

## *Legionella pneumophila* Major Acid Phosphatase and Its Role in Intracellular Infection

VIRGINIA ARAGON, SHERRY KURTZ, AND NICHOLAS P. CIANCIOFFO\*

Department of Microbiology and Immunology, Northwestern University  
Medical School, Chicago, Illinois 60611

Received 24 July 2000/Returned for modification 4 October 2000/Accepted 15 October 2000

*Legionella pneumophila* is an intracellular pathogen of protozoa and alveolar macrophages. This bacterium contains a gene (*pilD*) that is involved in both type IV pilus biogenesis and type II protein secretion. We previously demonstrated that the PilD prepilin peptidase is crucial for intracellular infection by *L. pneumophila* and that the secreted *pilD*-dependent proteins include a metalloprotease, an acid phosphatase, an esterase/lipase, a phospholipase A, and a *p*-nitrophenyl phosphorylcholine hydrolase. Since mutants lacking type IV pili, the protease, or the phosphorylcholine hydrolase are not defective for intracellular infection, we sought to determine the significance of the secreted acid phosphatase activity. Three mutants defective in acid phosphatase activity were isolated from a population of mini-Tn10-mutagenized *L. pneumophila*. Supernatants as well as cell lysates from these mutants contained minimal acid phosphatase activity while possessing normal levels of other *pilD*-dependent exoproteins. Genetic studies indicated that the gene affected by the transposon insertions encoded a novel bacterial histidine acid phosphatase, which we designated Map for major acid phosphatase. Subsequent inhibitor studies indicated that Map, like its eukaryotic homologs, is a tartrate-sensitive acid phosphatase. The *map* mutants grew within macrophage-like U937 cells and *Hartmannella* amoebae to the same degree as did wild-type legionellae, indicating that this acid phosphatase is not essential for *L. pneumophila* intracellular infection. However, in the course of characterizing our new mutants, we gained evidence for a second *pilD*-dependent acid phosphatase activity that, unlike Map, is tartrate resistant.

*Legionella pneumophila*, the etiologic agent of Legionnaires' disease, is a gram-negative, facultative intracellular bacterium (10). Normally, *L. pneumophila* is an inhabitant of fresh water, where it lives as part of biofilms or as a parasite of protozoa (28). However, it can reach the human respiratory tract following inhalation or aspiration of contaminated water (20, 26, 60). In the lung, the legionellae replicate within alveolar macrophages, eventually causing much cell death and damage to lung tissue (24, 60).

The identification of an *L. pneumophila pilD* gene (31, 32) afforded new insight into the molecular pathogenesis of legionellosis. In a variety of gram-negative bacteria, PilD is an inner membrane peptidase that cleaves and methylates the pilin and pilin-like proteins that will form the type IV pili (2, 3, 21, 38, 49, 57). In addition, PilD processes a second set of pilin-like molecules which contribute to the formation of a type II protein secretion system (9, 21, 22, 33, 43, 48, 56). The two functions of PilD have been confirmed in *L. pneumophila*; i.e., a mutation in *pilD* renders bacteria nonpiliated and deficient for protein secretion (5, 31). The existence of the *Legionella* type II secretion system was later confirmed by the identification of some of the genes encoding components of the secretion apparatus (23). Importantly, the *L. pneumophila pilD* mutant is severely defective (i.e., ca. 1,000-fold) for replication within freshwater protozoa and human macrophages (31). Although type IV pili are modestly involved in the attachment of the bacteria to host cells, they are not critical for intracellular

growth and survival (31, 55). Consequently, it became more significant to identify and characterize the secreted activities that are absent in the *pilD* mutant. Several *Legionella* enzymatic activities have recently been shown to be secreted in a *pilD*-dependent manner, including a zinc metalloprotease, phospholipase A, acid phosphatase, *p*-nitrophenyl phosphorylcholine (*p*NPPC) hydrolase, RNase, and esterase/lipase (5, 31). Since mutants specifically defective for the production of either the *p*NPPC hydrolase activity or the metalloprotease proved not to be impaired for intracellular infection (5, 58), the critical *pilD*-dependent factors remain to be identified.

For three reasons, we targeted the acid phosphatase activity for further analysis. First, phosphatase activity is diminished in type II secretion mutants that are defective for intracellular growth (O. Rossier and N. P. Cianciotto, submitted for publication). Second, our observations in *Legionella* represent the first connection between an acid phosphatase and PilD or type II secretion. Third, in other pathogens, acid phosphatases have been postulated to have a role in intracellular infection (7, 11, 30, 45, 46, 50, 51). In this work, we provide evidence that *L. pneumophila* possesses two acid phosphatase activities and report the identification and mutation of the major, *pilD*-dependent acid phosphatase. Although the major acid phosphatase proved to be unique among bacterial acid phosphatases in terms of its sequence and secretion, it was not essential for the intracellular replication of *L. pneumophila* in macrophages or amoebae.

### MATERIALS AND METHODS

**Bacteria and media.** *L. pneumophila* serogroup 1 strain 130b (ATCC BAA 74), a virulent clinical isolate, and NU243, a direct derivative of 130b that contains a stable mini-Tn10 insertion in the *Legionella pilD* gene, were described previously

\* Corresponding author. Mailing address: Department of Microbiology and Immunology, Northwestern University Medical School, 320 East Superior Street, Chicago, IL 60611-3010. Phone: (312) 503-0385. Fax: (312) 503-1339. E-mail: n-cianciotto@northwestern.edu.

(19, 31). A population of 130b randomly mutagenized with mini-Tn10 was also reported before (41). Legionellae were routinely cultured on buffered charcoal yeast extract (BCYE) agar for 3 days at 37°C (17). In order to determine the levels of secreted enzymatic activities and compare the growth rates between strains, bacteria were cultured in buffered yeast extract (BYE) broth (5). The extent of bacterial growth was assessed by measuring the optical density of the cultures at 660 nm (OD<sub>660</sub>) (31). To ultimately screen for mutants deficient in acid phosphatase activity, colonies of randomly mutagenized 130b bacteria were taken from BCYE plates and inoculated into 100 µl of BYE contained in wells of 96-well plates. After overnight incubation at 37°C with shaking, 10-µl aliquots from each culture were assayed for acid phosphatase activity (see below). *Escherichia coli* DH5α was used as the host for recombinant plasmids (6). *E. coli* was grown in Luria-Bertani broth or agar, with ampicillin (100 µg/ml) added when needed.

**Preparation of supernatants and cell lysates.** To test for secreted or cell-associated enzymes, supernatants and lysates from *L. pneumophila* cultures were prepared as before (5). Briefly, supernatants were obtained by centrifugation followed by filtration (0.22 µm pore size) and in some cases were concentrated 100-fold through a Millipore YM10 ultrafiltration cell. Lysates were prepared by treatment with Triton X-100 and lysozyme.

**Phosphatase and other enzymatic assays.** Phosphatase activity was measured as the ability of the sample to release *p*-nitrophenol (*p*NP) from *p*-nitrophenyl phosphate (*p*NPP) (Sigma Chemical, St. Louis, Mo.) (5). Bacterial supernatant or cell lysates were incubated with 7.6 mM *p*NPP in 50 mM citric acid buffer (pH 5) or 0.2 M acetate buffer (pH 5.5) at 37°C. Since *p*NP is colorless at acid pH, concentrated NaOH was added to the reactions after the appropriate incubation at 37°C, and the production of *p*NP was monitored at 410 nm. In some experiments, sodium tartrate and sodium molybdate, compounds that usually inhibit acid phosphatases, were assayed throughout a concentration range from 0.001 to 10 mM (15, 29, 50, 54). To measure alkaline phosphatase activity, the *p*NPP hydrolysis reactions were performed in 100 mM Tris (pH 10) (5). One unit of acid or alkaline phosphatase activity was defined as that which releases 1 nmol of *p*NP in 1 min. Acid phosphatase activity was also determined in samples electrophoresed through polyacrylamide gels (47, 52). Proteins in 100-fold-concentrated supernatants were separated in a discontinuous system with a 10% polyacrylamide separating gel under nondenaturing conditions. After the electrophoresis, the gel was equilibrated in 0.2 M acetate buffer (pH 5.5) and incubated with 7.6 mM *p*NPP in the same buffer at room temperature. To then detect the release of *p*NP, the solution was made alkaline by the addition of concentrated NaOH. The reactive band was isolated from the gel by electroelution under nondenaturing conditions following the manufacturer's recommendations (Bio-Rad Laboratories, Hercules, Calif.), and the proteins contained within were examined for *p*NPP hydrolysis and for size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27).

Bacterial supernatants were assayed for their ability to release *p*NP from *p*-nitrophenyl caprylate or palmitate (indicative of a lipase/esterase activity) and from *p*NPPC as described previously (5).

**PCR, sequencing, and clone analysis.** Genomic DNA from *L. pneumophila* was extracted as previously described (42). Based upon data from the *L. pneumophila* Philadelphia I genome project (<http://genome3.cpmc.columbia.edu/~legion/>), three DNA primers were designed for the amplification of an acid phosphatase gene: 1, 5'-GCCATCTTCCAAGGTATAGC, corresponding to a site 1,020 bp upstream of the gene; 2, 5'-ACCAACGGTGGCAAGATACG, from a site 74 bp upstream; and 3, 5'-ATTCCGAGCAGACCACAAC, from a site 201 bp downstream of the gene. To determine the approximate position of the mini-Tn10 in several acid phosphatase mutants, a standard PCR was performed using primer 3 and a primer designed from the end of the transposon (5'-CCTTAACCTAATGATTTTTAC) (32). PCR products obtained with wild-type DNA and primers 1 plus 3 and 2 plus 3 were used for sequencing the 130b acid phosphatase gene. Sequencing reactions were performed using the BigDye terminator cycle sequencing mix from PE Applied Biosystems (Foster City, Calif.). Primer synthesis and automated sequence analysis on an ABI Prism 373 DNA sequencer (Applied Biosystems) were performed at the Biotech Facility at Northwestern University Medical School, Chicago, Ill. Sequence database searches were performed using programs based on the BLAST algorithm (4). The nucleotide sequence was analyzed for promoter prediction (44). The predicted protein was analyzed with the SignalP program (36) and Psort (35) for a signal sequence and for protein motifs with the PROSITE database (8). The PCR fragments that were used in sequencing experiments were also cloned into the pGem-T Easy vector (Promega, Madison, Wis.). The 2.3-kb fragment derived using primers 1 and 3 generated plasmid pVA13, whereas the 1.3-kb fragment obtained with primers 2 and 3 generated pVA12.

**Intracellular infection of U937 cells and *Hartmannella amoebae*.** U937, a human cell line that differentiates into macrophage-like cells after treatment with phorbol esters, served as a host for in vitro infection by *L. pneumophila* (13). The cell line was infected as previously described (31), with some modifications. To quantitate intracellular growth, monolayers containing 10<sup>6</sup> macrophages were inoculated with approximately 10<sup>5</sup> CFU, incubated for 0, 6, 18, 24, 48, or 72 h, and then lysed. Serial dilutions of the lysates were plated on BCYE agar, supplemented with kanamycin for the mutants, to determine the number of bacteria per monolayer. To establish the cytopathic effect of *L. pneumophila* on U937 cells, the viability of infected monolayers was tested by their ability to reduce alamar blue, as recommended by the manufacturer (Biosource International, Vacaville, Calif.). Briefly, at 0, 24, 48, and 72 h, the wells were thoroughly washed to eliminate the extracellular bacteria. Medium (100 µl) with 10 µl of alamar blue was added to each well and incubated at 37°C for 4 h. After this time, the fluorescence (excitation, 540 nm; emission, 584 nm) was read in a Spectra Max Gemini fluorescence reader (Molecular Devices, Sunnyvale, Calif.). To examine the ability of legionellae to grow within a protozoan host, *Hartmannella vermiformis* was infected as previously indicated (14, 31). Approximately 10<sup>5</sup> CFU were added to wells containing 10<sup>5</sup> amoebae, and at 0, 24, 48, or 72 h postinoculation, the numbers of bacteria within the coculture were determined by serial dilution.

**Statistical analysis.** To study the statistical significance of the differences observed between samples, Student's *t* test was used. The *P* values are given when appropriate.

**Nucleotide accession number.** The *L. pneumophila* map locus sequence has been deposited in the GenBank database at the National Center for Biotechnology Information under accession number AF299349.

## RESULTS

**Isolation of *L. pneumophila* acid phosphatase mutants.** With the purpose of determining the role of the acid phosphatase(s) in *L. pneumophila* intracellular infection, we sought to isolate and characterize mutants specifically deficient in this enzymatic activity. Toward that end, 1,767 colonies from a randomly mutagenized population of *L. pneumophila* 130b were inoculated into 100 µl of BYE in microtiter plates. After overnight growth at 37°C, an aliquot from each culture was tested for phosphatase activity with *p*NPP at pH 5. Five mutants with reduced activity, which was visually apparent, were selected and grown in standard culture tubes for retesting. The three mutants that appeared to produce a reduced level of acid phosphatase upon reexamination were designated NU254, NU255, and NU256. These mutants replicated in BYE broth with a growth profile similar to that of wild-type 130b (data not shown), indicating that the reduction in acid phosphatase activity was not due to a general growth defect.

Previously, we had shown that the highest level of acid phosphatase activity in *L. pneumophila* supernatants occurs at late log phase (5). Therefore, late-log-phase BYE cultures of NU254, NU255, and NU256 were analyzed for phosphatase activity at pH 5 in order to confirm and quantitate their enzymatic defect. The supernatants from the mutants presented a level of acid phosphatase activity significantly reduced compared with the wild type (*P* < 0.001) (Fig. 1A). Indeed, the level of acid phosphatase activity found for NU254, NU255, and NU256 supernatants was similar to the level found for the secretion-defective *pilD* mutant (Fig. 1A). To determine whether the reduction in *p*NPP hydrolysis was due to the absence of one or more secreted proteins, concentrated late-log-phase supernatants were electrophoresed through polyacrylamide under nondenaturing conditions, and then the gel was incubated in the presence of *p*NPP. After 5 min of incubation, wild-type samples yielded a single reactive band, whereas the samples from the phosphatase mutants and the *pilD* mutant

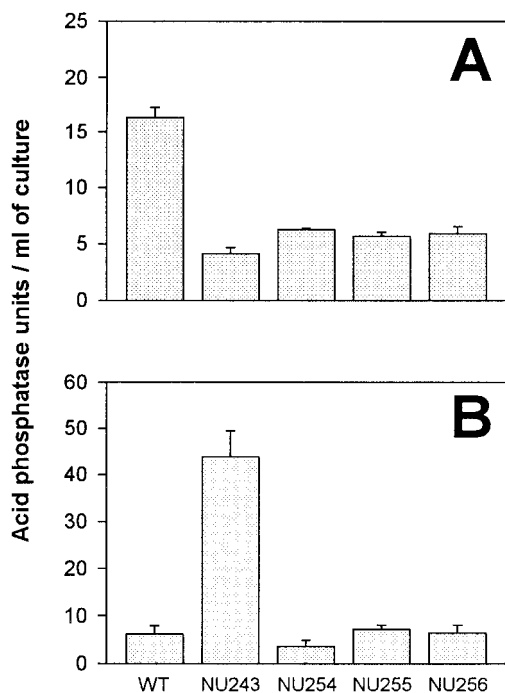


FIG. 1. Acid phosphatase activity produced by *L. pneumophila* strains. Late-log-phase ( $OD_{660} = 1.8$  to  $1.9$ ) supernatants (A) and cell lysates (B) of wild-type strain 130b (WT), *pilD* mutant NU243, NU254, NU255, and NU256 were examined for their ability to release pNP from pNPP at pH 5. Bars represent the means  $\pm$  standard deviation of the activity found in three cultures and are representative of the results seen in three independent experiments.

failed to exhibit any reactive band (data not shown). When the reactive band in wild-type samples was electroeluted from the gel, it showed hydrolytic activity against pNPP at pH 5 (5.6 U/ml) but not at pH 10 ( $-0.6$  U/ml). These data confirm that NU254, NU255, and NU256 lack most of the secreted acid phosphatase activity.

To begin to examine the reason for the observed losses of acid phosphatase activity, cell lysates from the mutants were tested for their ability to hydrolyze pNPP. Unlike the *pilD* mutant, NU254, NU255, and NU256 did not show accumulated acid phosphatase activity in their lysates, suggesting that they are not secretion mutants (Fig. 1B). The supernatants of the new mutants were next tested for esterase/lipase and pNPPC hydrolase activities, two functions known to be secreted in a *pilD*-dependent fashion (5). The levels of these activities were similar for the mutants and the wild type (Fig. 2), confirming that NU254, NU255, and NU256 do not have a general defect in secretion. Incidentally, the fact that these mutants had wild-type levels of pNPPC hydrolysis confirms our earlier conclusion that the *L. pneumophila* pNPPC hydrolase activity is not due to any major acid phosphatase activity (5). To address the possibility that the mutants had a regulatory defect, affecting the stage at which they produce the acid phosphatase, supernatants from log-phase ( $OD_{660} = 0.8$  to  $0.9$ ), late-log-phase ( $OD_{660} = 1.8$  to  $1.9$ ), and stationary-phase ( $OD_{660} = 2.1$  to  $2.2$ ) cultures were examined for the presence of acid phosphatase. At log phase, the acid phosphatase level in wild-type cultures was barely detectable, with only  $3.8$  ( $\pm 1.2$ )

U/ml, but was already higher than that in the supernatant from the *pilD* mutant ( $1.6 \pm 0.1$ ;  $P < 0.05$ ) or NU254 and NU255 ( $1.5 \pm 0.2$  and  $1.6 \pm 0.2$ , respectively;  $P < 0.05$ ). The difference in the amount of activity present in wild-type and mutant supernatants was greater at late log and stationary phases, similar to the results presented in Fig. 1. Taken together, these results suggest that NU254, NU255, and NU256 contain a mutation in the gene encoding an *L. pneumophila* acid phosphatase.

**Nucleotide sequence of the major *L. pneumophila* acid phosphatase (*map*) gene.** To establish the genetic basis of the defect in NU254, NU255, and NU256, we sought to determine the sequences into which the transposon had inserted in each mutant. At the time we were starting this analysis using inverse PCR, as we have done in the past for other mutants (32), the sequence of an open reading frame (ORF) with homology to acid phosphatases became available in the developing *L. pneumophila* Philadelphia I genome database. To determine if this was the gene mutated in our 130b acid phosphatase mutants, PCR experiments were performed using a primer corresponding to sequences just 3' of the ORF and a primer for the mini-Tn10. The PCR fragments amplified from DNA of the three mutants indicated that this was the gene interrupted; i.e., we amplified fragments of 430 bp for NU254, 260 bp for NU255, and 960 bp for NU256. The exact position of the

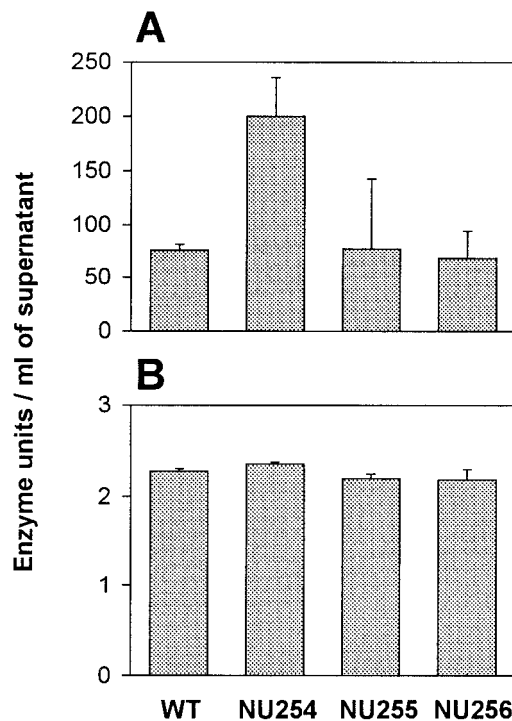


FIG. 2. Lipase/esterase and pNPPC hydrolase activity secreted by *L. pneumophila* strains. Late-log-phase ( $OD_{660} = 1.8$  to  $1.9$ ) supernatants of wild-type 130b (WT), NU254, NU255, and NU256 were tested for their ability to release pNP from p-nitrophenyl palmitate (A) and pNPPC (B). Bars represent the means  $\pm$  standard deviation of the activity found in three cultures and are representative of the results obtained in three independent experiments. Similar to the results in panel A, the three mutants were not defective in the hydrolysis of p-nitrophenyl caprylate (data not shown)

transposon in NU255 was confirmed by sequencing of the PCR fragment (see below).

Using genomic DNA from strain 130b, the gene was sequenced in its entirety, along with up and downstream regions (Fig. 3). The ORF had 1,059 bp, encoding a predicted protein of 352 amino acids (39.4 kDa). This size was in agreement with the migration observed for the electroeluted acid phosphatase (see above) when it was analyzed by SDS-PAGE (39 kDa) (data not shown). Analysis of the protein sequence showed the presence of a signal peptide (Fig. 3), supporting the secreted nature of the *L. pneumophila* acid phosphatase activity. Importantly, the predicted protein contained the motif [LIVM]-X-X-[LIVMA]-X-X-[LIVM]-X-R-H-[GN]-X-R-X-[PAS] characteristic of histidine acid phosphatases (Fig. 3). An alignment with the amino acid sequence of the well-studied *Escherichia coli* periplasmic acid phosphatase indicated that the *Legionella* protein had all the residues that are essential for enzymatic activity (39) (bold asterisks in Fig. 3). Interestingly, the protein showed its greatest overall homology to eukaryotic acid phosphatases, such as a lysosomal acid phosphatase from mouse (protein accession number P24638), the human prostatic acid phosphatase (2HPA), the *Drosophila* acid phosphatase (S64682), and the *Leishmania donovani* acid phosphatases (AAC79513 and AAC47744), with identities ranging from 28 to 30% and similarities ranging from 45 to 46%. To confirm that *map* encodes a functional acid phosphatase, we examined two recombinant *E. coli* clones that contain the entire gene for *pNPP* hydrolysis. DH5 $\alpha$ (pVA12) had  $110.3 \pm 0.58$  U/ml of culture, and DH5 $\alpha$  (pVA13) had  $92 \pm 1.9$  U/ml, while DH5 $\alpha$  containing the pGem-T Easy vector had just  $1.6 \pm 0.17$  U/ml of culture. Given this result, the sequence data, and the behavior of the *L. pneumophila* mutants, we named this new *L. pneumophila* gene *map* for major acid phosphatase gene.

Immediately upstream of *map*, a putative promoter was detected, as well as a ribosome-binding sequence (Fig. 3). Two hundred sixty-eight base pairs upstream of the *map* start codon, and oriented in the opposite direction, the beginning of an ORF with high homology to the D-stereospecific peptide hydrolase of *Bacillus cereus* (protein accession number AJ011526) was detected. Downstream of *map*, and also oriented in the opposite direction, the beginning of an ORF without clear homology to known proteins was detected. These data suggest that *map* is expressed as a monocistronic message. Therefore, the transposon insertions in NU254, NU255, and NU256 would not be expected to produce polar effects. The GC content in the *map* locus was 39%, similar to the %GC of the *L. pneumophila* genome (10).

**Identification of a minor acid phosphatase activity produced by *L. pneumophila*.** Although an acid phosphatase gene was interrupted in NU254, NU255, and NU256, the supernatants from the mutants always showed a low level of activity in the *pNPP* assay (Fig. 1A) which could be eliminated by 10 min of incubation at 100°C. To determine whether this activity was simply cell-associated alkaline phosphatase that was released by spontaneous lysis of some cells in the culture (5, 25), supernatants and cell lysates from the wild type and *map* mutants were assayed for hydrolysis of *pNPP* at both pH 5 and 10 (Fig. 4). The supernatants showed a small amount of phosphatase activity at pH 10. However, the cell lysates, which were very rich in alkaline phosphatase, showed a minimal *pNPP* reaction

at pH 5, indicating that alkaline phosphatase cannot be responsible for the activity in the supernatants from the mutants. Since two of the mutants had transposon insertions in domains of the protein that are likely essential for activity (Fig. 3), and all of them have the same level of *pNPP* hydrolytic activity (Fig. 1A and 4), it is unlikely that the residual activity is due to low levels of truncated *Map*. Consequently, we suspected that the phosphatase activity remaining in the supernatants of the *map* mutants was due to another (minor) acid phosphatase activity, since bacteria can have multiple acid phosphatases (47, 50).

To explore this possibility, we examined the effect of molybdate and tartrate on the *pNPP* hydrolysis mediated by mutant and wild-type supernatants, since acid phosphatases are commonly molybdate sensitive but only some are sensitive to tartrate (15, 29, 50, 54). The activity in 130b supernatants was completely inhibited by molybdate (Fig. 5). In contrast, it was only partially inhibited by tartrate; even a concentration of 10 mM tartrate inhibited the activity only up to 75% (Fig. 5). The activity present in the supernatants of the *map* mutants was completely inhibitable by sodium molybdate but interestingly, was not affected by sodium tartrate (Fig. 5). The level of activity remaining in NU254 and NU255 corresponded to the level of tartrate-resistant activity found in the wild type, demonstrating that the mutants indeed lack a tartrate-sensitive acid phosphatase activity while retaining a tartrate-resistant one. In support of these data, the activity expressed by recombinant, *map*-containing *E. coli* (Fig. 6), as well as the electroeluted *pNPP*-reactive protein band (data not shown), was completely inhibited by either tartrate or molybdate. Altogether, these results indicate that *L. pneumophila* has two different acid phosphatase activities: one that is molybdate and tartrate sensitive and is completely missing in NU254, NU255, and NU256, and one that is molybdate sensitive but tartrate resistant and is still present in the supernatants of the mutants. Since the level of tartrate-resistant activity is reduced in the supernatant of NU243 ( $P < 0.01$  at 1 to 10 mM tartrate; Fig. 5), this second acid phosphatase activity is probably *pilD* dependent.

**Intracellular infection by *L. pneumophila map* mutants.** To determine the role of the major acid phosphatase in intracellular infection, we tested the *map* mutants for their relative ability to infect macrophages and protozoa. It is well documented that the intracellular growth of *L. pneumophila* results in the destruction of the macrophage host (1, 12, 40). Therefore, the acid phosphatase mutants, together with the wild type and the *pilD* mutant, were first inoculated into U937 cells and examined for cytopathic effects at different times after infection (Fig. 7). The macrophages infected with the wild type showed a substantial decrease in viability after 48 and 72 h of infection, only 19 and 9% viable macrophages, respectively ( $P < 0.001$ ). Contrary to these results, the macrophages infected with the *pilD* mutant showed little, if any, reduction in viability after 72 h, as has been seen previously (5, 31). The *map* mutants showed a pattern of cytopathicity that was identical to that of the wild type (Fig. 7). To determine if this cytopathic effect correlated with a normal capacity to replicate inside macrophages, the mutants were inoculated into U937 monolayers, and the number of bacteria was recorded after various incubation intervals (Fig. 8A). Wild-type 130b showed the typical intracellular growth pattern, increasing 10,000- to

```

1  AAAGACAATAAAAAATGAAAAACGTATAAATCAATTTCCACTTGCATACATAACGGCTCATTGCAACTCCA
   F V I F I F F T Y I L K W K C V Y R S M SD
71  ATGTGGCAAGGGTTTTCTCACCCACCAATGAGCTATATCTTAATTCTAGTCGATTTCCAGGATAATTTCT
   -10 -35
141 TTACTTCCTGTATAAAATAGATTTCTCCTCCAGGTTTCAAATGAATCAGGGAATACAAAAGCCGATGTC
211 TCGATTTTTATTTGTGTCTTCGTCATAAGTGGCAAACCGAGATTAACCAACGGTGGCAAGATACAAACAAGAA
   -35 -10 map→ ACCAACGGTGGCAAGATACG→
281 TTTTTTACGAAGCCTCATGTTTACTTTAACTCAAGGAGTATGAATACATGGTCAACAAGATTTGCCTGAG
   SD M V N K I C L S
351 TTTTCGCACTATTAATTAGCGCACCCCTCCATTTTATTAGCACAAAGACAAACTTTTGTGCTGGACATT
   F A L L I S A P S I L L A Q D K L I F A V D I
421 ATTCGTTCATGGCGACAGAACGCCGATTGTTGCTTTGCCCTACTGTAAATTACCAATGGCAAGAGGGCTTG
   I R H G D R T P I V A L P T V N Y Q W Q E G G L
491 G Q L T A E G M Q Q E Y K M G V A F R K K Y I E
   GCCAGCTTACGGCAGAAGGCATGCAACAAGAATACAAAATGGGTGTGGCATTTCGCCAAAAATATATTGA
   NU256↓ *
561 E L H L L P E H Y E Y G T I Y V R S T D Y A R
   AGAGTTGCATTTATTACCTGAACACTATGAATACGGAACCATCTATGTCCGATCTACGGATTATGCACGT
   T L M S A Q S L L M G L Y P P G T G P S I P A
631 ACTCTGATGAGCGCCAGTCATTTGTTAATGGGCTATATCCACCTGGAAGTGGACCATCAATTCCTGCAG
   G T S A L P H A F Q P I P V F S A P S K Y D E V
701 GGACCTCCGCTTTACCTCATGCTTTTCAACCTATTCCCGTATTTAGCGCACCGTCTAAGTAGCAGCAGGT
   I I Q Q V D R K E R K K L M E Q Y V F S T R E
771 TATCATTAGCAGGTAGATCGTAAGGAACGGAAAAAGCTAATGGAGCAATATGTTTTTCTACCAGGGAG
   W Q Q K N N E L K D K Y P L W S R L T G I N I
841 TGGCAACAAAAAATAATGAATTGAAAAGACAAATACCCATTTATGGAGCCGCTGACTGGCATTAAATTTG
911 D T L E D L E T V G H T L Y V H Q I H N A P M P
   ATACTCTGGAGGATCTGGAAACAGTTGGTCATACCTTATATGTTTCATCAAATCCATAATGCTCCTATGCC
981 E G L A S N D I E T I I N S A E W A F F M A Q E
   GGAAGATTAGCTTCTAACGATATTGAAACAATTATCAATTCTGCTGAGTGGGCTTTTATGGCACAAAGAA
1051 K P Q Q I A N V Y S S K L M T N I A D Y L N S
   AAACCTCAACAAATAGCGAATGTTTATAGTTCAAAGCTGATGACTAACATAGCTGATTATCTGAATTCGG
   NU254↓ * *
1121 G S M K K S K L K Y V L L S A H D T T I A S V L
   GAAGTATGAAAAATCTAAACTGAAATATGTTTTGCTATCTGCTCATGATACTACAATAGCCAGTGTATT
1191 S F L G A P L E K S P P Y A S N V N F S L Y D
   AAGTTTTTTAGGTGCTCCTTTGGAAAAATCCCCACCTTATGCCTCAAATGTTAATTTTTCTCTCTATGAT
   NU255↓
1261 N G A N Y Y T V K I T Y N G N P V L I P A C G
   AATGGAGCAAATATTACACAGTAAAAATTACCTATAATGGTAATCCGGTACTTATTCTGCTTGGCGGT
1331 G S V C E L Q Q L V N L V H D S K N #
   GCTCAGTATGTGAGTTACAACAGCTTGTAATTTGGTACATGATTTCAAAAATTGAGTGAATCAGGAGA
1471 TATTTGGCTTGGGTGAGTAATCCTAACCATCTCAAACATCTGTTGTTGCAAAAACAGCAATAGCAGATGG
1541 TTTGAAGTAATTCAAATAATCGATCAATTCAAAAAGATCTGGGCACATGAATGGGATACTCTAATAATCA
1611 AAACCTCTTCAAGATAAGAATTTGTTGCGGTGCTGCTGGGAATGCAACCCTTGATAGAGTACACACAAA
   ←CAACACCAGCACGACCTTA
1681 TCCGGATGGAAAAATTTATAAATTAATATGACCTGACAGATACCTTTGAATAGCTGGTAACGTGCAGAT
1751 AAAACTAGAATAACTAAGTCTTATATTTGCTAAGATTAGTTTTTAGCCTTGGTTTCAAGATTCAACTTTT
   # N K A K T E L N L K Q
1821 GATTTAAATCAATATCTAATCTTGATTTGG
   ←Orf
   N L D I D L E Q N P

```

FIG. 3. Nucleotide sequence of the *L. pneumophila* 130b *map* locus. The deduced amino acid sequences of the various ORFs and the termination codons (#) are indicated. For the predicted Map protein, the putative signal peptide is indicated in bold, and conserved protein domains are shadowed. Within the conserved domains, asterisks indicate conserved amino acids, and bold asterisks indicate amino acids that are essential for activity in the *E. coli* periplasmic acid phosphatase. Putative promoter sequences are indicated by the -10 and -35 designations, and the Shine-Dalgarno sequence is indicated (SD). The mini-Tn10 insertions are indicated by an arrow alongside the name of the corresponding mutant. The positions of the insertions are approximate for NU254 and NU256. Primers 2 and 3 (see Materials and Methods) are shown in italics at their binding regions.

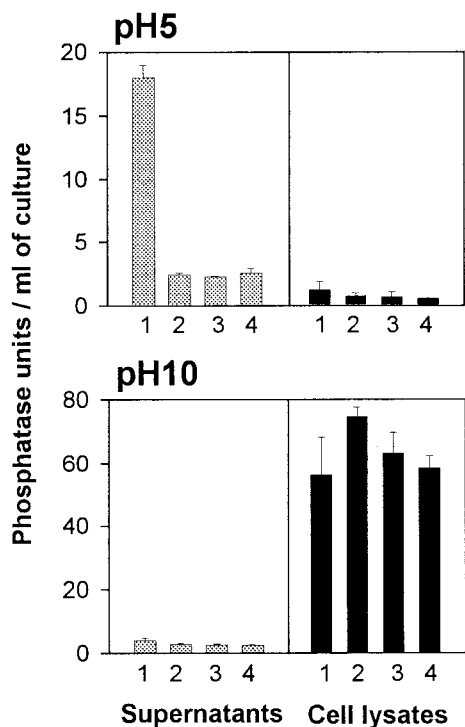


FIG. 4. Acid and alkaline phosphatase activity in *L. pneumophila* cultures. Supernatants and cell lysates from the wild type (bar 1) and *map* mutants NU254 (bar 2), NU255 (bar 3), and NU256 (bar 4) were examined for their ability to hydrolyze *p*NPP at pH 5 and 10. Bars represent the means  $\pm$  standard deviation of three cultures. The only significant difference between the wild type and mutants was observed with supernatants tested at pH 5 ( $P < 0.01$ ).

100,000-fold by 48 h after infection. Interestingly, the same results were obtained with the acid phosphatase mutants, indicating that these strains are not defective for intracellular replication in U937 cells. Moreover, the mutants did not seem to have a defect in entry, since the numbers recovered after the uptake period were the same as for the wild type (see  $t = 0$  in Fig. 8A). To determine the role of the major acid phosphatase in amoeba infection, *H. vermiformis* cultures were inoculated with the wild type and *map* mutants, and the number of bacteria was recorded at different times. As in the macrophage infection, the same numbers of bacteria were recovered from cocultures with the wild type or with the mutants (Fig. 8B). Together, these results indicate that the major acid phosphatase is not essential for macrophage or amoeba infection by *L. pneumophila*.

#### DISCUSSION

An acid phosphatase activity was first described in 1981 for the *Legionella* genus (34). That activity was detected in all the strains tested, i.e., representatives of *L. pneumophila* serogroups 1 to 6 and *Legionella* species *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. longbeachae*, and *L. micdadei* (34, 37, 59). Subsequently, two cell-associated acid phosphatases, ACP<sub>1</sub> and ACP<sub>2</sub>, were purified from *L. micdadei* (50). Recently, we demonstrated that the acid phosphatase activity of *L. pneumophila* is actually secreted in a *pilD*-dependent fashion (5). Our cur-

rent data indicate that the secreted acid phosphatase activity of *L. pneumophila* is the result of at least two different enzymes, which can be discriminated by their different sensitivity to tartrate. Mutant and sequence analyses indicated that the majority of activity is due to a tartrate-sensitive histidine acid phosphatase, which we have named Map. The Map protein is notable among bacterial acid phosphatases in two aspects. First, it is an extracellularly secreted enzyme, while the rest of the bacterial acid phosphatases characterized to date are periplasmic or membrane-bound proteins (47). Second, Map aligned with significant homology only with eukaryotic histidine acid phosphatases that are also secreted. Although several bacterial acid phosphatases have been sequenced (47), they did not show homology with *L. pneumophila* Map as determined by the NCBI BLAST programs. Thus, our data represent the first evidence for a bacterial extracellularly secreted histidine acid phosphatase.

*L. pneumophila* mutants containing insertions in *map* did not show a defect in infection of U937 cells or amoebae. Therefore, Map either has no role in *L. pneumophila* intracellular replication or has a dispensable one. As we have demonstrated, *L. pneumophila* has at least one other acid phosphatase, and this, or other enzymes, may compensate for the lack of Map. Also, even though Map is not essential for U937 cell

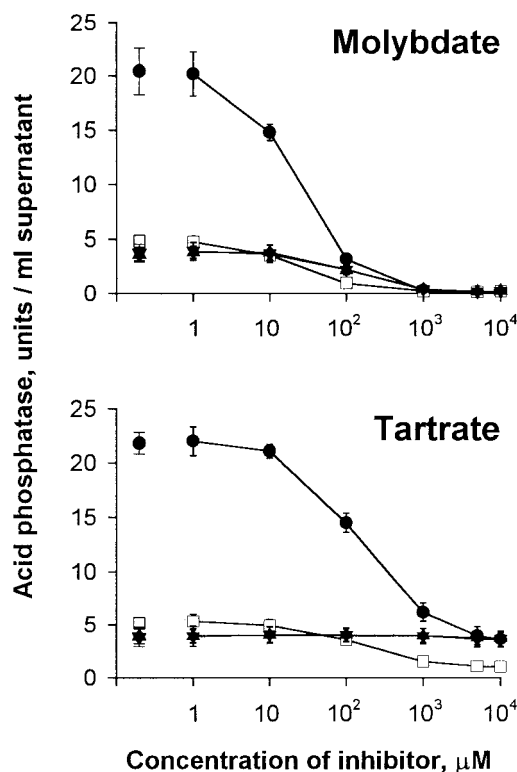


FIG. 5. *L. pneumophila map* mutants lack a tartrate-sensitive acid phosphatase. Supernatants from wild-type 130b (●), the *pilD* mutant NU243 (□), and the mutants NU254 (▲) and NU255 (▼) were examined for their ability to hydrolyze *p*NPP in the presence of different concentrations of molybdate or tartrate. The isolated symbols represent the level of activity without inhibitor. The results are the means  $\pm$  standard deviation of the activity found in three cultures. Similar results were obtained in two independent experiments using concentrated supernatants (data not shown).

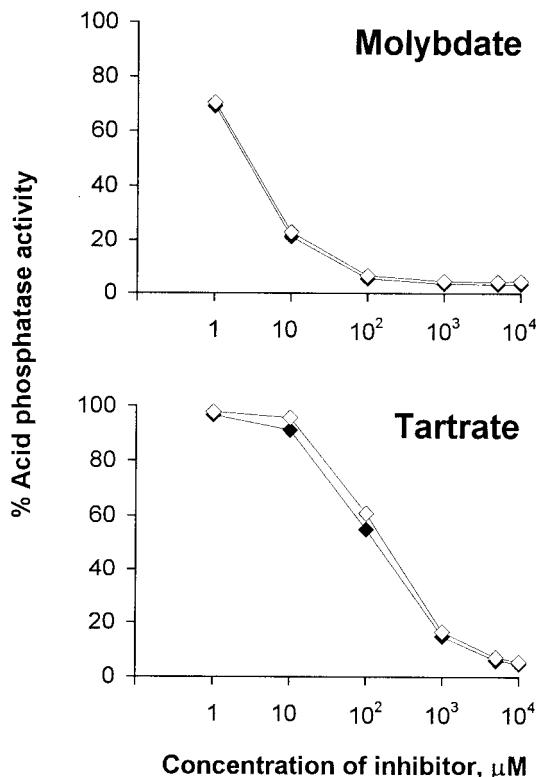


FIG. 6. Effect of molybdate and tartrate on the cloned *L. pneumophila* Map activity. Whole cultures of *E. coli* DH5α(pVA12) (◇) and DH5α(pVA13) (◆) were tested for acid phosphatase activity in acetate buffer (pH 5.5) in the presence of different concentrations of sodium molybdate (top panel) or sodium tartrate (bottom panel). The results are expressed as the percentage of refractive activity and are the means ± standard deviation of the activity found in three cultures and representative of two independent experiments. The error bars are too small to be seen.

infection, it may play a role in *Legionella* survival within other host cells, such as activated macrophages or neutrophils. Although there is no bacterial precedent for Map, two secretory histidine acid phosphatases, SAcP-1 and SAcP-2, have been identified in the macrophage parasite *Leishmania donovani* (53). It is believed that the parasitic phosphatases do have a role in infection, because they are produced during human infection (18). Since Map and the SAcP enzymes showed significant homology, they may have a common target or function during intracellular infection. On the other hand, it is entirely possible that Map and its parasite analog promote extracellular survival and spread in vivo.

Although the loss of Map secretion was not the reason for the intracellular infectivity defect of the *pilD* mutant, it is possible that the *pilD*-dependent tartrate-resistant acid phosphatase is important for U937 cell or amoeba infection. Indeed, in addition to *L. micdadei* ACP<sub>2</sub> noted above, tartrate-resistant acid phosphatases from *Francisella tularensis*, *Coxiella burnetii*, and *Leishmania donovani* have been implicated in the resistance of these intracellular pathogens to the oxidative burst of phagocytes; the purified enzymes inhibit superoxide production by fMet-Leu-Phe-activated cells (7, 30, 45, 46, 50). In the case of *L. micdadei* and *Leishmania donovani*, the enzymes interfere in phosphoinositide metabolism

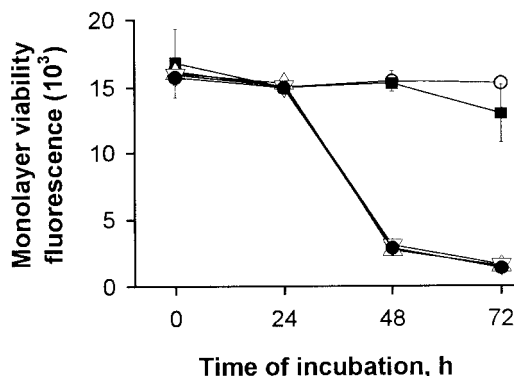


FIG. 7. Cytopathic effect of *L. pneumophila* on U937 cells. At different times after inoculation, the viability of the macrophage monolayer that had been infected at a multiplicity of infection of 0.1 with 130b (●), *pilD* mutant NU243 (■), NU254 (△), or NU255 (▽) was measured by fluorescence associated with alamar blue reduction. As a control, an uninfected monolayer (○) was processed in parallel. Results represent the means ± standard deviation of triplicate wells and are representative of two independent experiments.

by degrading phosphatidylinositol diphosphate and inositol trisphosphate (16, 51). Although the tartrate-resistant activity is a minor one in *L. pneumophila* BYE culture supernatants, it may be a major secreted factor when the bacteria are exposed to host cells or tissue. Thus, the tartrate-resistant acid phosphatase activity that we have uncovered, as well as other *pilD*-

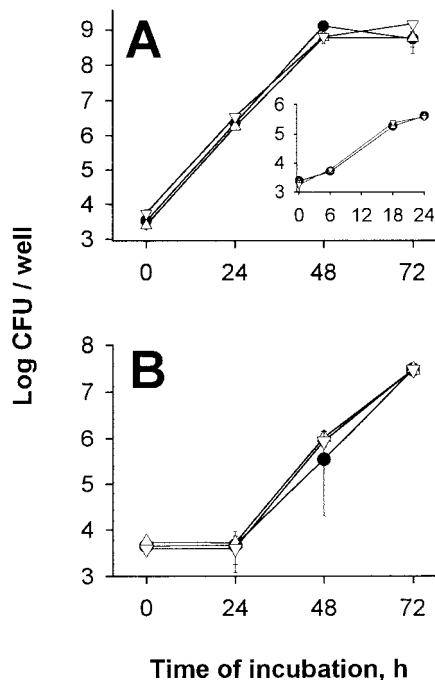


FIG. 8. Intracellular infection by wild-type and mutant *L. pneumophila*. U937 cells (A) and *H. vermiformis* amoebae (B) were infected at multiplicities of infection of 0.1 and 1, respectively, with wild-type 130b (●), NU254 (△), or NU255 (▽). The number of bacteria in each well was quantitated at 0, 24, 48, and 72 h by plating aliquots on BCYE agar. Results represent the means ± standard deviation of triplicate wells and are representative of two independent experiments. In a third U937 experiment, the numbers of bacteria were also determined at 6 and 18 h (inset).

dependent factors, may be quite relevant for the pathogenesis of *L. pneumophila*.

#### ACKNOWLEDGMENTS

We thank Ombeline Rossier for first noticing the acid phosphatase gene in the *Legionella* database and for helpful discussions.

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

#### REFERENCES

- Abu Kwaik, Y. 1998. Fatal attraction of mammalian cells to *Legionella pneumophila*. *Mol. Microbiol.* **30**:689–695.
- Alm, R. A., J. P. Hallinan, A. A. Watson, and J. S. Mattick. 1996. Fimbrial biogenesis genes of *Pseudomonas aeruginosa*: *pilW* and *pilX* increase the similarity of type 4 fimbriae to the GSP protein-secretion systems and *pilY1* encodes a gonococcal PilC homologue. *Mol. Microbiol.* **22**:161–173.
- Alm, R. A., and J. S. Mattick. 1996. Identification of two genes with prepilin-like leader sequences involved in type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* **178**:3809–3817.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Aragon, V., S. Kurtz, A. Flieger, B. Neumeister, and N. P. Cianciotto. 2000. Secreted enzymatic activities of wild-type and *pilD*-deficient *Legionella pneumophila*. *Infect. Immun.* **68**:1855–1863.
- Ausubel, F. M., R. B. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. *Current protocols in molecular biology*. John Wiley & Sons, New York, N. Y.
- Baca, O. G., M. J. Roman, R. H. Glew, R. F. Christner, J. E. Buhler, and A. S. Aragon. 1993. Acid phosphatase activity in *Coxiella burnetii*: a possible virulence factor. *Infect. Immun.* **61**:4232–4239.
- Bairoch, A., P. Bucher, and K. Hofmann. 1997. The PROSITE database: its status in 1997. *Nucleic Acids Res.* **25**:217–221.
- Bally, M., B. Wretling, and A. Lazdunski. 1989. Protein secretion in *Pseudomonas aeruginosa*: molecular cloning and characterization of the *xcp-1* gene. *J. Bacteriol.* **171**:4342–4348.
- Brenner, D. J., A. G. Steigerwalt, R. E. Weaver, J. E. McDade, J. C. Feeley, and M. Mandel. 1978. Classification of the Legionnaires' disease bacterium: an interim report. *Curr. Microbiol.* **1**:7–10.
- Chhatwal, G. S., M. J. Walker, H. Yan, K. N. Timmis, and C. A. Guzman. 1997. Temperature dependent expression of an acid phosphatase by *Bordetella bronchiseptica*: role in intracellular survival. *Microb. Pathog.* **22**:257–264.
- Cianciotto, N., B. I. Eisenstein, N. C. Engleberg, and H. Shuman. 1989. Genetics and molecular pathogenesis of *Legionella pneumophila*, an intracellular parasite of macrophages. *Mol. Biol. Med.* **6**:409–424.
- Cianciotto, N. P., B. I. Eisenstein, C. H. Mody, G. B. Toews, and N. C. Engleberg. 1989. A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. *Infect. Immun.* **57**:1255–1262.
- Cianciotto, N. P., and B. S. Fields. 1992. *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. *Proc. Natl. Acad. Sci. USA* **89**:5188–5191.
- Colon, L. S., N. M. Jimenez, and H. Zlotnik. 1992. Properties of a partially purified acid phosphatase from pathogenic *Nocardia brasiliensis*. *Mycopathologia* **118**:85–93.
- Das, S., A. K. Saha, A. T. Remaley, R. H. Glew, J. N. Dowling, M. Kajiyoshi, and M. Gottlieb. 1986. Hydrolysis of phosphoproteins and inositol phosphates by cell surface phosphatase of *Leishmania donovani*. *Mol. Biochem. Parasitol.* **20**:143–153.
- Edelstein, P. H. 1981. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *J. Clin. Microbiol.* **14**:298–303.
- Ellis, S. L., A. M. Shakarian, and D. M. Dwyer. 1998. *Leishmania*: amastigotes synthesize conserved secretory acid phosphatases during human infection. *Exp. Parasitol.* **89**:161–168.
- Engleberg, N. C., D. J. Drutz, and B. I. Eisenstein. 1984. Cloning and expression of *Legionella pneumophila* antigens in *Escherichia coli*. *Infect. Immun.* **44**:222–227.
- Fields, B. S. 1996. The molecular ecology of legionellae. *Trends Microbiol.* **4**:286–290.
- Filloux, A., G. Michel, and M. Bally. 1998. GSP-dependent protein secretion in Gram-negative bacteria: the Xcp system of *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.* **22**:177–198.
- Finlay, B. B., and S. Falkow. 1997. Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* **61**:136–169.
- Hales, L. M., and H. A. Shuman. 1999. *Legionella pneumophila* contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. *Infect. Immun.* **67**:3662–2666.
- Horwitz, M. A. 1992. Interactions between macrophages and *Legionella pneumophila*. *Curr. Top. Microbiol. Immunol.* **181**:265–282.
- Kim, M. J., J. E. Rogers, M. C. Hurley, and N. C. Engleberg. 1994. Phosphatase-negative mutants of *Legionella pneumophila* and their behavior in mammalian cell infection. *Microb. Pathog.* **17**:51–62.
- Kramer, M. H., and T. E. Ford. 1994. Legionellosis: ecological factors of an environmentally 'new' disease. *Zentralbl. Hyg.* **195**:470–482.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Lee, J. V., and A. A. West. 1991. Survival and growth of *Legionella* species in the environment. *J. Appl. Bacteriol. Symp. Suppl.* **70**:121S–129S.
- Li, H. C., J. Chernoff, L. B. Chen, and A. Kirschonbaum. 1984. A phosphotyrosyl-protein phosphatase activity associated with acid phosphatase from human prostate. *Eur. J. Biochem.* **138**:45–51.
- Li, Y. P., G. Curley, M. Lopez, M. Chavez, R. Glew, A. Aragon, H. Kumar, and O. G. Baca. 1996. Protein-tyrosine phosphatase activity of *Coxiella burnetii* that inhibits human neutrophils. *Acta Virol.* **40**:263–272.
- Liles, M. R., P. H. Edelstein, and N. P. Cianciotto. 1999. The prepilin peptidase is required for protein secretion by and the virulence of the intracellular pathogen *Legionella pneumophila*. *Mol. Microbiol.* **31**:959–970.
- Liles, M. R., V. K. Viswanathan, and N. P. Cianciotto. 1998. Identification and temperature regulation of *Legionella pneumophila* genes involved in type IV pilus biogenesis and type II secretion. *Infect. Immun.* **66**:1776–1782.
- Martinez, A., P. Ostrovsky, and D. N. Nunn. 1998. Identification of an additional member of the secretin superfamily of proteins in *Pseudomonas aeruginosa* that is able to function in type II secretion. *Mol. Microbiol.* **28**:1235–1246.
- Muller, H. E. 1981. Enzymatic profile of *Legionella pneumophila*. *J. Clin. Microbiol.* **13**:423–426.
- Nakai, K., and M. Kanehisa. 1991. Expert system for predicting protein localization in Gram-negative bacteria. *Proteins Struct. Funct. Genet.* **11**:95–110.
- Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**:1–6.
- Nolte, F. S., G. E. Hollick, and R. G. Robertson. 1982. Enzymatic activities of *Legionella pneumophila* and *Legionella*-like organisms. *J. Clin. Microbiol.* **15**:175–177.
- Nunn, D. N., and S. Lory. 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. *Proc. Natl. Acad. Sci. USA* **88**:3281–3285.
- Ostanin, K., E. H. Harms, P. E. Stevis, R. Kuciel, M.-M. Zhou, and R. L. V. Etten. 1992. Overexpression, site-directed mutagenesis, and mechanism of *Escherichia coli* acid phosphatase. *J. Biol. Chem.* **267**:22830–22836.
- Pearlman, E., A. H. Jiwa, N. C. Engleberg, and B. I. Eisenstein. 1988. Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line. *Microb. Pathog.* **5**:87–95.
- Pope, C. D., L. Dhand, and N. P. Cianciotto. 1994. Random mutagenesis of *Legionella pneumophila* with mini-Tn10. *FEMS Microbiol. Lett.* **124**:107–111.
- Pope, C. D., W. O'Connell, and N. P. Cianciotto. 1996. *Legionella pneumophila* mutants that are defective for iron acquisition and assimilation and intracellular infection. *Infect. Immun.* **64**:629–636.
- Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* **57**:50–108.
- Reese, M. G., N. L. Harris, and F. H. Eeckman. 1996. Large scale sequencing specific neural networks for promoter and splice site recognition, p. 737–738. *In* L. Hunter and T. E. Klein (ed.), *Biocomputing: proceedings of the 1996 Pacific symposium*. World Scientific Publishing Co., Singapore, Singapore.
- Reilly, T. J., G. S. Baron, F. E. Nano, and M. S. Kuhlenschmidt. 1996. Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. *J. Biol. Chem.* **271**:10973–10983.
- Remaley, A. T., D. B. Kubus, R. H. Basford, R. H. Glew, and S. S. Kaplan. 1984. Leishmanial phosphatase blocks neutrophil O<sub>2</sub> production. *J. Biol. Chem.* **259**:11173–11175.
- Rossolini, G. M., S. Schippa, M. L. Riccio, F. Berlutti, L. E. Macaskie, and M. C. Thaller. 1998. Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. *Cell. Mol. Life Sci.* **54**:833–850.
- Russel, M. 1998. Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. *J. Mol. Biol.* **279**:485–499.
- Russell, M. A., and A. Darzins. 1994. The *pilE* gene product of *Pseudomonas aeruginosa*, required for pilus biogenesis, shares amino acid sequence identity with the N-termini of type 4 prepilin proteins. *Mol. Microbiol.* **13**:973–985.
- Saha, A. K., J. N. Dowling, K. K. LaMarco, S. Das, A. T. Remaley, N. Olomur, M. T. Pope, and R. H. Glew. 1985. Properties of an acid phosphatase from *Legionella micdadei* which blocks superoxide anion production by human neutrophils. *Arch. Biochem. Biophys.* **243**:150–160.
- Saha, A. K., J. N. Dowling, A. W. Pasculle, and R. H. Glew. 1988. *Legionella micdadei* phosphatase catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate in human neutrophils. *Arch. Biochem. Biophys.* **265**:94–104.
- Salvano, M. A., and C. E. Domenech. 1999. Kinetic properties of purified



- Pseudomonas aeruginosa* phosphorylcholine phosphatase indicated that this enzyme may be utilized by the bacteria to colonize in different environments. *Curr. Microbiol.* **39**:1–8.
53. **Shakarian, A. M., S. L. Ellis, D. J. Mallinson, R. W. Olafson, and D. M. Dwyer.** 1997. Two tandemly arrayed genes encode the (histidine) secretory acid phosphatases of *Leishmania donovani*. *Gene* **196**:127–137.
54. **Stankiewicz, P. J., and M. J. Gresser.** 1988. Inhibition of phosphatase and sulfatase by transition-state analogues. *Biochemistry* **27**:206–12.
55. **Stone, B. J., and Y. Abu Kwaik.** 1998. Expression of multiple pili by *Legionella pneumophila*: identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. *Infect. Immun.* **66**:1768–1775.
56. **Strom, M. S., D. Nunn, and S. Lory.** 1991. Multiple roles of the pilus biogenesis protein PilD: involvement of PilD in excretion of enzymes from *Pseudomonas aeruginosa*. *J. Bacteriol.* **173**:1175–1180.
57. **Strom, M. S., D. N. Nunn, and S. Lory.** 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and *N*-methylation of proteins belonging to the type IV pilin family. *Proc. Natl. Acad. Sci. USA* **90**:2404–2408.
58. **Szeto, L., and H. A. Shuman.** 1990. The *Legionella pneumophila* major secretory protein, a protease, is not required for intracellular growth or cell killing. *Infect. Immun.* **58**:2585–2592.
59. **Thorpe, T. C., and D. Miller.** 1981. Extracellular enzymes of *Legionella pneumophila*. *Infect. Immun.* **33**:632–635.
60. **Winn, W. C. J.** 1988. Legionnaires' disease: historical perspective. *Clin. Microbiol. Rev.* **1**:60–81.

---

Editor: J. T. Barbieri