Secreted Enzymatic Activities of Wild-Type and *pilD*-Deficient *Legionella pneumophila*

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Legionella pneumophila*, the agent of Legionnaires’ disease, is an intracellular pathogen of protozoa and macrophages. Previously, we had determined that the *Legionella pilD* gene is involved in type IV pilus biogenesis, type II protein secretion, intracellular infection, and virulence. Since the loss of pili and a protease do not account for the infection defect exhibited by a *pilD*-deficient strain, we sought to define other secreted proteins absent in the mutant. Based upon the release of *p*-nitrophenol (pNP) from *p*-nitrophenyl phosphate, acid phosphatase activity was detected in wild-type but not in *pilD* mutant supernatants. Mutant supernatants also did not release either pNP from *p*-nitrophenyl caprylate and palmitate or free fatty acid from 1-monopalmitoylglycerol, suggesting that they lack a lipase-like activity. However, since wild-type samples failed to release free fatty acids from 1,2-dipalmitoylglycerol or to cleave a triglyceride derivative, this secreted activity should be viewed as an esterase-monoacylglycerol lipase. The mutant supernatants were defective for both release of free fatty acids from phosphorylcholine and degradation of RNA, indicating that *pilD*-negative bacteria lack a secreted phospholipase A (PLA) and nuclease. Finally, wild-type but not mutant supernatants liberated pNP from *p*-nitrophenylphosphorylcholine (*pNPPC*). Characterization of a new set of mutants defective for *pNPPC*-hydrolysis indicated that this wild-type activity is due to a novel enzyme, as opposed to a PLC or another known enzyme. Some, but not all, of these mutants were greatly impaired for intracellular infection, suggesting that a second regulator or processor of the *pNPPC* hydrolase is critical for *L. pneumophila* virulence.

*L. pneumophila* is the agent of Legionnaires’ disease, a potentially fatal form of pneumonia (76). *L. pneumophila*, a gram-negative inhabitant of fresh water, enters the respiratory tract following either the inhalation of contaminated aerosols generated by air conditioners and other devices or the aspiration of contaminated potable water (22, 38, 76). Once in the alveoli, the bacterium invades and replicates to high numbers within macrophages (32, 76). Ultimately, host cell death and lysis as well as bacterial degradative enzymes result in damage to lung tissue. We recently discovered that *L. pneumophila* possesses a gene (*pilD*) whose analogs, in other gram-negative bacteria, promote both pilus biogenesis and protein secretion (42). The inner membrane PilD-related proteins facilitate type IV pilus formation in two ways (15, 28, 35, 40, 43, 44, 51, 56, 77). First, they cleave the signal sequence from type IV prepilin and methylate the amino terminus of the resultant mature pilin (52, 71). Second, they cleave and methylate six prepilin-like proteins that, once processed, help form the shaft through which pilin is assembled into a pilus (1, 2, 23, 61). The PilD peptidases also facilitate the passage of proteins through the main terminal branch of the general secretory pathway, a form of protein export that is commonly known as type II secretion (9, 23, 25, 45, 56, 60, 70). PilD and its analogs promote secretion by processing another set of pseudopilins, which constitute part of the type II secretion apparatus (8, 10, 53). Factors whose secretion is PilD dependent include the aerolysin and protease of *Aeromonas hydrophila*, the exotoxin A, lipase, and phospholipase C (PLC) of *Pseudomonas aeruginosa*, and the cholera toxin of *Vibrio cholerae* (24, 44, 56, 58, 63, 70).

Given the pivotal role that PilD-like proteins can have in virulence, we recently began an analysis of an *L. pneumophila pilD* mutant (41). It was first observed that the mutant was nonpiliated, confirming a role for PilD in the biogenesis of *L. pneumophila* type IV pili (41, 42, 69). Examination of the mutant’s supernatants revealed the loss of several protein species, confirming that PilD is required for *Legionella* protein secretion (41). This latter observation indicated that *L. pneumophila* possesses a type II secretion system, a hypothesis that was later confirmed by the identification of genes encoding components of the secretion apparatus, including the pseudopilins (29). Most importantly, the *pilD* mutant was defective for intracellular infection and virulence (41). The strain was ca. 1,000-fold impaired in its ability to infect a human macrophage (U937) cell line and a *Hartmannella* strain of fresh water amoebae. In addition, it did not replicate within the lungs of guinea pigs, displaying a 50% lethal dose that was at least 100-fold greater than wild type. Since the *Legionella* type IV pilus is not critical for intracellular growth (69), we reasoned that the mutant’s attenuation was due to the loss of PilD-dependent, secreted proteins (41). However, the only exoprotein known to be lacking in the PilD-negative strain was a metalloprotease, an enzyme that is not required for intracellular infection and has only a minor role in pulmonary disease (41, 48, 72). Shortly after its discovery, *L. pneumophila* was found to exhibit phosphatase, lipase, nuclease, and PLC-like activities (7, 16, 49, 50, 73). Because some of these activities are linked to type II secretion in other bacteria, they served as a starting point in our search for new PilD-dependent exoproteins. Here, we report that the *L. pneumophila pilD* mutant is defective for the...
secretion of an acid phosphatase, monoucglycerol lipase, RNase, PLA, and p-nitrophosphorylcholine (pNPC)-hydrolyase.

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MATERIALS AND METHODS

Bacterial strains and media. The wild-type L. pneumophila used in this study was serogroup 1 strain 130b (Wadsworth), a virulent clinical isolate (21). Mutant NU243, a direct derivative of mini-Tn10-resistant insertion in the Legionella pilD gene (41). The strains NU243 (pMRL13), NU243 (pBBR1MCS), and 130b (pBBR1MCS) that were used for trans-complementation analysis were also previously described (41). To ultimately screen for mutants deficient in specific secretion activities, strain 130b was mutagenized with mini-Tn10pho3, as previously described (46, 57). After mini-Tn10 mutagenesis, at least 96% of L. pneumophila mutants contain single DNA insertions (57). Bacteria were generally cultured on buffered charcoal yeast extract agar for 3 days at 37°C (18). However, to facilitate the detection of certain lytic enzymes, legionellae were also cultured on buffered starch yeast extract agar containing 5% egg yolk (7, 73). Finally, in preparation for assessing secreted enzymatic activities, bacteria were grown in buffered yeast extract (BYE) broth, the standard liquid medium for culturing L. pneumophila. Growth was assayed by measuring the optical density of the culture at 660 nm (OD660) (41).

Preparation of supernatants and cell lysates. Supernatants from L. pneumophila cultures to be secreted enzymes were prepared in the following manner. First, bacteria from buffered charcoal yeast extract agar were suspended in 25 ml of BYE broth, contained within 125-ml flasks, at an OD660 of approx- imately 0.2. Then, after overnight growth at 37°C, the broth-adapted legionellae were subcultured into 25 ml of fresh medium, and the cultures were returned to the 37°C shaking incubator. At various times postinoculation, a 1.5-ml portion of the culture was removed and centrifuged for 5 min at 12,000 × g at 4°C. Finally, after careful removal from the centrifuge tube, the supernatant was sterilized by passage through a 0.2-μm-pore-size filter and either assayed immediately or stored at −20°C. Frozen samples retained all activities tested for up to at least 6 months. In order to detect some activities, ca. 200 ml of chilled supernatants were concentrated 40-fold by passage through Millipore YM10 ultrafiltration cells (41). To assay for cell-associated activities, the pellet obtained from centrifugation of the culture sample was lysed by resuspension in 300 μl of phosphate-buffered saline containing 0.1% Triton X-100 and 0.2 mg of lysozyme per ml. After repeated passage through a 26-gauge needle, the lysate was tested immediately or stored at −20°C.

Enzymatic assays. To detect phosphatase activity, samples were assayed, as is routinely done, for their ability to release p-nitrophenol (pNP) from p-nitrophenylphosphate (Sigma Chemical, St. Louis, Mo.) (24, 37, 67, 70, 73). Briefly, 10 μl of sample was added to 0.1 ml of a 50 mM p-NPP solution with acid buffer containing 10 μl of p-nitrophenylphosphate, and then after for 1 h at 37°C. The release of pNP was monitored spectrophotometrically at 405 nm. Three strains of L. pneumophila were used in this study: strain 130b (21), NU243 (41), and its wild-type parent 130b (21). For all of these strains, 50% infective doses (ID50) were determined after a 3-day incubation period (41). In a number of studies, the ID50 value has proven to be a reliable predictor of a given strain’s intracellular growth capacity (12, 30, 54). To quantitate intracellular growth, monolayers containing 105 macrophages were incubated with approximately 107 CFU, incubated for 0, 24, 48, 72, or 96 h, and then lysed. Concentrations of sample was added to 100 μl of 0.1% Triton X-100 and 0.2 mg of lysozyme per ml. After repeated passage through a 26-gauge needle, the lysate was tested immediately or stored at −20°C.

RESULTS

For the following experiments, the pilD mutant NU243 and its wild-type parent 130b were grown in BYE broth, and then supernatants were analyzed for enzymatic activities. Since NU243 and 130b have identical growth patterns in BYE, ex-cept for slightly reduced viability and/or recoverability in very late stationary phase (41), culture supernatants could be di-rectly compared.

Acid phosphatase secretion by L. pneumophila strains. Early studies reported acid and alkaline phosphatase activities for 10 different strains of L. pneumophila (49, 50, 73). Although the alkaline phosphatase is now known to be a periplasmic enzyme that is not critical for intracellular infection (37), the location and significance of the acid phosphatase has remained unclear. Thus, we began our study by determining whether filter-sterilized supernatants from wild-type cultures effectively released pNP from p-nitrophosphosphate at pH 5. The initial experiment indicated that an acid phosphatase activity was detectable at any time after mid-log phase (Fig. 1A and B). Super-natants from late-log-phase 130b cultures also had acid phosphatase activity (Fig. 2). For three reasons, we believe that...
this activity is reflective of a secreted enzyme rather than a cell-associated protein that had simply leaked into the medium. First, the supernatants containing acid phosphatase lacked appreciable alkaline phosphatase activity (data not shown). Second, the acid phosphatase was easily detected by using unconcentrated supernatants. Third, it was apparent in mid log phase, a stage of growth lacking significant cell lysis.

With the realization that the *L. pneumophila* acid phosphatase is secreted, we next examined the supernatants from NU243 cultures for loss of activity. The *pilD* mutant’s supernatants exhibited a level of phosphatase activity that was significantly less than that of wild type (Fig. 1B and 2). The loss of acid phosphatase was most apparent when mid- to late-log-phase culture supernatants were compared. In a similar way, the general protein secretion defect of NU243 was most clearly seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis when log-phase cultures were analyzed (41). Since type II secreted proteins generally accumulate within *pilD* mutants (24, 56, 70), we compared mutant and wild-type cell lysates for differences in acid phosphatase activity. Upon examination of late-log-phase lysates, NU243 contained fivefold more acid phosphatase activity than did strain 130b (Fig. 2). Taken together, these data indicate that the *L. pneumophila* acid phosphatase is a secreted enzyme whose export is dependent upon the prepilin peptidase.

**Protease secretion by *L. pneumophila* strains.** During these experiments, we took the opportunity to assess the kinetics of metalloprotease secretion. Although earlier work had determined the protease to be secreted and *pilD* dependent (17, 41), these studies apparently did not monitor when, in the course of growth, the enzyme was released. As presented in Fig. 1C, the kinetics of protease production paralleled that of acid phosphatase secretion.

**Esterase-lipase secretion by *L. pneumophila* strains.** All 13 strains of *L. pneumophila* previously tested possessed lipase-like activities (5, 7, 49, 50, 73). Since there has been some debate as to whether these activities reflect a true lipase or a simple esterase (49, 50), we examined wild-type legionellae in three different lipase assays. First, we assessed their supernatants’ ability to release pNP from *p*-nitrophenyl caprylate. Strain 130b supernatants, but not cell lysates, contained signif-
significant levels of reactivity (Fig. 3A). As was noted for the phosphatase and protease activities, the lipase-like activity first appeared in mid-log phase and peaked by late-log phase (data not shown). For the same reasons cited above, we believe that this activity also represents a bona fide secreted enzyme. With the confirmation of the lipase-like activity in *Legionella* supernatants, we reinvestigated the capacity of the enzyme to process long-chain fatty acids by using *p*-nitrophenyl palmitate as the substrate (73, 75). Indeed, supernatants from strain 130b split the palmitate substrate, albeit not as well as the caprylate substrate (Fig. 3B), suggesting the existence of a lipase. To test this hypothesis, we determined whether the 130b product could release free fatty acid from 1-MG and 1,2-DG substrates. The *Legionella* sample cleaved 1-monoacylglycerol (Fig. 4) but not the diacylglycerol substrate (data not shown), suggesting that *L. pneumophila* only possesses a monoacylglycerol lipase. Finally, we employed a new spectrophotometric assay that uses an artificial triglyceride with long-chain fatty acids in the sn-1 and sn-2 positions, i.e., measurement of the release of resorufin from 1,2-O-dilauryl-rac-glycero-3-glutaric acid resorufin ester by lipases (35). Neither concentrated nor unconcentrated 130b supernatants released resorufin from the triglyceride derivative, demonstrating that *L. pneumophila* indeed lacks a triacylglycerol lipase.

With the clarification of an esterase-lipase activity in wild-type supernatants, we could examine the *pilD* mutant for another secretion defect. NU243 was >10-fold impaired for esterase secretion, regardless of whether the substrate was *p*-nitrophenyl caprylate or *p*-nitrophenyl palmitate (Fig. 3). Similarly, the strain’s supernatants had a diminished ability to release free fatty acid from 1-MG (Fig. 4). However, esterase activity was apparent within the NU243 cell, again indicating that the strain is a secretion mutant (Fig. 3A). In sum, these data indicate that *L. pneumophila* secretes an esterase-monoacylglycerol lipase whose export is dependent upon *pilD*.

**PLA secretion by *L. pneumophila* strains.** Using methods such as thin-layer chromatography and mass spectrometry, Flieger et al. recently found an *L. pneumophila* PLA (26). Interestingly, the PLA was evident in log-phase cultures and peaked during late-log to early-stationary phase (26), suggesting that its expression is controlled in a manner similar to that of the acid phosphatase, protease, and esterase-lipase activities. Thus, we examined supernatants from strain 130b and its *pilD*-negative derivative for the presence of PLA (Fig. 5). Strain 130b, like other wild-type legionellae, secreted an enzyme that was capable of releasing free fatty acid from phosphatidylcholine. In contrast, NU243 was defective for PLA secretion, indicating that the processing of this newly described enzyme is also influenced by the prepilin peptidase.

**Nuclease secretion by *L. pneumophila* strains.** It had been reported that *L. pneumophila* secretes nuclease activities (73). Thus, we compared supernatants from late-log-phase cultures of 130b and NU243 for their ability to clear agar matrices impregnated with either RNA or DNA (64). On three occasions, the wild-type samples completely cleared the RNA-containing agar, yielding hydrolysis zones that were approximately 30 mm in diameter. The 130b supernatants also exhibited a DNase activity, yielding zones that were ca. 20 mm in diameter and were nearly clear. The supernatants from strain NU243 showed a consistent, albeit modest, reduction in RNase activity, yielding zones that were about 30% smaller and less clear compared to those of the wild type. On the other hand, the mutant was not impaired for DNA-degrading activity, as evidenced by the normal size and the clarity of its hydrolysis zones. In sum, the secretion of only one of the *L. pneumophila* nucleases was notably diminished by the loss of *pilD*.
Secretion of a pNPPC-hydrolase by \textit{L. pneumophila} strains. Since its colonies produce a zone of opacity on egg yolk plates and its supernatants release pNP from pNPPC, \textit{L. pneumophila} has long been believed to possess a PLC (6–8). Indeed, strain 130b secreted a factor during log phase that cleaves pNPPC (Fig. 1D). Importantly for us, supernatants from \textit{pilD} mutant cultures were lacking in pNPPC hydrolysis (Fig. 1D), while mutant lysates showed elevated pNPPC-hydrolase activity (data not shown). Although our interest in the pNPPC-hydrolase activity had been piqued, new data raised doubts about its molecular basis. First, acid and alkaline phosphatases and GPC-phosphodiesterases can also release pNP from pNPPC (27, 66, 68). Second, a very recent study concluded that \textit{L. pneumophila} does not produce a PLC (26). Since we found that strain 130b did not express a GPC-phosphodiesterase activity (data not shown), it now seemed plausible that the \textit{pilD}-dependent, pNPPC-hydrolyzing activity was another manifestation of the secreted acid phosphatase and/or esterase.

As one approach to determining whether the pNPPC-hydrolyzing activity reflects a known \textit{Legionella} enzyme, we sought a 130b mutant that is specifically defective for one of the secreted activities. Toward that end, transposon-mutagenized legionellae were first screened for alterations on egg yolk plates. Nine mutants were obtained that had diminished iridescence. Interestingly, these mutants, designated as strains NU245 through NU253, produced supernatants that had reduced pNPPC-hydrolase activity (data not shown), it now seemed plausible that the \textit{pilD}-dependent, pNPPC-hydrolyzing activity was another manifestation of the secreted acid phosphatase and/or esterase.

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**Complementation of the \textit{L. pneumophila} \textit{pilD} mutation.** To confirm that the secretion defects of NU243 were caused by the loss of \textit{pilD} and not a second site mutation, we examined the supernatant activities from NU243 harboring a plasmid (i.e., pMRL13) that contains as its \textit{Legionella} DNA component only \textit{pilD}. For all activities tested, NU243(pMRL13) exhibited, as expected, a level of activity that was comparable to that of 130b but greater than that of mutant bacteria containing only the pBBR1MCS vector (Table 1). Thus, we believe that the altered secretion phenotype displayed by the \textit{pilD} mutant is indeed due to the loss of prepilin peptidase.

**Intracellular infection by \textit{L. pneumophila} strains.** As a first step to ultimately determining which, if any, of the newly defined exoproteins promote intracellular infection and virulence, we assayed the nine pNPPC-hydrolase mutants for their ability to infect U937 cells. Based upon ID$_{50}$ analysis, seven of the mutants did not show a significant defect in macrophage infection (data not shown), indicating that the pNPPC-hydrol...
The present study provides seven basic conclusions about L. pneumophila secretion (Table 1). First, L. pneumophila does indeed secrete an acid phosphatase, PLA, DNase, and RNase.

**DISCUSSION**

The pNPPC-hydrolase activity is not required for intracellular infection. Interestingly, however, two of the mutants, NU247 and NU253, exhibited ID_{50}s that were at least 100-fold greater than wild type, suggesting that they are notably impaired for macrophage infection. To confirm this hypothesis, U937 cells were infected with equal amounts of wild-type and mutant bacteria and then, at various times, the bacteria within the monolayers were quantitated. Both NU247 and NU253 displayed a dramatic intracellular growth defect, which was slightly greater in magnitude to that of the pilD mutant (Fig. 7). Following an apparently normal uptake period, the numbers of mutant bacteria did not significantly increase for 2 days. Although replication was evident by the third day, the mutants ultimately produced 1,000-fold fewer progeny than did strain 130b. Inoculation of U937 cell monolayers with a low multiplicity of infection of L. pneumophila generally results in death and lysis of host cells (12). To determine whether these pNPPC-hydrolase mutants were also defective for cytopathic effect, we examined the viability of the infected monolayers with vital stains (Fig. 8). Within the first 72 h of incubation, strain 130b destroyed 75% of host cells, while inoculation with at least fourfold-greater numbers of NU243, NU247, and NU253 failed to reduce monolayer viability. By 96 h postinoculation, the mutants did elicit a significant cytopathic effect, albeit one that was still less than that of the wild type. Given the strong similarities that exist between Legionella macrophage and protozoan infection, we finally assessed the ability of NU247 and NU253 to infect Hartmannella amoebae (Fig. 9). The two pNPPC-hydrolase mutants were greatly impaired for protozoan infection, even more so than the pilD mutant, i.e., the numbers of NU247 and NU253 never increased during the 72-h incubation. In summary, NU247 and NU253 lack a factor that is necessary for optimal intracellular infection. Since the pNPPC-hydrolase activity is not required for intracellular infection, we suspect that this factor is a regulator of pNPPC-hydrolase expression or secretion.

**TABLE 1. Secreted activities of wild-type, pilD mutant, and complemented mutant bacteria**

<table>
<thead>
<tr>
<th>Activity assayed</th>
<th>Level of enzymatic activity within strain supernatants (mean ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>130b and 130b(pBBR1MCS)</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+ (5.14 ± 0.61)</td>
</tr>
<tr>
<td>Proteasec</td>
<td>+ (0.45 ± 0.02)</td>
</tr>
<tr>
<td>Esterase-lipase</td>
<td>+ (37.46 ± 2.85)</td>
</tr>
<tr>
<td>PLA</td>
<td>+</td>
</tr>
<tr>
<td>DNase</td>
<td>+</td>
</tr>
<tr>
<td>RNase</td>
<td>+</td>
</tr>
<tr>
<td>pNPPC-hydrolase</td>
<td>+ (0.28 ± 0.04)</td>
</tr>
</tbody>
</table>

* a, Wild-type level of activity; −, a significantly reduced or undetectable level of activity.
  b, The values in parentheses represent the mean and standard deviations from triplicate late-log cultures of either 130b(pBBR1MCS), NU243(pBBR1MCS), or NU243(pMRL13) and are representative of two additional complementation experiments. In all trials, the secreted activities exhibited by the uncomplemented mutant were significantly less than those of the wild type and the complemented strain (P < 0.0001, Student’s t test). The complemented mutant behaved as did the wild type (P > 0.5).
  c, As measured by the azocasein assay.
  d, As measured by the release of pNP from p-nitrophenyl caprylate.
  e, ND, not determined.

**FIG. 7.** Macrophage infection by wild-type and mutant L. pneumophila. U937 cell monolayers were infected with approximately 5 × 10^6 CFU of wild-type 130b (●), pilD mutant NU243 (□), and pNPPC-hydrolase mutants NU247 (○) and NU253 (△). CFU per well were quantitated at 0, 24, 48, and 72 h. Each datum point represents the mean and standard deviation for three monolayers. Significant differences in recovery between 130b and its mutant derivatives were evident at 24 h (P < 0.05) and beyond (P < 0.001). These differences were seen in three additional experiments (data not shown).

**FIG. 8.** Cytopathic effect of L. pneumophila strains on U937 cells. Replicate monolayers (n = 6) were either not infected (●) or were infected with 10^6 CFU of strain 130b (●), 5 × 10^6 CFU of NU243 (□), 8 × 10^6 CFU of NU247 (○), or 6.5 × 10^6 CFU of NU253 (△). After various periods of incubation, the viability of the host cells was measured by neutral red uptake. Since the pilD mutant does not elicit any cytopathic effect within the typical 72-h infection assay (41), the monolayers were monitored for 96 h and were purposely infected with greater numbers of mutant relative to wild-type bacteria. Datum points represent the mean OD_{540}, and vertical bars indicate the standard deviations. Differences in cytopathic effect between 130b and its mutant derivatives were significant at 72 and 96 h postinoculation (P < 0.001, Student’s t test). Similar conclusions were obtained from two additional experiments with neutral red and a third trial with alamar blue (data not shown).
Second, the organism secretes an esterase-monoacylglycerol lipase. Third, the pNPPC-hydrolase activity of *Legionella*, originally ascribed to a PLC, is not, as yet, accounted for by known major enzymatic activities. Fourth, the export of the acid phosphatase, zinc metalloprotease, esterase-monoacylglycerol lipase, and pNPPC-hydrolase was detectable during log phase. A companion study demonstrated that the PLAs is similarly expressed (26). Although nucleic release was not monitored over the entire growth cycle, it was apparent in late-log-phase cultures. Fifth, secretion of the acid phosphatase, esterase-monoacylglycerol lipase, PLA, RNase, and pNPPC-hydrolase, like that of the protease, is deficient in a *L. pneumophila* pilD mutant (Table 1). Sixth, as in other bacteria, the mutation in pilD results in the intracellular accumulation of the oxoenzymes (24, 56, 70). Seventh, the effect of the pilD mutation may only be evident when the comparisons between wild type and mutant utilize mid- to late-log-phase bacteria. Thus, we suspect that our earlier study overlooked the effect of PilD on phosphatase expression because stationary-phase cultures had been examined (41). Based upon analyses of other gram-negative bacteria, the changes in secretion activity in the *L. pneumophila* pilD mutant are likely due, at least in part, to the absence of a type II secretion apparatus, some of whose components are substrates for the PilD (23, 60).

The finding of up to six pilD-dependent exoenzymes in *L. pneumophila* adds considerably to an expanding appreciation for PilD and type II secretion in bacterial physiology. The oxoenzymes previously found lacking from pilD or other type II secretion mutants include the following: the esterase and lipase of *Acinetobacter calcoaceticus*; the acyltransferase, aerolysin, and protease of *A. hydrophila*; the lipases of *Burkholderia* sp.; the pectate lyase and cellulase of *Erwinia chrysanthemi*; the pullulanase of *Klebsiella oxytoca*; the alkaline phosphatase, lipase, elastase, exotoxin A, LasA protease, and PLC of *P. aeruginosa*; the cholera toxin, protease, and endochitinase of *V. cholerae*; and the amylase, cellulase, endoglucanase, and protease of *Xanthomonas campestris* (14, 20, 24, 33, 36, 44, 55, 56, 58, 63, 70). Thus, there is precedent for a linkage between pilD and an esterase-lypase and protease. However, this study is the first to document how the loss of pilD is associated with changes in acid phosphatase, PLA, and RNase activity. Furthermore, we believe that *L. pneumophila* has other PilD-dependent activities, including the factor responsible for pNPPC hydrolysis. Indeed, visualization of supernatant proteins by Coomassie staining of polycrylamide gels suggested that the pilD mutant is missing at least eight exoproteins (41). In addition, the *L. pneumophila* pilD mutant displays an altered colony morphology that is not simply due to the loss of pil, suggesting that PilD influences the expression of surface components (41). We do not believe, however, that all *Legionella* exoproteins are controlled by PilD and type II secretion. For example, our current data suggest that the *L. pneumophila* DNAse is not dependent upon PilD, a finding that has a precedent in the *V. cholerae* system (63).

Along with the increased understanding of the gram-negative secretion machinery, recent attention has been directed toward defining the role of PilD- and type II secretion-dependent exoproteins in pathogenesis. For example, our previous study was the first to implicate PilD-dependent secretion in intracellular infection (41), and the present study signifies an initial step toward identifying those secreted proteins that potentiate *L. pneumophila* macrophage infection and overall virulence. Although the characterization of a new panel of mutants indicated that the pNPPC-hydrolase activity is not required for U937 cell infection, the acid phosphatase, esterase-monoacylglycerol lipase, PLA, and RNase constitute potential cell infectivity determinants. Alternatively, the newly defined activities, including that of pNPPC-hydrolase, might promote virulence by fostering extracellular survival as opposed to or in addition to intracellular infection. Interestingly, acid phosphatasases, due to their ability to inhibit superoxide anion production, have been implicated in the intracellular survival of *Francisella tularensis*, *L. micdadei*, and *Leishmania donovani* (59, 62). In addition, PLAs promote the pathogenesis of *A. hydrophila* and *Yersinia enterocolitica*, and an RNase is required for the virulence of *Shigella flexneri* (11, 47, 65). The characterization of additional mutants that are defective for single exoenzymes will show if and how the various secreted products promote *L. pneumophila* pathogenesis.

Unlike other pNPPC-hydrolase mutants, NU247 and NU253 were greatly impaired for intracellular infection. Although other scenarios exist, we hypothesize that these two strains represent regulatory or processing mutants, i.e., they lack a factor(s) that, in addition to affecting the expression of the pNPPC-hydrolase, influences the production of molecules that do promote intracellular growth. Since NU247 and NU253 were not deficient in the acid phosphatase, esterase, PLA, and protease activity, this factor would have to be acting with a certain degree of specificity. However, there are many examples of transcriptional regulators that coordinately control the expression of some but not all secreted activities, including PilD- or type II-dependent exoproteins, e.g., ToxRS/T in *Vibrio* sp. and *Fur* in *Pseudomonas* sp. (25). Similarly, a periplasmic chaperone can promote the secretion of some but not all exoproteins, e.g., Lif specifically influences lipase secretion in *Pseudomonas* sp. (23, 34). Thus, further examination of these mutants should provide yet additional new insights into *L. pneumophila* regulation, secretion, and pathogenesis.

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REFERENCES


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