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

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Received 2 September 2003/Returned for modification 3 October 2003/Accepted 7 October 2003

**Legionella pneumophila**, the gram-negative agent of Legionnaires’ disease, possesses type IV pilis 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 on 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 pilID 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 pilis (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 lspGHIIK, which encodes the pseudopilins LspGHIIK (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 (pilE1) 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 castellani* 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 pilin 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. (Portions of this work were presented at the 102nd General Meeting of the American Society for Microbiology [O. Rossier...)}
TABLE 1. Wild-type Legionella strains used in this study

<table>
<thead>
<tr>
<th>Species and strain*</th>
<th>Serogroup</th>
<th>Source</th>
<th>Reference</th>
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<td>BAA-74</td>
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<td>L. parisiensis 35299</td>
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<td>L. spiritensis 35249</td>
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</table>

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

MATERIALS AND METHODS

Bacterial strains and media. L. pneumophila serogroup 1 strain 130b (ATCC BAA-74), which serves as the wild-type strain in this study, and its derivatives NU243, NU258, NU259, BS100, and AA200, which contain stable insertions of kanamycin resistance (Km') gene in pilD, lspDE, pilG, pilE2, and proA, respectively, were described previously (25, 52, 67, 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 600 nm (OD600). Escherichia coli strains NovaBlue (Novagen, Madison, Wis.) and DH5a (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-1 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, ORSppB (5'-CCTCCAGGATCTGGCGGTA), and ORSppH (5'-GCTGTTTGTTAG AACCAAGCGGTT) yielding a 1,972-bp fragment containing lspF and lpsG, OR33piQ (5'-CAAACCTGAGCTGACGATA) and OR34piQ (5'-GCCAG CAGGCGCTCTAAATA) yielded a 2,857-bp fragment encoding pilQ, and OR32ppA (5'-CATGAGGATCTGGCGGTT) and OR33ppA (5'-GCTGAA CCAGTCCTACC) yielded an 1,830-bp fragment containing pspA. Sequencing reactions were performed using 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-
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. 10^8 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 (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 abilities of strains to replicate and survive in mice lungs, mice (n = 4 or 5) were infected separately with 10^5 CFU of wild type or mutant strain, 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 detected with rabbit anti-*L. pneumophila* sera harvested 3 weeks after inoculation. Chemiluminescence Western blotting (Amersham Biosciences) and a charge-coupled device camera (Chemi Imager 5500; Alpha Innotech, San Southern Lab, Calif.) was used to estimate 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 NU243, NU272, NU273, and NU274. Each contained a Gm cassette inserted into the same site in pilD as the Km 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,
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' 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, lspD, 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, 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

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 triplicate 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' mutant NU243 (□), Gm' 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).
TABLE 2. Secreted activities of L. pneumophila strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Protease</th>
<th>Activity (% of supernatant)</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Acid phosphatase</td>
</tr>
<tr>
<td>130b</td>
<td>WT</td>
<td>+</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>NU243</td>
<td>pilD'</td>
<td>-</td>
<td>21 ± 1d</td>
</tr>
<tr>
<td>NU272</td>
<td>pilD</td>
<td>-</td>
<td>19 ± 1d</td>
</tr>
<tr>
<td>NU258</td>
<td>lspDE</td>
<td>-</td>
<td>31 ± 2d</td>
</tr>
<tr>
<td>NU259</td>
<td>lspG</td>
<td>-</td>
<td>20 ± 2d</td>
</tr>
<tr>
<td>NU275</td>
<td>lspF</td>
<td>-</td>
<td>17 ± 3d</td>
</tr>
<tr>
<td>130b(pMMB2002)</td>
<td>+</td>
<td>101 ± 5</td>
<td>102 ± 5</td>
</tr>
<tr>
<td>130b(pMF)</td>
<td>+</td>
<td>103 ± 9</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>NU275(pMMB2002)</td>
<td>+</td>
<td>16 ± 1d</td>
<td>27 ± 4d</td>
</tr>
<tr>
<td>AA200</td>
<td>proA</td>
<td>-</td>
<td>154 ± 7d</td>
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<tr>
<td>BS100</td>
<td>pilE</td>
<td>+</td>
<td>100 ± 7</td>
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<tr>
<td>NU279</td>
<td>pilQ</td>
<td>-</td>
<td>94 ± 2</td>
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<td>NU280</td>
<td>psaA</td>
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<td>106 ± 3</td>
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<td>16 ± 0d</td>
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<td>dotA</td>
<td>-</td>
<td>109 ± 5</td>
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<td>GGI262</td>
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<td>GN142</td>
<td>icmL</td>
<td>ND</td>
<td>122 ± 2</td>
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* 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* in L. pneumophila supernatants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Increase in A260 (mean ± SD)</th>
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<tr>
<td>130b</td>
<td>0.013 ± 0.021</td>
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<tr>
<td>NU258</td>
<td>0.362 ± 0.040</td>
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<td>NU259</td>
<td>0.042 ± 0.023</td>
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<tr>
<td>NU275</td>
<td>0.056 ± 0.019</td>
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<tr>
<td>NU275(pMMB2002)</td>
<td>0.418 ± 0.045</td>
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<tr>
<td>NU275(pMF1)</td>
<td>0.396 ± 0.058</td>
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</table>

* 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 A260 over a 40-min period. Values are the means ± standard deviations from three samples.

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 known 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 lspGHJK, 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 pilation is related to the XepQ secretin involved in secretion, and the PilE, PilVX, and FimT pseudopilins that help assemble pili are akin to XepTUUV 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 pilation but appears essential for intestinal colonization (49). Since our
previous work showed that the pilE<sub>1</sub> gene encoding <i>L. pneumophila</i> 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 <i>L. pneumophila</i> 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 <i>L. pneumophila</i> genome database, we confirmed the identity of that ORF by cloning and completely sequencing the gene from strain 130b. The <i>L. pneumophila</i> pilQ gene encoded a 77-kDa protein with 36% identity and 56% similarity to <i>P. aeruginosa</i> PilQ. BLAST searches of the <i>L. pneumophila</i> 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 (MRLQLMKTGFTLET [PROSITE PS00409]). We designated this ORF pspA for pseudopilin gene A. In order to examine the function of <i>Legionella</i> PilQ and PspA, we used allelic exchange to isolate 130b mutants containing an antibiotic resistance gene inserted into either pilQ or pspA. Two Km<sup>+</sup> pilQ mutants (NU277 and NU278), one Gm<sup>+</sup> pilQ mutant (NU279), two Km<sup>+</sup> pspA mutants (NU280 and NU281), and one Gm<sup>+</sup> 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 <i>L. pneumophila</i> pilE<sub>1</sub> 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<sub>1</sub> is not required for the secretion of known type II exoproteins (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 lspD 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 <i>L. pneumophila</i> type II secretion.

Upon coculture with <i>H. vermiformis</i>, 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 <i>L. pneumophila</i> intracellular infection in vitro.

### TABLE 4. Natural transformation frequency of <i>L. pneumophila</i> strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transformation frequency (mean ± SD)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>Wild-type 130b</td>
<td>(8.8 ± 3.2) × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>lspG mutant NU259</td>
<td>(3.1 ± 0.2) × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>pilE&lt;sub&gt;1&lt;/sub&gt; mutant BS100</td>
<td>(6.3 ± 0.4) × 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>pilQ mutant NU279</td>
<td>(3.1 ± 5.3) × 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>pspA mutant NU280</td>
<td>(3.3 ± 1.7) × 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> 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 ± standard deviations from three samples. The data presented are representative of at least two independent experiments.
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., $10^7$ 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 *pilEL* 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 4B), 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-
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 pilEL mutant, the lspF mutant NU275, or the pilD mutant NU272. 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 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.
Distribution of lsp genes in L. pneumophila serogroups and other Legionella spp. Given the importance of type II secretion for serogroup I 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 lspFGHJK, 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 pilEL 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 pilEL, 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 pilEL-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 tspA mutants showed no significant replication defect in U937 cells or H. vermiformis. When tested in vitro, strains lacking pilE\textsubscript{2} 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 peptidase 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 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.

ACKNOWLEDGMENTS

We are grateful to Paul Edelstein for sharing the partial sequence of pilQ; Alan Hauser for providing pX1918GT; and Youssef Abu Kwaik for providing strains BS100, GG105, QG262, and GN142. For technical assistance with the competition assays, we thank Kimberly Allard, Virginia Aragon, Antje Flieger, Joseph Garonski-Salerno, and Marianna Hartaney. We also thank Bethany Boardman for assistance with generating the lspF mutant. We are extremely grateful to past and present members of the laboratory for helpful discussions and comments.

This work was supported by NIH grant AI43987 awarded to N.P.C.

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