 ± 9[d]	38 ± 3[d]	12 ± 1[d]	13 ± 5[d]
NU275(pMF)		+	107 ± 7	96 ± 4	125 ± 11	95 ± 13	88 ± 12	95 ± 12	91 ± 6	81 ± 5
AA200	*proA*	−	154 ± 7[d]	149 ± 7[d]	132 ± 11	ND	ND	60 ± 2[d]	54 ± 3[d]	25 ± 4[d]
BS100	*pilE*L	+	100 ± 7	99 ± 4	118 ± 3	111 ± 3	110 ± 3	ND	127 ± 3	136 ± 10
NU279	*pilQ*	+	94 ± 2	93 ± 1	99 ± 8	92 ± 8	97 ± 2	109 ± 7	95 ± 11	110 ± 16
NU280	*pspA*	+	106 ± 3	100 ± 7	105 ± 9	120 ± 9	103 ± 0	118 ± 11	121 ± 5	147 ± 16
NU283	*lspDE pilQ*	−	16 ± 0[d]	19 ± 0[d]	9 ± 5[d]	21 ± 1[d]	22 ± 1[d]	34 ± 3[d]	15 ± 0[d]	15 ± 6[d]
GG105	*dotA*	ND	109 ± 5	108 ± 1	123 ± 17	98 ± 12	99 ± 6	104 ± 6	99 ± 4	102 ± 10
GQ262	*dotDCB*	ND	130 ± 15	119 ± 2	ND	ND	91 ± 7	100 ± 7	104 ± 4	99 ± 4
GN142	*icmJB*	ND	122 ± 2	110 ± 4	146 ± 6	ND	100 ± 15	103 ± 5	104 ± 4	106 ± 5

[a] Presence (+) and absence (−) of clearing on casein agar; ND, not determined.

[b] Culture supernatants (*n* = 3) were incubated with appropriate substrates. Activities are expressed as percentage of wild-type activity (means ± standard deviations). ND, not determined.

[c] As described in the text, strain NU243 has an unmarked, second-site mutation(s) in addition to the marked mutation in *pilD*.

[d] Significant differences were obtained between enzymatic activities of the wild-type and the mutant strains (Student's *t* test, $P < 0.005$).

comparing supernatants from 130b and its isogenic *proA* mutant AA200. The mutant supernatants were reduced in their activity on monoacylglycerol, *p*-NP caprylate, and *p*-NP palmitate, suggesting that the protease may promote activation of lipolytic enzymes (Table 2). In contrast, AA200 exhibited a slight increase in secreted acid phosphatase and PLC activity (Table 2), indicating that the protease might partially degrade some secreted enzymes.

Previously, *lspDE*, *lspG*, and *lspK* mutants of strain 130b were shown to be defective for growth in *Hartmannella* amoebae and in human macrophages and monocytes (70, 75) (Fig. 1C). Similarly, an *lspGH* mutant of strain Philadelphia-1 is impaired for growth in acanthamoebae (39). When assessed for its replication in *H. vermiformis* and U937 macrophages, the *lspF*-negative mutants exhibited a growth defect similar to that of the other secretion mutants (data not shown). This defect was fully complemented by *lspF*-containing pMF1 (Fig. 2), proving that *lspF* is required for intracellular infection.

TABLE 3. Ribonuclease activity[a] in *L. pneumophila* supernatants

Sample	Increase in A_{260} (mean ± SD)
BYE	0.013 ± 0.021
130b[b]	0.362 ± 0.040
lspDE mutant NU258	0.042 ± 0.023[c]
lspF mutant NU275	0.056 ± 0.019[c]
130b(pMMB2002)	0.418 ± 0.045
NU275(pMMB2002)	0.070 ± 0.055[c]
NU275(pMF1)	0.396 ± 0.058

[a] Filtered supernatants from cultures of the indicated bacterial strains or BYE medium were incubated with Baker's yeast RNA, and the release of nucleotides was measured by the increase in A_{260} over a 40-min period. Values are the means ± standard deviations from three samples.

[b] When RNA was omitted from the 130b sample reaction, the increase in absorbance was only 0.020 ± 0.049, a value that was not different from that obtained with the BYE negative control.

[c] The differences in activity between 130b and this mutant were significant (Student's *t* test, $P < 0.005$) and were observed in an additional experiment.

These data represent the first genetic proof for the importance of a type II secretion gene in *L. pneumophila* infection of both protozoan and human cells. The *Legionella* Lsp pathway remains the only type II secretion system shown to promote intracellular infection.

Given the novel functions associated with *L. pneumophila* type II secretion, we sought additional *lsp* genes by examining the developing *L. pneumophila* Philadelphia-1 genome database (http://genome3.cpmc.columbia.edu/~legion/). Thus, we performed a BLAST search (2) of the current database using as query sequences the proteins that are conserved in the type II secretory pathways of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Erwinia carotovora*. Besides the known *lspDE*, *lspF*, and *lspGHIJK*, we found two loci, designated *lspC* and *lspLM*, that encode proteins with greatest homology (49, 43, and 52% similarity) with *Aeromonas hydrophila* ExeC, *Erwinia chrysanthemi* OutL, and *A. hydrophila* ExeM, respectively (42, 46, 53). Thus, *L. pneumophila* has analogs of most of the genes that have been implicated in type II secretion in other bacteria (78, 79).

The role of Tfp assembly genes in *L. pneumophila* secretion and intracellular infection. For several reasons, we wished to explore whether Tfp assembly genes also have a role in *L. pneumophila* secretion. First, there is similarity between proteins involved in Tfp assembly and those involved in type II secretion; e.g., in *P. aeruginosa*, the outer membrane PilQ secretin required for piliation is related to the XcpQ secretin involved in secretion, and the PilE, PilVWX, and FimT pseudopilins that help assemble pili are akin to XcpTUVW secretion pseudopilins (67, 76). Second, in enteropathogenic *E. coli*, the accumulation of ≥11 proteins in culture supernatants depends on the Tfp secretin BfpB (81). Third, in *Vibrio cholerae*, Tfp assembly genes, including the *tcpC* secretin gene, control the export of a protein that is not necessary for piliation but appears essential for intestinal colonization (49). Since our
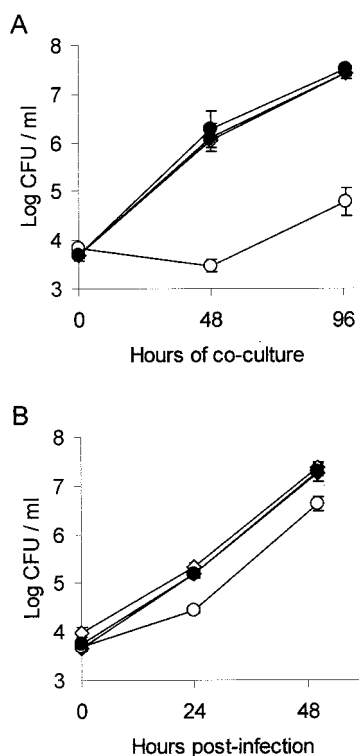
FIG. 2. Intracellular infection of *H. vermiformis* amoebae and U937 cell macrophages with an *L. pneumophila lspF* mutant and its complemented derivative. Amoebae (A) and U937 cells (B) were inoculated with strains 130b(pMMB2002) (◊), 130b(pMF1) (♦), NU275 (pMMB2002) (○) and NU275(pMF1) (●) at a multiplicity of infection of 0.1, and then the numbers of bacteria in each well were determined at various times postinoculation. For both panels, results are the means and standard deviations (error bars) of triplicate wells and are representative of two independent experiments. (B) The differences in CFU recovery between 130b(pMMB2002)- versus NU275(pMMB2002)-infected monolayers were significant at both 24 and 48 h postinoculation (Student's *t* test, $P < 0.005$).

TABLE 4. Natural transformation frequency of
L. pneumophila strains

Strain	Transformation frequency (mean $\pm$ SD)[a]
Wild-type 130b	$(8.8 \pm 3.2) \times 10^{-6}$
lspG mutant NU259	$(3.1 \pm 0.2) \times 10^{-6}$
pilE$_L$ mutant BS100	$(6.3 \pm 0.4) \times 10^{-9}$
pilQ mutant NU279	$(3.1 \pm 5.3) \times 10^{-9}$
pspA mutant NU280	$(3.3 \pm 1.7) \times 10^{-9}$

[a] Following incubation with a 5-μg/ml concentration of either pGD::Gm (see Materials and Methods) or pVA14-1 (5) at 30°C for 20 h, the indicated bacterial suspensions in BYE broth were plated on BCYE agar with the appropriate antibiotics. Values are the means $\pm$ standard deviations from three samples. The data presented are representative of at least two independent experiments.

pilQ mutants (NU277 and NU278), one Gm^r *pilQ* mutant (NU279), two Km^r *pspA* mutants (NU280 and NU281), and one Gm^r *pspA* mutant (NU282) were derived. As do Tfp mutants of other gram-negative bacteria (32, 33, 37, 44, 48, 95), the *pilQ* and *pspA* mutants were defective for natural transformation (Table 4). Indeed, they were as defective as the *L. pneumophila pilE*$_L$ mutant. An *lspG* mutant exhibited normal competence (Table 4), indicating that genes involved in type II protein secretion do not influence transformation. All of the new mutants grew in BYE broth similarly to wild type (data not shown), indicating that *pilQ* and *pspA* are not required for extracellular replication. When grown on BCYE agar, they appeared as typical colonies (data not shown). All further experiments were done with multiple *pilQ* and *pspA* mutants with similar results; however, for simplicity, findings will only be presented for one *pilQ* mutant (i.e., NU279) and one *pspA* mutant (i.e., NU280).

To test whether *pilQ* or *pspA* play a role in type II protein secretion, NU279 and NU280 were grown in BYE broth to late exponential phase, and then filtered supernatants were assayed as described previously (4, 75). Both strains had wild-type levels of activities (Table 2), indicating that *pilQ* and *pspA* do not have an essential role in the secretion of known activities. We also extended our previous observations (75) indicating that the pilin gene *pilE*$_L$ is not required for the secretion of known type II exoproducts (Table 2). Finally, we explored the possibility that the Tfp secretin and the type II secretory secretin have redundant roles in secretion. Thus, we used allelic exchange to introduce the *pilQ* mutation into an *lspDE* mutant. Three double mutants (NU283, NU284, and NU285) were obtained and found to behave similarly. Culture supernatants of these mutants had levels of activity that were comparable to those of the type II secretion mutants (Table 2), confirming that Tfp assembly genes are not involved in *L. pneumophila* type II secretion.

Upon coculture with *H. vermiformis*, the Tfp mutants grew comparably to wild type, indicating that neither *pilQ* nor *pspA* is required for infection of amoebal hosts (Fig. 3A and B). When U937 cells were infected, the mutants continued to behave like wild type (Fig. 3C and D). Finally, in both protozoa and macrophages, the *lspDE pilQ* double mutants had the same replication defect as the *lspDE*-negative strain (Fig. 3A and C). These results indicate that *pilQ*, *pspA*, and, by extension, the Tfp assembly apparatus, are not required for *L. pneumophila* intracellular infection in vitro.

previous work showed that the *pilE*$_L$ gene encoding *L. pneumophila* pilin is not required for secretion of known type II exoproteins (75), we focused the present effort on Tfp secretins and pseudopilins.

Sequencing near the cloned *L. pneumophila aroB* gene (23) revealed an incomplete open reading frame (ORF) predicted to encode a protein with homology to the PilQ secretin (P. Edelstein, personal communication). Following a BLAST search (2) of the *L. pneumophila* genome database, we confirmed the identity of that ORF by cloning and completely sequencing the gene from strain 130b. The *L. pneumophila pilQ* gene encoded a 77-kDa protein with 36% identity and 56% similarity to *P. aeruginosa* PilQ. BLAST searches of the *L. pneumophila* database also found an ORF encoding a putative 18-kDa pseudopilin, based upon the presence of a conserved cleavage and methylation site (underlined) in its N-terminal sequence (MRLQLMKI<u>TGFTLET</u> [PROSITE PS00409]). We designated this ORF *pspA* for pseudopilin gene A. In order to examine the function of *Legionella* PilQ and PspA, we used allelic exchange to isolate 130b mutants containing an antibiotic resistance gene inserted into either *pilQ* or *pspA*. Two Km^r
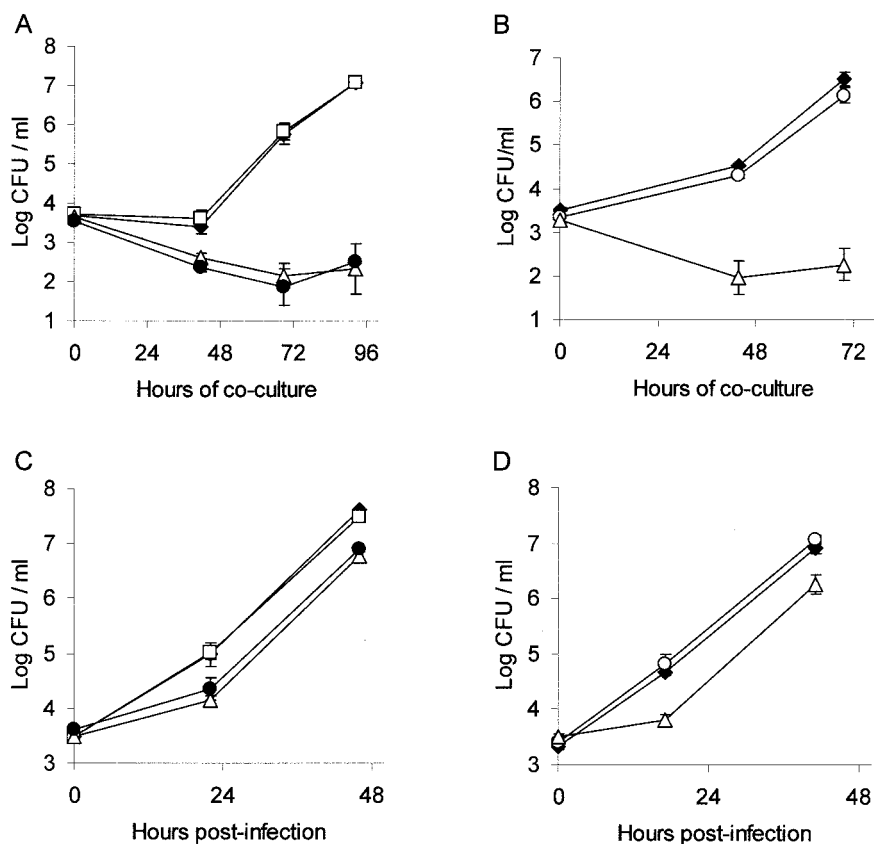
FIG. 3. Intracellular infection of *H. vermiformis* amoebae and U937 cell macrophages by *L. pneumophila pilQ*, *pspA*, and *lsp* mutants. Amoebae (A and B) and U937 cells (C and D) were infected at a multiplicity of infection of 0.1 with various strains, and then the numbers of bacteria in each well were determined at various times postinoculation. (A and C) Infection profiles for strains 130b (♦), *pilQ* mutant NU279 (□), *lspDE* mutant NU258 (●), and *lspDE pilQ* double mutant NU283 (△). (B and D) Infectivities of strains 130b (♦), *pspA* mutant NU280 (○), and *lspG* mutant NU259 (△). The results are the means and standard deviations (error bars) of triplicate wells and are representative of two independent experiments. In panels C and D, significant differences in recovery were obtained between 130b and NU258, 130b and NU283, and 130b and NU259 (Student's *t* test, $P < 0.005$).

The role of *dot/icm* genes in *L. pneumophila* type II protein secretion. In addition to its type II secretion system, *L. pneumophila* possesses a type IV secretion system that is involved in intracellular infection (43, 56, 65, 66, 83, 97–99). To test whether this secretion apparatus influences type II protein secretion, we analyzed the enzymatic activities in culture supernatants of strains GG105, GQ262, and GN142, which contain an insertion mutation in *dotA*, *dotDCB*, and *icmJB*, respectively (34). The three mutants secreted normal levels of acid phosphatase, PLC, PLA, LPLA, and lipase (Table 2), indicating that *dot/icm* genes are not required for *Legionella* type II secretion.

Virulence of *L. pneumophila* secretion and piliation mutants in A/J mice. To determine whether PilD, Tfp, and type II secretion are important in vivo, we tested the wild type and isogenic mutants in the A/J mouse model of legionellosis (1, 16, 19, 74). As was done before (73, 74), we performed an in vivo competition assay in which a sublethal dose of bacteria (i.e., 10^5 CFU) containing a 1:1 mixture of 130b and a mutant are inoculated by intratracheal injection. In such assays, increases in the ratio of wild type to mutant recovered from infected lungs reflect a growth and/or survival defect for the

mutant. At 24 h postinoculation, the ratio of 130b to the *lspDE* mutant NU258 and that of 130b to the *lspF* mutant NU275 increased to 3.2 and 4.2, respectively (Fig. 4A). By 72 h, the ratios of wild type to *lsp* mutant had further increased to 11 to 14 (Fig. 4B), showing that type II secretion mutants have a competitive disadvantage in vivo. In contrast, the ratios of 130b to the *pilE_L* mutant BS100 and to the *pilQ* mutant NU279 did not change significantly at 24 h postinoculation and increased only slightly at 72 h postinoculation (Fig. 4A and B), indicating that *L. pneumophila* Tfp do not confer a growth advantage in mouse lungs. The *pilD* mutant NU272 behaved as the type II secretion mutants did (Fig. 4A and B), supporting the belief that type II secretion promotes *L. pneumophila* replication in the A/J lung. Incidentally, strain NU243, which contains a second site mutation in addition to an insertion in *pilD*, was very defective in the competition assay (Fig. 5). Indeed, the ratio of 130b to NU243 increased to 38 and 1,266 at 24 and 72 h postinoculation, respectively, indicating that the gene(s) affected by the secondary mutation plays a crucial role for in vivo infections. To confirm the importance of type II protein secretion in the mouse lung, we monitored the survival and replication of 130b and NU275 following intratracheal inocu-
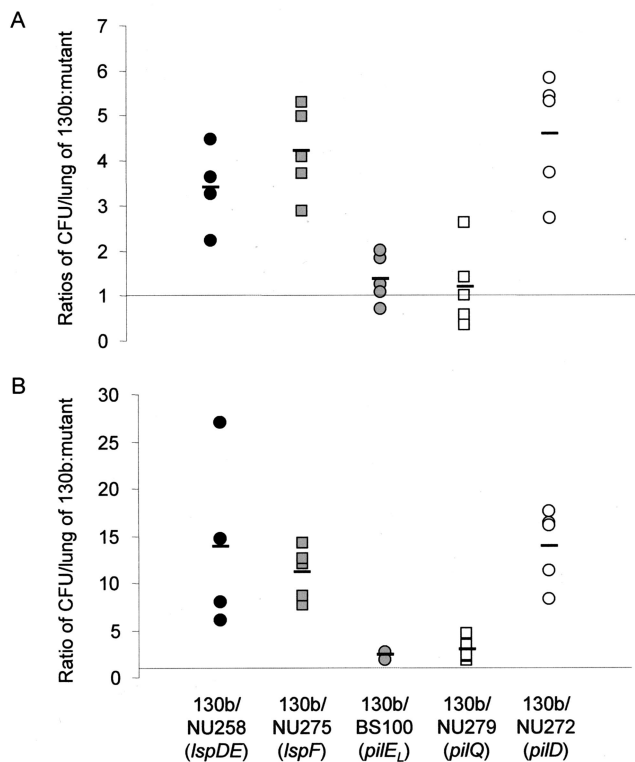
FIG. 4. In vivo competition between wild-type *L. pneumophila* and mutants lacking type II protein secretion and Tfp. A mixture of 130b and mutant were introduced into the lungs of A/J mice by intratracheal inoculation. At 1 day (A) and 3 days (B) postinoculation, the ratio of 130b to mutant in infected lungs was determined and normalized to the ratio of wild type to mutant in the inoculum. Data are representative of actual values obtained per mouse ($n = 4$ to 6), and the solid bars represent the mean values. Strains tested in competition with wild type were from left to right: *lspDE* mutant NU258 (black circles), *lspF* mutant NU275 (gray squares), *pilE_L* mutant BS100 (gray circles), *pilQ* mutant NU279 (white squares), and *pilD* mutant NU272 (white circles). For the trials done with NU275, BS100, NU279, and NU272, significant differences were obtained between ratios observed at day 1 and day 3 (Student's *t* test, $P < 0.05$).

FIG. 5. In vivo competition between wild-type *L. pneumophila* and mutant NU243. A mixture of 130b and the Km[r] mutant NU243 was introduced into the lungs of A/J mice by intratracheal inoculation. At 1 and 3 days postinoculation, the ratio of 130b to mutant in infected lungs was determined. Data are representative of the actual values obtained per mouse ($n = 4$), and a solid bar represents the mean value. The 130b to mutant ratio increased significantly between days 1 and 3 (Student's *t* test, $P < 0.05$).

the *L. pneumophila* type II secretion is operating during in vivo growth, we investigated whether A/J mice infected with wild-type 130b developed antibodies that reacted with type II exoproteins. Sera were obtained from two infected animals 3 weeks after their inoculation with a sublethal dose of 10^6 CFU and were incubated with blots containing concentrated culture supernatants from strain 130b and its *lspF*-negative derivative NU275, as well as ATCC 33155, a representative of *L. pneumophila* serogroup 3 (Fig. 7). The sera reacted with at least five proteins in the 130b supernatants whose sizes were ca. 72, 65, 55, 42, and 27 kDa. The 65- and 42-kDa proteins, as well as a 74-kDa species, were detected in the supernatants of strain 33155. All reactive proteins were absent in the supernatant of the *lspF* mutant. Thus, *L. pneumophila*-infected mice develop antibodies against multiple type II-secreted proteins, and therefore, we conclude that *Legionella* type II secretion is functioning during bacterial infection of the mammalian lung.

lation into separate groups of mice. Whereas the wild type multiplied over the first 48 h postinoculation, as previously observed (16, 20), the *lspF* mutant exhibited a 17-fold decrease in CFU (Fig. 6). At 4 days postinoculation, there was still a fivefold difference in CFU recovery between the two strains (Fig. 6). In the A/J mouse model, doses of *L. pneumophila* 130b greater than 10^7 CFU result in death within 48 h, with this acute lethality being ascribed to a bacterial toxin that is distinct from endotoxin (1, 16). Thus, we challenged groups of mice with 10^9 CFU of 130b, the *pilE_L* mutant, the *lspF* mutant NU275, or the *lspDE* mutant NU258. All mice died acutely and within the same 48-h time frame. Thus, Tfp and type II secretion are not required for rapid killing by high-dose inoculation. However, the results of both the competition assays and the clearance study indicate that type II secretion is critical for the multiplication and survival of *L. pneumophila* in mammalian lungs.

Detection of antibodies against type II-secreted proteins in the antisera of *L. pneumophila*-infected mice. To confirm that
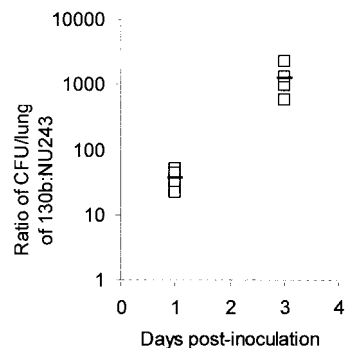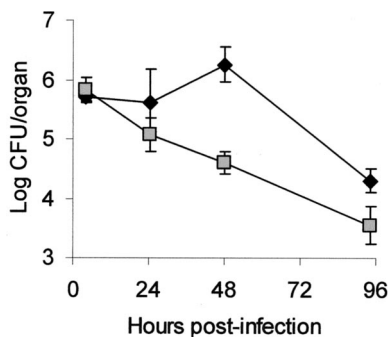

FIG. 6. Growth and survival of wild-type and *lspF* mutant *L. pneumophila* in the lungs of infected A/J mice. Mice were intratracheally inoculated with equal numbers of either 130b (black diamonds) or *lspF* mutant NU275 (gray squares), and then at various time points, the CFU in infected lungs were determined by plating. Data are the means and standard deviations (error bars) obtained for four to six infected animals. Significant differences were obtained between the CFU recovered from mice infected with 130b and those infected with NU275, at 48 and 96 h postinoculation (Student's *t* test, $P < 0.01$).
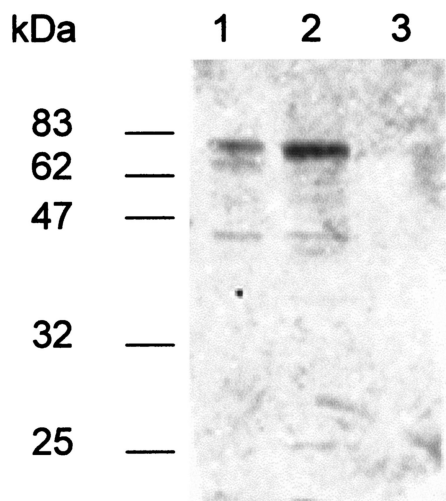
FIG. 7. Immunodetection of *L. pneumophila* type II-secreted proteins by infected mouse sera. Concentrated supernatants from cultures of *L. pneumophila* strains 33155 (lane 1) and 130b (lane 2) and *lspF* mutant NU275 (lane 3) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and reacted with the diluted serum of a 130b-infected A/J mouse. Similar results were obtained using the serum of a second infected mouse.

Distribution of *lsp* genes in *L. pneumophila* serogroups and other *Legionella* spp. Given the importance of type II secretion for serogroup 1 strains of *L. pneumophila*, we sought to evaluate the distribution of *lsp* genes in other *L. pneumophila* serogroups as well as other *Legionella* spp. Thus, Southern hybridizations were performed using probes derived from *lspC*, *lspD*, *lspFG*, and *lspLM* of 130b. Under high-stringency conditions (10% base pair mismatch allowed), sequences homologous to all of the probes were observed in all *L. pneumophila* strains tested, i.e., representatives of serogroups 1 to 8, 13, and 14 (Table 1). Under low-stringency conditions (30% bp mismatch allowed), sequences homologous to all of the probes were detected in all *Legionella* species tested, i.e., *L. cherrii*, *L. feeleii*, *L. gormanii*, *L. longbeachae*, *L. micdadei*, *L. parisiensis*, and *L. spiritensis* (Table 1). Thus, type II secretion genes seem to be distributed throughout the genus *Legionella*. With the exception of *L. cherrii*, the *Legionella* spp. were tested for protease and lipolytic activities on casein and egg yolk agar (data not shown). Most strains produced both types of activities. However, the *L. micdadei* strains failed to show any activity, even though they contained the *lsp* genes. The *L. spiritensis* strain did not produce clearing on the egg yolk agar, suggesting that it does not secrete a PLA. Finally, the *L. feeleii* isolate did not produce iridescence on the egg yolk agar, indicating that it lacks a secreted lipase. Taken together, these data indicate that a type II secretion system is present and operative in most type of legionellae.

DISCUSSION

Using thorough complementation analysis, we have now formally established that the Lsp type II secretion system promotes infection of amoebae and macrophages, the two host cells most relevant to *L. pneumophila* ecology and pathogenesis. To our knowledge, this *L. pneumophila* pathway remains the only type II secretion system to be implicated in intracellular infection. In a previous study, we had demonstrated that protease, acid phosphatase, PLC (*p*-NP phosphorylcholine hydrolase), multiple lipase, PLA, and LPLA activities were lacking in the supernatants of *lspG* and *lspDE* mutants (75). With additional mutant constructions and a corresponding complementation analysis, we have confirmed that the *L. pneumophila* type II secretion system governs the secretion of all of these activities as well as tartrate-resistant and tartrate-sensitive acid phosphatase and an RNase. Thus, *L. pneumophila lsp* genes promote the secretion of at least eight extracellular enzymatic activities, a workload that matches if not exceeds that of other known type II systems (79). SDS-PAGE analysis of bacterial supernatants confirms that the *L. pneumophila* type II system controls the secretion of multiple protein species, some of which may facilitate yet-to-be-defined enzymatic activities (39, 52). *L. pneumophila* genes encoding protease (*proA*), tartrate-sensitive acid phosphatase (*map*), lipase (*lipA* and *lipB*), LPLA (*plaA*), and PLC (*plcA*) activities have been identified (3, 5, 30, 62, 90), although the corresponding mutants are not impaired in intracellular infection. Thus, it will be important to continue to pursue the genes encoding the PLA, RNase, tartrate-resistant acid phosphatase, as well as the lipase and PLC activities not associated with *lipA*, *lipB*, or *plcA*. Besides its influence on the release of proteins and enzymes into supernatants, the Lsp system had appeared to affect the morphology of *L. pneumophila* colonies (75). This supposition has also now been confirmed, suggesting that the *L. pneumophila* type II system may also mediate the localization of cell envelope proteins. It is possible that the type II-dependent factors that promote intracellular infection include outer membrane proteins.

In addition to *pilD*, *lspDE*, and *lspFGHIJK*, we have now identified in the *L. pneumophila* genome three more genes, *lspC*, *lspL*, and *lspM*, which encode conserved components of type II secretion pathways. Although components associated with some type II systems (e.g., homologs of *V. cholerae* EpsA, EpsB, and EpsN) have not yet been identified in the *L. pneumophila* genome, the *Legionella* organism does have a set of genes that matches the *xcp* genes of *P. aeruginosa* (78, 79). Unlike *P. aeruginosa* (7), the Philadelphia-1 database does not reveal a second type II secretion system. In *L. pneumophila*, the 12 *lsp* genes are located in five loci scattered throughout the chromosome, a feature rarely seen in other bacteria, where most type II secretion genes are clustered in one or two loci (76, 78, 79). Further studies are needed in order to establish whether the additional *lsp* genes are required for protein secretion.

Stone and Abu Kwaik had established that the *L. pneumophila pilE_L* gene is required for Tfp-mediated natural transformation (85). We have now identified two additional genes, *pilQ* and *pspA*, that are necessary for DNA transformation. No cross talk between Tfp and type II protein secretion was evident, since disruption of *lspG* did not abolish transformation and loss of *pilE_L*, *pilQ*, or *pspA* and did not influence the secretion of enzymatic activities. In extracellular gram-negative pathogens, Tfp are often important virulence factors that promote adherence to and colonization of the host (11, 41, 91). Although an *L. pneumophila pilE_L*-negative strain exhibits a 50% reduction in adherence to mammalian and protozoan

cells (84), it replicates like the wild type within these cells in vitro (75, 84). Similarly, the *pilQ* and *pspA* mutants showed no replication defect in U937 cells or *H. vermiformis*. When tested in vivo, strains lacking *pilE*$_L$ and *pilQ* strains were only slightly outcompeted by the wild type in A/J mouse lungs, suggesting that Tfp are not a significant virulence determinant in the mammalian host. However, it is possible that Tfp have a role that cannot be easily monitored with the A/J mouse model. Nonetheless, Tfp appear to be quite significant for *L. pneumophila* persistence in the environment; i.e., pilus mutants are defective for colonization of aquatic biofilms (C. E. Lucas, E. Brown, T. S. Forster, R. Murga, R. M. Donlan, N. P. Cianciotto, Y. Abu Kwaik, and B. S. Fields, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol 2002, abstr. Q-258, p. 422, 2002).

In contrast to the Tfp mutants, type II secretion mutants exhibited a severe defect in the competition assay in A/J mouse lungs. That the *pilD* mutant NU272 had a defect similar to that of the *lsp* mutants implies that the importance of the prepilin peptidase in vivo is due to its role in promoting protein secretion. By monitoring the kinetics of bacterial growth in vivo, we firmly established that the *lspF* mutant is impaired for replication and survival in the animal lungs. Thus, we strongly believe that type II secretion genes have a significant role in *L. pneumophila* pathogenesis. The in vivo relevance of type II secretion was also evidenced by the presence of antibodies specific to Lsp-dependent proteins in the sera of infected mice. This finding is compatible with a previous study in which the ProA metalloprotease was shown to be expressed in the lungs of guinea pigs infected with *L. pneumophila* (21). Moreover, individuals diagnosed with Legionnaires' disease have antibodies specific to the protease, indicating that factors secreted by the type II pathway are also expressed during human infection (47, 72).

The reduced survival of the *lsp* mutants in the A/J mouse lung is likely due, at least in part, to diminished growth in alveolar macrophages, since the mutants are also defective for in vitro infection of U937 cells. However, the number of *lspF* mutant CFU did not increase within the mouse lungs, whereas they did, albeit not optimally, in vitro. Several factors may be responsible for this difference. First, the mouse alveolar macrophages and the U937 cell macrophages may provide different responses to *L. pneumophila* infection. On the one hand, mouse and human macrophages may have significant differences in permissiveness, and on the other, resident macrophages may be much more restrictive than a macrophage cell line; e.g., an *lspK* mutant exhibits a modest defect in U937 cells but is unable to replicate in human blood monocyte-derived macrophages (70). Second, since *L. pneumophila* can also infect lung epithelial cells (35, 61), it is possible that type II secretion is needed for effective intracellular spread beyond the alveolar macrophage. Third, the *lsp* mutants may be defective for extracellular processes that are operative in the lungs. In attempting to reconcile in vitro and in vivo findings, our experience with the *lsp* mutants is somewhat reminiscent of observations made with protease mutants, i.e., a *proA* (*msp*) mutant, while not defective for in vitro intracellular infection (62, 90), elicits less necrosis and more macrophage infiltration in the lungs of guinea pigs inoculated by the intratracheal route (62). In light of our results with the *lspF* mutant, all type II effector mutants should be examined in the animal model of disease, rather than just pursuing those that display intracellular growth defects in vitro.

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