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Phosphatase-negative mutants of *Legionella pneumophila* and their behavior in mammalian cell infection

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Microbial phosphatases are known or suspected to play a role in the pathogenesis of several intracellular pathogens, including *Legionella micdadei*. *Legionella pneumophila* also possess phosphatase activites, but their possible roles in cellular infection are unknown. We generated mutants of a serogroup 1 isolate of *L. pneumophila* that lack the major phosphatase. Isolation of a Pho mutant after random mutagenesis with transposon MudII4041 allowed us to dissociate the major alkaline phosphatase (pH optimum ~8) from a minor acid phosphatase activity. Both activities were concentrated in the bacterial periplasm. The gene encoding the major alkaline phosphatase (*pho*) was cloned by expression in *E. coli* and used to generate a site directed mutation in two *L. pneumophila* strains. Each parent–mutant pair was compared in a U937 cell tissue culture assay for capacity to infect, lyse, and grow within mammalian cells. Although the parental stains differed in their U937 cell cytopathicity, neither was significantly more infective than its Pho derivative, suggesting that the alkaline phosphatase activity is not essential for cellular infection. Because they are not attenuated, Pho mutants can be used to generate gene fusions with *E. coli* alkaline phosphatase to study and secretion and cellular infectivity in *L. pneumophila*.

Key words: Legionella pneumophila; phosphatase; Pho mutants; mammalian cell.

Introduction

The Legionellae are facultative intracellular pathogens which multiply within free-living protozoa and mammalian phagocytic cells. In humans, infection of pulmonary alveolar macrophages can result in an atypical form of pneumonia, Legionnaires' disease. The phenomenology of host cell infection has been amply described, but relatively little is known about the specific molecular events involved in bacterial survival and growth within cells.[1,2]

It has been suggested that bacterial phosphatases may have a role in these events, since some of these enzymes can catalyze the dephosphorylation of molecules involved in mammalian cell signal transduction. Within the genus *Legionella, L.*

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micdadei has been shown to possess an acid phosphatase that can catalyze the dephosphorylation of inositol phosphates and blocks neutrophil superanion production *in vitro*.[3,4] However, the localization of this enzyme within the bacteriurn is uncertain, and its role in cellular infection is unclear. *L. pneumophila*, a species much more commonly associated with human disease than *L. micdadei*[5], also possesses phosphatase activities, although very little is known about their functional roles during infection.[6,7] The roles of phosphatases in the pathogenesis of certain other intracellular pathogens is better established. The tyrosine phosphatase of *Yersinia pseudotuberculosis* (YopH) is an outer-membrane enzyme that dephosphorylates macrophage proteins during intracellular infection.[8] This molecule is required for expression of bacterial virulence. Similarly, a tartrate-resistant acid phosphatase of *Leishimania donovani* interferes with superoxide and peroxide production by phagocytes.[9] Virulent *L. donovani* clones have been shown to express a higher level of this enzyme on their surfaces than avirulent clones.[10]

In studies from this laboratory, we have used translational fusions with the *E. coli* alkaline phosphatase to identify and study secreted proteins of *L. pneumophila*.[11,12] These experiments are possible because the *E. coli* enzyme in *L. pneumophila* does not alter cellular infectivity *per se*. However, the isolation of phosphatase fusions would be technically facilitated if the experimental *L. pneumophila* strain lacked background phosphatase activity. For these reasons, we began studies to examine the properties of the *L. pneumophila* phosphatases and to define any roles they might have in cellular infection.

Results

Isolation and characterization of L. pneumophila *phosphatase mutants using mini-Mu transposon mutagenesis*

Mu*dI*I4041 was transposed to vector pRTP1 following the method of Castilho *et al.*[13] and then transferred conjugally to *L. pneumophila* AA103 with counterselection of the vector plasmid. Based on Southern hybridization analysis of genomic DNA from 16 randomly selected transconjugants, we determined that Mu*dI*I4041 had transposed in an apparently random manner, with a frequency of two to three transpositions in each transconjugant (data not shown). Sixty-five (97%) of 67 randomly selected transconjugant colonies probed with pRTP1 were negative for vector sequences, confirming that the delivery plasmid had been lost in most of the isolates. Finally, we screened several thousand transconjugants on XP indicator plates at pH 8.5. One phosphatase-negative mutant was found and designated as AA120. After multiple passages of AA120, we obtained a spontaneous Pho$^+$ isolate, AA121.

L. pneumophila AA103, AA120, and AA121 were assayed qualitatively for phosphatase activity on XP indicator plates buffered to pH 8.5 or 5.0 (Fig. 1). Colonies of strain AA103 contained phosphatase activity on both media. In contrast, colonies of strains AA120 and AA121 lacked activity on the alkaline pH plates. In other experiments, the alkaline phosphatase activity in sonicates of strain AA103 and recombinant *E. coli* CC118 expressing the *L. pneumophila pho* gene (see below) were found to have a pH optimum $\sim$8 with a functional range from pH 6 to 11 (not shown). Therefore, the specific activity of phosphatase (units/mg of protein) in cellular fractions were measured at pH 5 and 10.05 to maximize the specificity of assays for the acid and alkaline activities, respectively (Fig. 2). Both alkaline and acid phosphatases were mainly localized to the osmotic shockates. Strain AA103 contained a high level of alkaline phosphatase that was absent in both AA120 and AA121. Figure 2 also shows that strain AA121 contained an altered, high level of acid phosphatase. We

Table 1 Bacterial strains and plasmids

Strain/plasmid	Genotype or phenotype	Source or reference
E. coli		
CC118	*araD 139* Δ (*ara,leu*) *7696* Δ*lacX 74 phoA* Δ*20 galE galK thi rspE rpoB argEam* recA1	Manoil and Beckwith[33]
χ2981	*E.coli* K-12 F- Δ*41* [*proB-lacYZ*] λ-*T3r* Δ*asdA4 zhf-2::Tn 10 cycA1*	Doggett and Curtiss[34]
BAC101	M8820 *recA56 srl300::Tn10* Mu *cts62* with Mu*dII*4041	Castilho *et al.*[13]
L. pneumophila		
AA103	Spontaneous Nal^r Str^r derivative of *L. pneumophila* SG1 130b, high-frequency conjugal receipient	
AA107	Str^r derivative of *L. pneumophila* SG1 130b	University of Michigan Medical School
AA120	AA103::Mu*dII*4041 Nal^r Str^r Km^r Pho	This work
AA121	derivative of AA120 with spontaneous reversion to high acid phosphatase activity	This work
AA122	AA103, *pho*::Tn*5*; Nal^r Str^r Km^r Pho	This work
AA123	AA107, *pho*::Tn5; Nal^r Str^r Km^r Pho	This work
Plasmids		
pRK212.1	Ap^r, Tc^r, Tra$^+$	Figurski *et al.*[35]
pRTP1	pBR322 derivative, Ap^r, *cos*, *rpsL* (Sm^s) *onT*	Stibitz *et al.*[36]
pTLP6	Cm^r, *rspL*$^{(Sms)}$, *oriT*	University of Michigan Medical School
pTLP7	Cm^r, *sacB*, *oriT*	Albano *et al.*[11]
pMJK1	Cm^r, *oriT*, *pho*$^+$	This work
pMJK2	Cm^r, *rspL* (Sm^s), *oriT*, *pho*$^+$	This work

concluded that *L. pneumophila* contains prominent alkaline phosphatase and minor acid phosphatase activities, both primarily concentrated in the periplasm. Both Mu*d* mutants lack alkaline phosphatase, but strain AA121 possesses increased activity of acid phosphatase.

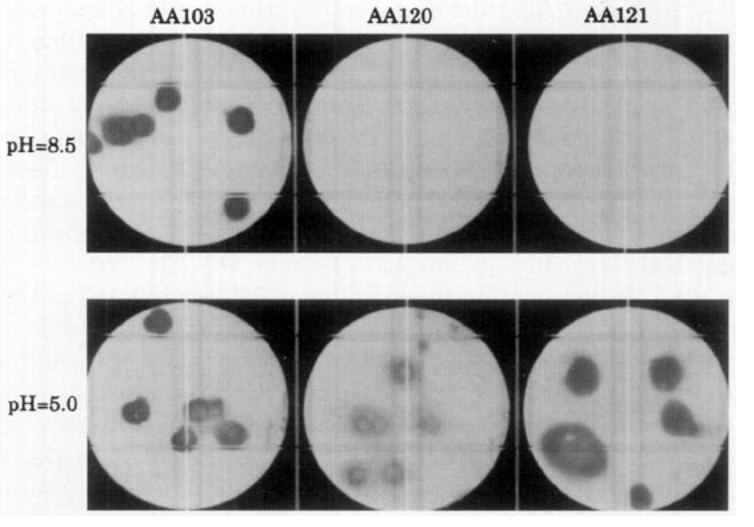

Fig. 1. *In situ* assay for phosphatase activity in *L. pneumophila* strains. Blue colonies (dark in black and white) indicate dephosphorylation of XP by whole bacteria at the indicated pH.

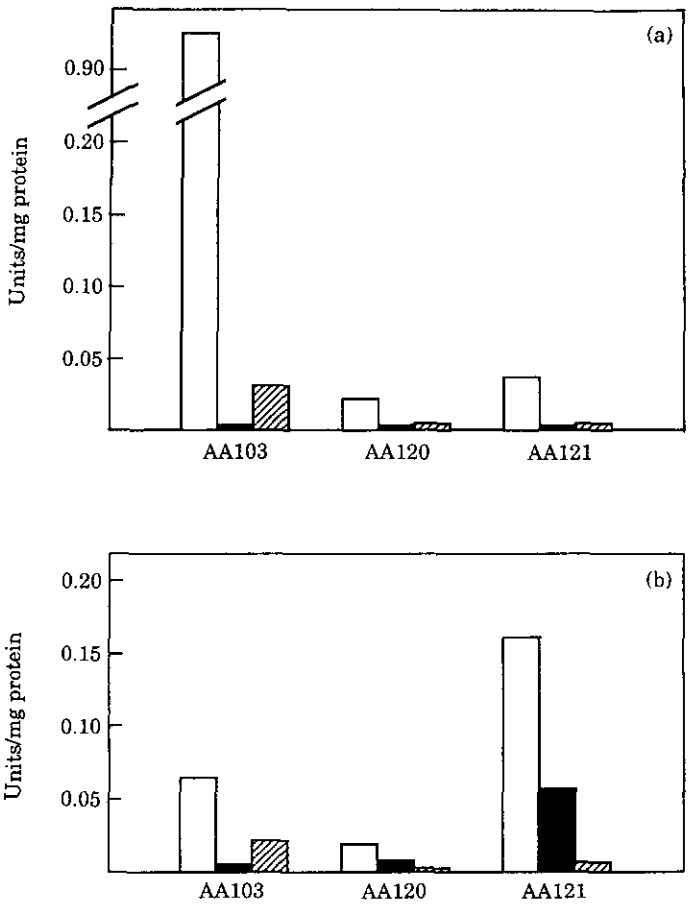

Fig. 2. Quantitative assay for phosphatase in subcellular fractions of *L. pneumophila* strains at pH of *(a) 10.5 and (b) 5.0. Symbols:* (□) *osmotic shockate;* (■) *cytoplasm;* (▨) *total membranes.*

Cloning of the L. pneumophila *alkaline phosphatase gene*

A cosmid library of *L. pneumophila* serogroup 1 (130b) was prepared in *E. coli* DH1 by inserting ~35 kb, *Sau*3A partial digestion fragments into cosmid vector pTLP6.[14] The library was screened on LB/Cm/XP media, and five intensely blue colonies were chosen for further study. Conjugal transfer of the cosmids from these clones to strain CC118 resulted in alkaline phosphatase activity, confirming that the activity was encoded on the cosmids. From one of these cosmids, we were able to delete large portions of the insert and still retain enzymatic activity, suggesting that the *pho* gene was situated adjacent to the vector junction. Complete digestion of this cosmid with *Sal*I or *Bam*HI, followed by recircularization resulted in Pho$^+$ subclones pMJK1 or pMJK2, respectively (Fig. 3).

To localize the *pho* gene within the subcloned DNA segments, pMJK1 or pMJK2 were subjected to transposon mutagenesis with Tn*phoA-oriT* or Tn*5*, respectively. Using these methods, the region encoding the phosphatase activity mapped to the extreme left end of the insert (Fig. 3). The loss of phosphatase activity associated with a Tn*5* insertion into the vector DNA adjacent to the *pho* gene suggests that expression depends on a vector promoter. In addition, the isolation of a Pho$^+$ Tn*phoA-oriT* insertion within the *pho* region is likely a *pho–phoA* fusion, confirming that the direction of transcription is from left to right.

The *Eco*RI fragment carrying the Tn*5* insertion (asterisk in Fig. 3) was isolated and

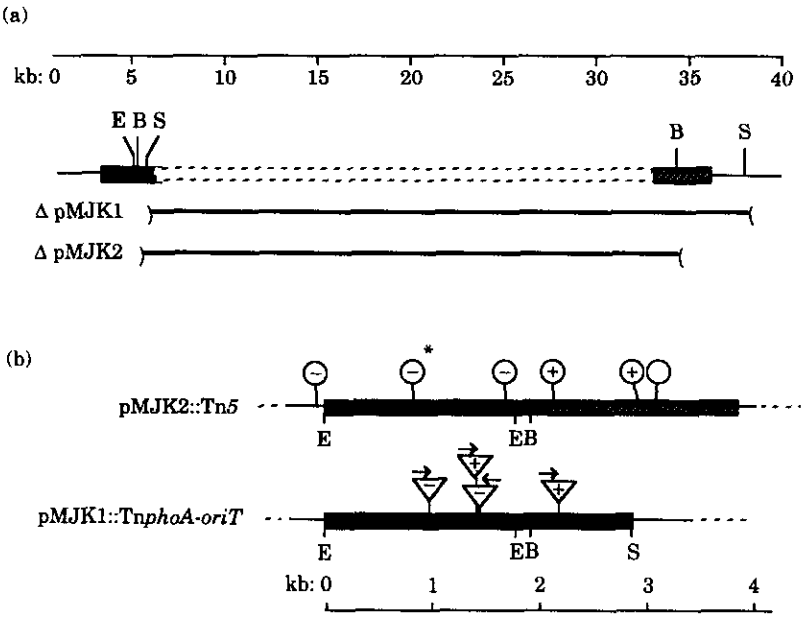

Fig. 3. (a) A partial map of a cosmid conferring alkaline phosphatase activity on *E. coli* CC118. Large deletions of this cosmid using restriction endonucleases *Sal* and *Bam*HI with recircularization resulted in subclones pMJK1 and pMJK2. The black and cross-hatched sequences indicate non-contiguous segments from the left and right junctions of the cosmid insert, respectively. (b) Transposon mutagenesis of these plasmids using Tn*5* (circles) or Tn*phoA-oriT* (triangles). (+) and (−) indicate the presence or absence of phosphatase activity associated with a specific insertion. The small arrows above the triangles indicate the direction of transcription of the *E. coli phoA* gene on Tn*phoA-oriT*.

recloned into another vector, pTLP7, to obtain pMJK3 for transfer to *L. pneumophila* and allelic exchange with the native gene. We have found in previous experiments that Tn*5* transposes poorly in *L. pneumophila*, and exchange of a cloned sequence with a Tn*5* insertion with the native gene by double homologous recombination occurs at a higher frequency than transposition. In this experiment, after transfer of pMJK3 to strain AA103, Km^r transconjugants were screened for Pho$^-$ mutants on XP indicator plates at pH 8.5. One Pho$^-$ mutant, designated AA122, was identified. We applied the same method to construct a second Pho mutant designated AA123 in strain AA107. As expected, introduction of pMJK1 into AA122 or AA123 restored phosphatase activity. In addition, pMJK1 was easily cured from these strains by serial passage on non-selective media, resulting in reversion to a Pho$^+$ phenotype. We confirmed the allelic exchange in these mutants by Southern hybridization analysis of genomic DNA.

Figure 4 shows a representative blot of chromosomal DNA from the parent strain AA103, AA122, and the Mu*d* mutants. *Eco*RI was used because there are no cleavage sites for this enzyme in either Mu*d*II4041 or Tn*5*. Using pMJK1 as a probe (Fig. 4), the hybridizing *Eco*RI fragment carrying *pho* gene was larger in AA120 and AA122 than AA103 by amounts predicted from the sizes of the transposons used, Mu*d*II4041 and Tn*5*, respectively. In contrast, the fragment from strain AA121 was reduced in size, indicating deletion or rearrangement of the *pho* gene. The same blot probed with a Mu*d*II4041 probe showed that AA120 and AA121 each contains bands corresponding to those that hybridized to pMJK1, indicating the Mu*d* had transposed into the *pho* gene region (data not shown).

U937 cell cytopathicity and intracellular growth kinetics

The ability of the Pho$^+$ parent and Pho$^-$ mutant strains to infect U937 cells were compared in assays for cellular cytopathicity and bacterial growth. In the first assays,

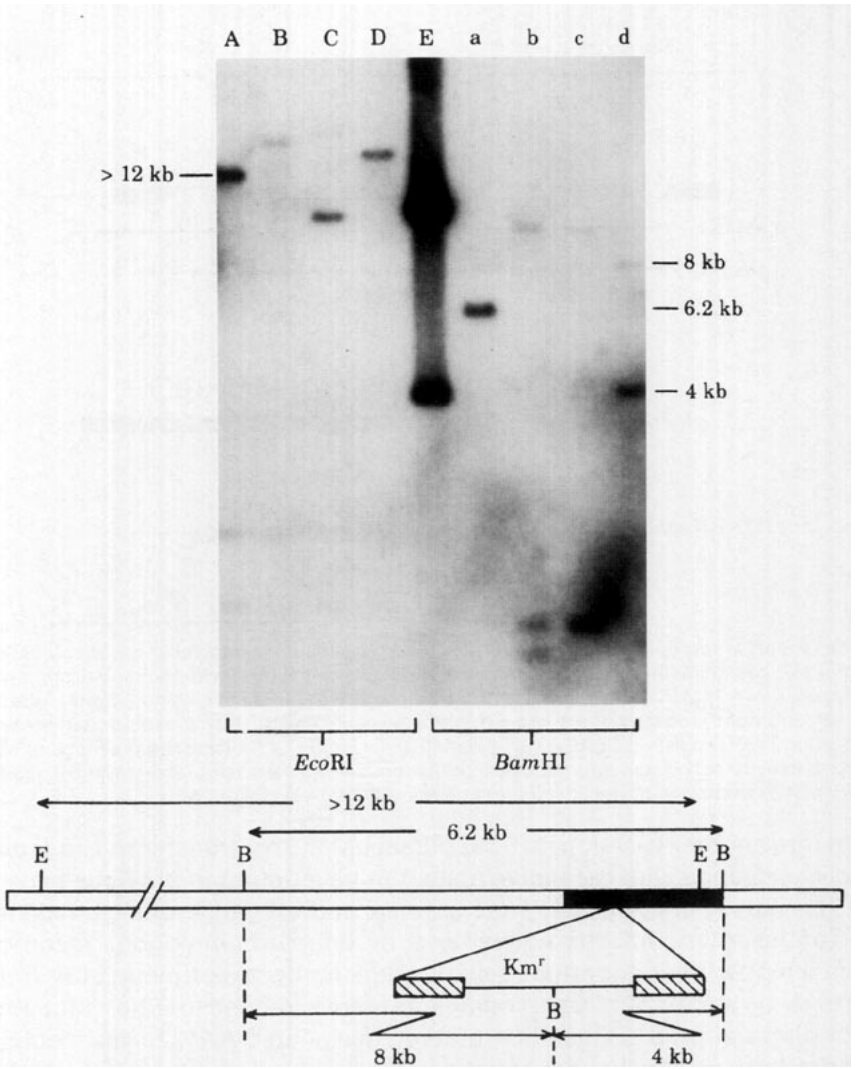

Fig. 4. Southern hybridization analysis of parental and phosphatase-negative mutant strains of *L. pneumophila*. DNA samples analyzed are as follows: lanes A and a, AA103; lanes B and b, AA120, lanes C and c, AA121, lanes D and d, AA122; lane E, pMJK1. Endonuclease digestion was performed as indicated below the blot. The cartoon below depicts the native *pho* locus and the position of the Tn*5* insertion in strain AA122. The black-filled region on the chromosomal map indicates the fragment cloned on pMJK1 which was used as the probe in this experiment.

the viability of infected U937 cells was measured at 48 and 72 h after inoculation. Both the mutant strains and the respective parental strains resulted in a 50–60% reduction in U937 cell viability at 48 h of infection (Fig. 5). In intracellular growth assays, we found that, like the parental strains, the Pho$^-$ mutant strains survived and multiplied normally for 120 h after infection (Fig. 6). The extent of bacterial replication correlated with the reduction in the survival of monolayers. Similar results were obtained from at least three independent experiments. These data indicate that the Pho$^-$ mutant strains were indistinguishable from their parent strains in terms of U937 cell cytopathicity and intracellular growth, and the alkaline phosphatase gene is not necessary for intracellular infection of macrophages.

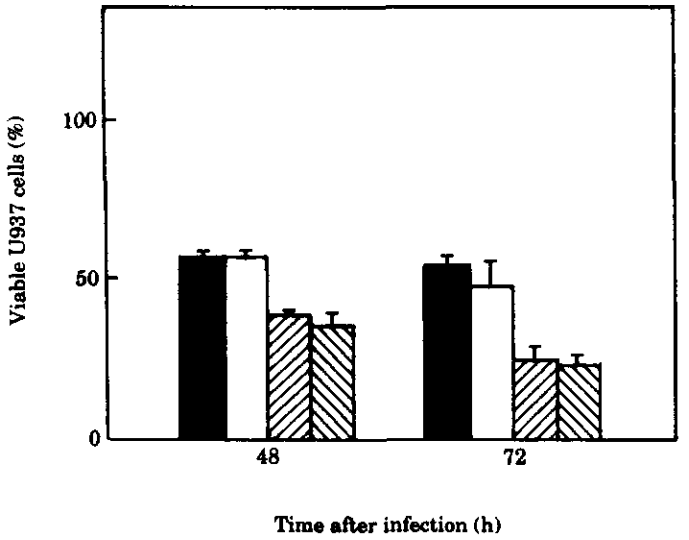

Fig. 5. Cytopathicity of *L. pneumophila* alkaline phosphatase mutants in differentiated U937 cells. (■) AA103 (Pho$^+$); (□) AA122 (Pho$^-$); (▨) AA107 (Pho$^+$); (▧) AA123 (Pho$^-$).

Growth of Pho$^-$ mutants with phosphate restriction

Strains AA103 and AA122 were cultivated in complete defined media (CDM),[15] modified by the substitution of ferric nitrate for ferric pyrophosphate, and growth was monitored by OD_{550}. Growth of AA103 was restricted at inorganic phosphate concentrations of 0.5mM or less. Strains AA103 (Pho$^+$) and AA122 (Pho$^-$) both grew equally well in CDM with any of the following as the sole source of phosphate: 1 mM sodium pyrophosphate, 1 mM adenosine triphosphate, 1 mM glucose-6-phosphate, 5 mM tyrosine phosphate. Strain AA103 grew normally with 5 mM β-glycerol phosphate, whereas strain AA122 required 10 mM for normal growth. We conclude that

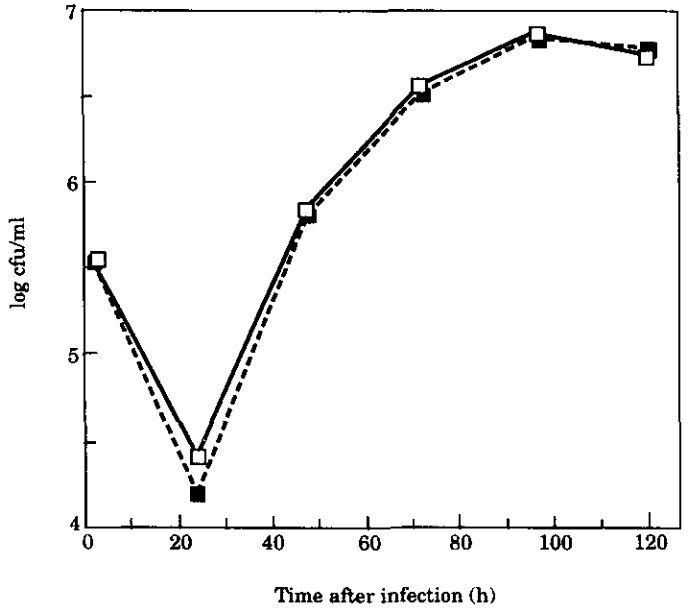

Fig. 6. Growth of alkaline phosphatase mutant AA122 and its immediate parent strain, AA103, in differentiated U937 cells. (■) AA103; (□) AA122.

the lack of alkaline phosphatase activity did not significantly impair the mutant in its ability to acquire phosphate from inorganic or some organic sources that might be encountered within the host cell.

Discussion

The presence of phosphatase activity has been described in several species of *Legionellae*.[16] *L. pneumophila, L. bozemani, L. gormanii*, and *L. dumoffi* all possess alkaline phosphatase. The role of this enzyme in mammalian cell infection is unknown; however, a rare, environmental species, *L. oakridgensis*, that naturally lacks alkaline phosphatase is pathogenic for guinea pigs.[17] We identified two phosphatase activities spanning a broad pH range in our strain of *L. pneumophila* serogroup 1, using mini-Mu transposon mutants with altered phosphatase activity. Similar observation on the broad pH range of phosphatases in *L. pneumophila* was made in earlier work.[18] The most abundant activity was observed at alkaline pH in parental strain AA103. This activity was isolated from a *L. pneumophila* cosmid library in *E. coli*. The mapping and orientation of the responsible gene at the vector-insert junction and the loss of activity associated with a transposon insertion in the adjacent vector sequences suggest that the expression in *E. coli* depends to some extent on transcriptional signals from the vector. This finding is consistent with the notion that the regulation of alkaline phosphatase in other bacteria depends on a cascade of regulatory factors.[19]

The cloning of the phosphatase structural gene permitted us to analyze the nature of the Pho$^-$ mutations obtained by direct transposon mutagenesis. We found that the loss of alkaline phosphatase activity in the Mu*dI*I4041-carrying strain AA120 resulted from an insertion directly into the phosphatase structural gene. A spontaneous pseudorevertant of this strain, AA121, also lacked this activity, but it expressed high levels of acid phosphatase activity. Southern hybridization analysis suggested that AA121 had undergone an additional deletion or rearrangement involving the phosphatase gene that may account for the altered expression of acid phosphatase, although we can only speculate about the mechanism of this up-regulation.

Several lines of evidence indicate that both phosphatases are periplasmic enzymes. They are efficiently released from the cells by osmotic shock procedures. Osmotic shockates contain the highest specific activity of these enzymes among subcellular fractions. Most of the phosphatase activity is measurable in intact cells using PNPP and 5-bromo-4-chloro-3-indolyl phosphate substrates that are unlikely to accumulate in the bacterial cytoplasm. Although we found some phosphatase activity in the cytoplasmic and membrane fractions, this may not reflect the native expression of this activity in these locations, since some periplasmic enzymes can be bound to cytoplasmic ribosomes or have some affinity to cell wall or membrane fragments produced during sonication.

The physiologic function of periplasmic phosphatases in *L. pneumophila* is assumed to be the scavenging of organic phosphorylated compounds that cannot otherwise pass the cytoplasmic membrane. The construction of a *L. pneumophila* alkaline phosphatase mutant permitted us to ask whether the organism is limited to an organic source of phosphate or whether phosphorylated organic compounds are essential during intracellular growth.

Two strains containing directed mutations in the native *L. pneumophila* phosphatase gene, AA122 and AA123, were generated from separate parental strains, AA103 and AA107, respectively. In experiments measuring the cytopathicity of these

strains for U937 cells, we detected no differences between the parent–mutant pairs. Moreover, there was no difference in the intracellular growth or survival of strain AA103 and its Pho⁻ derivative, AA122. We conclude that alkaline phosphatase has no essential role in intracellular bacterial survival and that neither the intracellular availability of phosphate nor the uptake of phosphorylated compounds are limited in the absence of this enzyme. In fact, strain AA122 was able to grow normally *in vitro at* neutral pH using several different organic sources of phosphate. This suggests that other enzymes may dephosphorylate some of these substrates (e.g. nucleotides[20,21]), or that some substrates (e.g. sugar-6-phosphates) may be transported to the cytoplasm intact.[22–24] Apart from the potential organic sources of phosphate in the cell, the intracellular milieu may also be rich in inorganic phosphate. Strain AA122 was also able to grow normally in as little as 1 mM inorganic phosphate, a concentration well below that measured in normal human leukocytes (1.7–2.4 mM).[25]

It is likely that *L. pneumophila* alkaline phosphatase has a more essential role, some other aspect of bacterial ecology than mammalian cell infection, perhaps in surviving nutritionally limited conditions in symbiosis with other aquatic microorganisms. However, the non-essential nature of this enzyme in mammalian cell infection suggests that we can confidently employ a Pho⁻ mutant strain for studies of cellular infection using *E. coli* phosphatase fusions in the future.

Conclusions

L. pneumophila serogroup 1 possesses at least two distinct periplasmic phosphatase activities. A site-directed mutation in the gene encoding the major, alkaline phosphatase activity has no detectable consequences for the bacterium during the infection of human, differentiated U937 cells. Consequently, a Pho⁻ *L. pneumophila* strain may be useful in studies of protein secretion and pathogenesis of this species.

Materials and methods

Bacterial strains and growth conditions. The strains and plasmids used in the study are described in Table 1. *L. pneumophila* AA103 is a spontaneous nalidixic and streptomycin-resistant derivative of a clinical isolate, strain 130b (Wadsworth), serogroup 1 and is a high-frequency conjugal recipient. *L. pneumophila* strains were grown on buffered charcoal yeast extract agar supplemented with α-keto-glutarate (BCYE-α).[26] *E. coli* strains were routinely cultured in Luria-Bertani(LB) medium. Antibiotics were added to the media as previously described.[11,27]

Plasmids, bacterial conjugations and genetic manipulations (see Table 1). Vector plasmids pTLP6 and pTLP7 were constructed in this laboratory for the purpose of gene transfer to *L. pneumophila.*[12] Both plasmids have a unique polylinker with multiple cloning sites, and a chloramphenicol acetyltransferse gene from pACYC184, which is selectable in *L. pneumophila.* Each plasmid also encodes a gene that is counterselectable in *L. pneumophila.* pTLP6 carries the *E.coli rpsL* allele, which confers streptomycin sensitivity on *L. pneumophila* AA103, and pTLP7 carries the sucrose-inducible levansucrase gene (*sacB*) of *Bacillus subtilis*[28] which confers intolerance to 6% sucrose. Both pTLP7 and pTLP6 carry the pRK2 origin of transfer (*oriT*) to facilitate conjugal transfer.

Recombinant plasmids were transferred from *E. coli* to *L. pneumoplila* by triparental matings as described previously.[27] Transconjugants were selected on appropriate antibiotic containing media and screened for phosphatase activity. *E. coli* strain EA103 was used as both a donor strain and helper strain for these matings (Table 1). EA103 is a recombination-defective derivative of a diaminopimelic acid (DAP) auxotroph (χ2981, Δ*asdA4*). This auxotrophy was useful in conjugations, since it permits efficient counterselection of the *E. coli* donor strains on BCYE-α medium without DAP supplementation. Conjugative plasmid pRK212.1 was introduced into EA103 by electroporation to generate a helper strain.

Chromosomal DNA was prepared from *L. pneumophila* using the method of Ausubel *et al.*[29] Plasmid DNA isolation, DNA cloning, and Southern hybridizations were performed using standard methods.[30] Qaiex kits (Qaigen, Chatsworth, CA) were used for the isolation of the DNA fragments from the agarose gels. DNA restriction enzymes were used as suggested by the manufacturers. [^{32}P]-labeled DNA probes were made with the multiprime kit (Amersham, Arlington Heights, IL).

In situ *plate assay for phosphatase activity.* To detect the presence of phosphatase activity in *L. pneumophila*, we used an *in situ* plate assay, as previously described.[11] Briefly, *L. pneumophila* were grown on BCYE-α agar and then lifted to nitrocellulose membranes. The membranes were then applied to agar indicator plates containing 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt (XP) 40 μg/ml. For separate experiments, these plates were buffered with 0.1 M sodium acetate to pH 5 or with 0.1 M Tris-HCl to pH 8.5 to detect acid or alkaline phosphatase activity, respectively. To detect *L. pneumophila* phosphatase activity in *E. coli* clones, bacteria were grown on LB agar supplemented with XP at pH 7.4. Colonies expressing phosphatase activity produce a blue color reaction in plates containing XP.

Preparation of cellular fractions. Periplasmic fractions were obtained using the method of Neu and Heppel.[31] *L. pneumophila* in stationary phase from BCYE-α plates or BYE broth culture were harvested and washed three times with 40 volumes of cold 0.01 M Tris-HCl (pH 8.1). The cell suspension adjusted to 1 at OD_{550} ($\sim 1 \times 10^9$ cells/ml) was resuspended in 1/10 volume of 20% sucrose; 0.03 M Tris-HCl (pH8.1); 1mM EDTA, rotated for 10 min and centrifuged at $13\,000 \times g$. The supernatant fluid was discarded and the pellet was rapidly dispersed with an equal volume of cold water on ice. The resulting mixture was stirred for 10 min and centrifuged at 4°C. The supernatant, 'osmotic shockate', was considered to be a periplasmic fraction. The well-drained pellet was sonicated four times for 40 s and then treated with DNase, RNase, lysozyme (0.1 mg/ml), and EDTA (10mM). Any unbroken cells were removed by low-speed centrifugation to obtain a cleared whole cell lysate. This lysate was then further centrifuged at $100\,000 \times g$ for 3 h and separated into a supernatant or 'cytoplasmic' fraction and a clear pellet or 'membrane' fraction. All subcellular fractions were concentrated using an Amicon Ultrafiltration Stirred Cell fitted with a YM100 membrane and stored at 4°C before use.

Quantitative phosphatase and protein assays. Colorimetric enzyme assays of various subcellular fractions were performed in 96-well microtiter plates with *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) as a substrate. Each reaction mixture consisted of 100 μl of sample and 50 μl of 3X substrate solution (final concentrations: 5 mM *p*-nitrophenyl phosphate; 1 mM $MgCl_2$; 1 mM $ZnCl_2$; 0.1 M sodium acetate (pH 5) or 0.1 M glycine (pH 10.05) for acid and alkaline phosphatase assays, respectively). During incubation at 37°C for 1 h, the plates were vigorously agitated using a microtiter plate agitator. The color change was read directly on an EAR 400 AT automatic ELISA reader at A_{410} and compared with a standard curve made with *E. coli* alkaline phosphatase (Sigma) in a range of 0.12 ~ 0.0039 U per well. For the pH optimization of phosphatase, the following buffers were prepared at different pH ranges; sodium acetate at pH 3 ~ 6, Tris-HCl at pH 7 ~ 9 and CAPS at pH 10 ~ 11. Protein concentration was estimated by the method of Bradford (Bio-Rad, Hercules, CA) using bovine serum albumin as standard.

Tissue culture model of infection. Cytopathicity, intracellular growth and survival of *L. pneumophila* strains were determined in U937 cells as previously described[32] with minor modifications. Monolayers containing $\sim 1 \times 10^6$ differentiated U937 cells per well were infected with the test strains at a ratio of 10:1 or 1:1 (bacteria:cells) in 24-well tissue culture plates and incubated at 37°C. To assay for cytophathic effect at 48 h and 72 h after infection, the infected monolayers were washed three times, and then treated with the vital stain tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-2,5-tetrazolium bromide (MTT; Sigma) for 4 h. Viable, remaining U937 cells were quantitated by measuring the optical density of the vital stain at 550 nm.

To examine intracellular growth and survival, monolayers were lysed at 2, 24, 48, 96 and 120 h after infection, and viable counts of bacteria were determined. At each time point, the supernatant from each well was transferred to a sterile tube, and the remaining monolayers were lysed by the addition of 2 ml of H_2O and repeated pipetting. The lysates were mixed with the supernatant and brought to an even volume.Bacterial counts were determined by diluting the lysates and spotting equivalent volumes onto BCYE-α plates.

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