INFECTION AND IMMUNITY, Sept. 1994, p. 4075–4080 Vol. 62, No. 9
0019-9567/94/$04.00+0
Copyright © 1994, American Society for Microbiology

Shuttle Mutagenesis of *Legionella pneumophila*: Identification of a Gene Associated with Host Cell Cytopathicity

JUAN ARROYO,[1]† MARY C. HURLEY,[1] MARK WOLF,[2] MARK S. McCLAIN,[1]
BARRY I. EISENSTEIN,[1,2]‡ AND N. CARY ENGLEBERG[1,2]*

Departments of Microbiology and Immunology[1] and Internal Medicine,[2] University of Michigan Medical School, Ann Arbor, Michigan 48109

Received 5 April 1994/Returned for modification 17 May 1994/Accepted 7 June 1994

We performed shuttle mutagenesis of *Legionella pneumophila*. Mutants were screened for reduced cellular infectivity. Approximately 10% of the mutants had decreased cytopathicity. The DNA sequence of one locus was determined; the inferred amino acid sequence revealed homology with transport proteins including *Escherichia coli* TolC, *Bordetella pertussis* CyaE, and *Alcaligenes eutrophus* CzcC and CnrC.

An approach to disclosing pathogenic mechanisms of *Legionella pneumophila*, the cause of a severe pneumonia (11, 14, 18), is to isolate mutants defective in virulence-associated traits. We concentrated mutagenesis of *L. pneumophila* on exported gene products because these proteins are likely to play a role in host cell interactions. We used Tn*phoA* to perform random mutagenesis and screened strains with PhoA$^+$ fusions for reduced cytopathicity (21). The use of Tn*phoA* was complicated by its inefficient transposition in *L. pneumophila*. We therefore employed shuttle mutagenesis (5, 30, 31). (Strains and plasmids used in this study are listed in Table 1.)

We constructed an *L. pneumophila* genomic library in cosmid pTLP5 (4); pTLP5 carries both selectable (i.e., chloramphenicol resistance) and counterselectable (i.e., streptomycin resistance) genes. DNA from *L. pneumophila* AA100 was partially digested with *Sau*3A, and DNA fragments were size fractionated on a sucrose density gradient. DNA fragments of ~35 kb were ligated with *Bam*HI-linearized pTLP5 and packaged by use of Gigapack II Gold extracts (Stratagene), according to the manufacturer's protocols. Transductions into *Escherichia coli* DH1 yielded ~10^5 CFU/μg of ligated vector-insert DNA (except where noted, *E. coli* strains were grown at 37°C in Luria-Bertani [LB] broth or agar medium and appropriate antibiotics [6]). A library of 1,000 individual transductants was saved in 96-well microtiter plates. Poisson distribution predicts a >99% probability that any given sequence will be represented in this collection.

For transpositional mutagenesis, we constructed a derivative of Tn*phoA* by inserting the *oriT* sequence of RK2 plasmids (a 0.7-kb *Bam*HI fragment from vector pEYDG1) into the unique *Bam*HI site of Tn*phoA*. The site of insertion inactivates the cryptic, streptomycin resistance gene. The *oriT* sequence allowed conjugal transfer of mutated cosmids into *L. pneumophila* (12). To facilitate the introduction of Tn*phoA-oriT* into strains of interest, a defective λ transducing vector was constructed to deliver the transposon, as has previously been done for delivery of Tn*5* and Tn*phoA* (21). Briefly, a temperature-sensitive λ573 was used to transduce *E. coli* LE392 to obtain a prophage, according to standard protocols (3). This lysogen was transformed with Tn*phoA-oriT* at 4°C to avoid temperature induction of the prophage. Kanamycin-resistant colonies were collected, and a large-scale lysis was induced at 42°C. λ particles were isolated from the lysate as described elsewhere (29), strain LE392 was transduced anew, and kanamycin-resistant lysogens were isolated. Five lysogens were induced at 42°C by the liquid lysate method described by Ausubel et al. (6). The lysate with the highest titer was used to make a final kanamycin-resistant, Tn*phoA-oriT* lysogen, which was immune to λ573. This final lysogen was used in all subsequent experiments to generate λ::Tn*phoA-oriT* transducing lysates.

Shuttle mutagenesis was performed by transducing 96 individual DH1 clones from the pTLP5 library with λ::Tn*phoA-oriT* at a time; replicate microtiter plates were prepared from the master library by use of a pin replicator. The clones were grown to early stationary phase and then transduced at a multiplicity of infection of ~5. After 30 min at 30°C without shaking, the cultures were diluted 1:10 with LB broth containing chloramphenicol and grown, shaking, at 30°C for 3 to 16 h. About 10^6 cells were plated from each transduction on LB agar containing kanamycin, chloramphenicol, and a substrate indicator for alkaline phosphatase, 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt (XP; 40 μg/ml). The LB-XP plates were incubated at 30°C for 48 h, and blue, PhoA$^+$ colonies were readily identified.

To distinguish transpositions in cosmids from those in the chromosome, small-scale matings into the *phoA* deletion strain, CC118, were performed. Briefly, donor, recipient, and helper plasmid-containing *E. coli* were grown in selective media to an optical density at 550 nm of 0.8. The cultures were mixed (1:1:1 ratio) in a 1.5-ml tube, pelleted, and spotted on a piece of nitrocellulose resting on an LB plate. After 3 h at 37°C, bacteria were resuspended in water and dilutions of 10^{-1} to 10^{-4} were spotted onto LB-XP containing chloramphenicol, kanamycin, and spectinomycin. If all three markers were mobilized simultaneously, we assumed that Tn*phoA-oriT* was on the cosmid. PhoA$^+$ transconjugants indicated cosmids with fusions in cloned *L. pneumophila* genes.

Cosmids with PhoA$^+$ fusions were transferred into *L. pneumophila* AA103 by triparental matings (except where noted, *L. pneumophila* was grown at 37°C with buffered charcoal yeast extract agar (BCYE) or buffered yeast extract broth (BYE)

* Corresponding author. Mailing address: 6643 Med Sci II, Box 0620, Univ. of Michigan Medical School, Ann Arbor, MI 48109-0620.

† Present address: Department of Pathology, Tufts University, Boston, MA 02111.

‡ Present address: Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285.

TABLE 1. Bacterial strains and plasmids

Bacterial strain, phage, or plasmid	Characteristics	Source or reference
E. coli		
DH1	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	16
LE392	F^- λ^- *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	23
CC118	*araD139* Δ(*ara leu*) *7696* Δ*lacX74 phoA* Δ20 *galE galK thi rpsE rpoB argE*(Am) *recA1*	21
HB101	F^- *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 supE44* λ^-	7
L. pneumophila		
AA100	*L. pneumophila* SG1 130b	
AA103	Nal^r Str^r derivative of AA100; high-frequency conjugation recipient	9
AA107	Str^r derivative of AA100	M. A. Albano
AA124	AA107 *I21*::Tn*phoA-oriT*	5
AA125	AA103 *hel*::Tn*phoA-oriT*	5
AA125a	AA107 *hel*::Tn*phoA-oriT*	5
AA126	Km^s derivative of AA125; *hel*::Tn*phoA-oriT* replaced by *hel*	This study
Bacteriophages and plasmids		
λ::Tn*phoA-oriT*	Derivative of λ573 with Tn*phoA-oriT* (Km^r Sm^s) inserted in an unknown location	This study
pEYDG1		35
pRK212.1	Ap^r Tc^r Tra^+	D. Figurski
pRTP1	Ap^r *rpsL* (Sm^s) *oriT cos*	32
pTLP5	Derivative of pTP1 with *cat* from pACYC184, Ap^r deleted, *oriT* deleted, and a unique polylinker added	This study
pJAI25	Cm^r Sm^s; vector, pTLP5; cloned *L. pneumophila* 35-kb insert with *I25* gene	This study
pJA6	2.2-kb *Hin*dIII fragment of pJAI25 containing *hel* cloned into pBluescriptKS$^+$; Ap^r	This study

and appropriate antibiotics [2, 12]). Kanamycin resistance was transferred to *L. pneumophila* at frequencies of 10^{-3} to 10^{-4} per recipient. Transconjugants were grown on BCYE containing streptomycin and kanamycin, and individual colonies were plated in replicate onto BCYE-kanamycin with or without 2.5 μg of chloramphenicol per ml to score for allelic exchanges (i.e., loss of the counterselectable vector pTLP5 results in chloramphenicol sensitivity). Passage of transconjugants on agar plates with streptomycin resulted in chloramphenicol sensitivity (i.e., loss of the cosmid) at frequencies of 10^{-1} to 10^{-2}. *L. pneumophila* colonies having the desired antibiotic sensitivities were tested for *E. coli* alkaline phosphatase activity by use of XP-agarose plates such that the endogenous *L. pneumophila* phosphatase was inactive (2).

Although mutagenesis of the library is not complete, 16% of 864 cosmids subjected to Tn*phoA-oriT* mutagenesis have genes that are targets for active alkaline phosphatase fusions in *E. coli*. Fifty-five percent of these fusions could be exchanged with the wild-type gene in *L. pneumophila*, suggesting that 45% of insertions isolated in *E. coli* may have occurred in essential genes of *L. pneumophila*.

Cytopathicity assays were performed in U937 cells as previously described (2), with the following modifications. Bacteria were grown on BCYE for 48 h at 37°C in the absence of antibiotics. To confirm the stability of the *phoA* gene fusions in strains grown under nonselective conditions, all mutants were tested on XP-agarose plates after growth on media without kanamycin. Among 44 PhoA$^+$ (*E. coli* PhoA$^+$) *L. pneumophila* mutants studied, 4 were significantly attenuated (i.e., ≥50% loss of cytopathic effect). This is consistent with results from screening over 80 *L. pneumophila* Mud*phoA* mutants (1). Figure 1 illustrates the results of a cytopathicity assay with a representative, fully infective mutant (AA124) and an attenuated mutant (AA125). The position of a single Tn*phoA-oriT* in the chromosome of these mutants was confirmed by Southern hybridization and presented elsewhere (5). Strain AA125 was found to be ~50% less cytopathic than its parent strain. This is comparable with the degree of attenuation observed with *mip* mutants in use of this assay (8).

We used two methods to confirm that the transposon

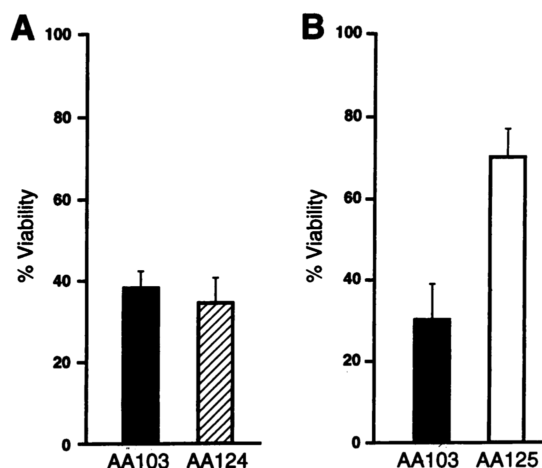


FIG. 1. U937 cell cytopathicity assay of PhoA$^+$ *L. pneumophila*. Bacteria were incubated with U937 cells at a 1:1 ratio for 30 min, and then the monolayers were washed and placed in fresh medium. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] vital staining was performed 72 h after infection. The viability of the infected monolayers was expressed as a proportion of the intensity of MTT staining in infected, versus uninfected, control monolayers (ordinate). Results are means for eight replicate wells; the error bars indicate standard deviations. The filled bars indicate monolayers infected with AA103. The hatched bar in panel A represents a fully infective PhoA$^+$ *L. pneumophila* fusion strain, AA124; the open bar in panel B represents an attenuated PhoA$^+$ fusion strain, AA125. The two panels represent separate experiments.

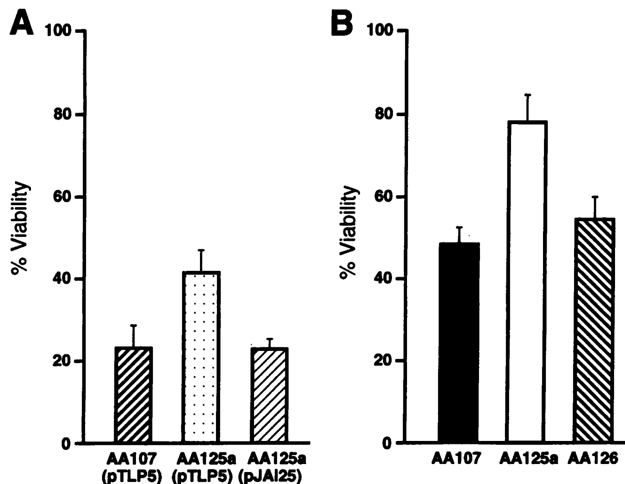


FIG. 2. Complementation and marker rescue of attenuated mutant AA125. (A) Monolayers were infected with (from left to right) AA107(pTLP5), AA125a(pTLP5), and AA125a(pJAI25). All three strains were grown in the presence of chloramphenicol (2.5 μg/ml) before inoculation. U937 cell monolayers were infected at a ratio of 10:1, and MTT staining was performed 48 h after infection. A higher multiplicity of infection was necessary, since growth in chloramphenicol adversely affected the cytopathicity of the inocula (unpublished observations). (B) Monolayers were infected with (from left to right) AA107, AA125a, and a PhoA$^-$ Km^s derivative of AA125a, AA126. A ratio of 0.5:1 was used, and MTT staining was performed 72 h after infection. Viability is expressed as described in the Fig. 1 legend.

insertion in AA125 was responsible for the cytopathic defect. First, we transferred the mutated cosmid (pJAI25::Tn*phoA-oriT*) from *E. coli* to *L. pneumophila* AA107. Exchange of the gene fusion for the native locus was repeated de novo, and the cytopathicity of this strain, AA125a, was tested. AA125a had precisely the same defect in the U937 cell assay as that seen with AA125, suggesting that the insertion at the *I25* locus, and not another unlinked mutation, was responsible for the defect.

Second, we complemented the mutation in *trans* with the *I25* locus from cosmid pJAI25. We transposed Tn*phoA-oriT* into the *rpsL* gene of cosmid pJAI25 in *E. coli* HB101, using kanamycin and streptomycin resistance to select for these particular insertions. Transfer of pJAI25 *rpsL*::Tn*phoA-oriT* to AA125a restored wild-type cytopathicity; the defect in cytopathicity was not complemented by transfer of vector pTLP5 alone (Fig. 2A).

By culturing *trans*-complemented AA125a in the absence of antibiotic selection, we identified PhoA$^-$ progeny at a frequency of 10^{-4} to 10^{-5}. Southern hybridization of one such isolate, AA126, probed with *I25* DNA showed that the gene fusion was lost and an intact copy of the *I25* gene was present on the chromosome, presumably resulting from homologous recombination with the insert on pJAI25 (4). In the cytopathicity assay, AA107 and AA126 had equivalent, wild-type cytopathicity, whereas AA125a was defective (Fig. 2B). These studies confirm that the insertion into the *I25* locus is the cause of the cytopathic defect in AA125a.

Using an oligonucleotide complementary to the 5′ end of *phoA*, we sequenced the fusion junction on pJAI25 (Sequenase Version 2.0 kit; U.S. Biochemicals). From this sequence, additional primers were synthesized (at the University of Michigan Medical School DNA Core Facility) to sequence the sense strand from a 2.2-kb *Hin*dIII fragment of the intact locus subcloned into pBluescript KS$^+$ (pJA6); the complementary strand was sequenced by making nested deletions (Erase-a-Base kit; Promega). The DNA sequence (Fig. 3) revealed an open reading frame (ORF) in frame with the Tn*phoA-oriT* insertion. Translation of the sequence predicted a 395-amino-acid polypeptide of 43,900 Da. A product of this size was identified by in vitro transcription/translation (*E. coli* S30 Extract System; Promega; data not shown). The sequence also shows an incomplete ORF that overlaps the stop codon of the target gene. A truncated product predicted by the sequence analysis of this downstream gene was also seen in the in vitro analysis.

Analysis of the inferred amino acid sequence revealed similarity with a family of transport proteins. DNA sequences were analyzed with MacVector (IBI-Kodak), and homology searches were obtained from the Swiss-Prot and GenBank/EMBL databases with the Genetics Computer Group (University of Wisconsin) version 7.3 VAX system. Among the most similar were proteins of *E. coli* (i.e., TolC), *Bordetella pertussis* (i.e., CyaE), and *Erwinia chrysanthemi* (i.e., PrtF) (Fig. 4). These proteins are required for export of alpha-hemolysin, a bifunctional hemolysin-adenylate cyclase, and a metalloprotease in their respective species (13, 17, 34). Each of these exported products (HlyA, CyaA, and PrtB) is a member of a family of proteins whose excretion is independent of N-terminal signal sequences.

Because of this functional similarity, we examined strains AA107, AA125a, and AA126 for proteolytic and hemolytic activities. The three strains exhibited similar zinc-metalloprotease activity as demonstrated by hide-powder azure assays (reference 10 and data not shown). For analysis of hemolytic activity, blood agar plates were prepared from BYE solidified with washed agar (28). On the basis of initial observations that indicated that only AA125a was not hemolytic towards human or sheep erythrocytes and of the fact that the mutated gene in AA125a does not encode an RTX toxin (such as HlyA [requiring TolC] and CyaA [requiring CyaE]), the mutated gene was designated *hel*, for hemolysis expression in *Legionella* (4).

Subsequent experiments suggested that the observed lack of hemolytic activity may be due to a subtle growth defect on media containing blood, rather than to the lack of hemolysin expression per se. However, strain AA125a did not exhibit a generalized growth defect; growth of strain AA125a was indistinguishable from growth of AA107 on BCYE plates or in BYE broth or chemically defined broth culture (reference 27 and data not shown). The subtle growth defect observed with strain AA125a in the presence of blood suggests that *hel* may be an inappropriate name. However, until a function has been defined for this locus, we will continue to use the *hel* designation.

The predicted *hel* gene product showed 25% identity to *Alcaligenes eutrophus* CzcC and CnrC (Fig. 4). The inferred amino acid sequence of the incomplete downstream ORF suggests homology with another family of transport proteins, MFP (membrane fusion proteins), which includes CzcB and CnrB from *A. eutrophus* (19, 20, 22, 24). The *czc* and *cnr* operons specify resistance to Co^{2+}, Cd^{2+}, and Zn^{2+} and to Co^{2+} and Ni^{2+}, respectively. However, strain AA125a grows as well as strains AA107 and AA126 in the presence of these ions (data not shown).

Whereas available information suggests that *hel* is involved in transport, its function remains to be determined, as does its role in infection of U937 cells. The homology of both the *hel* gene product and the product of the downstream ORF

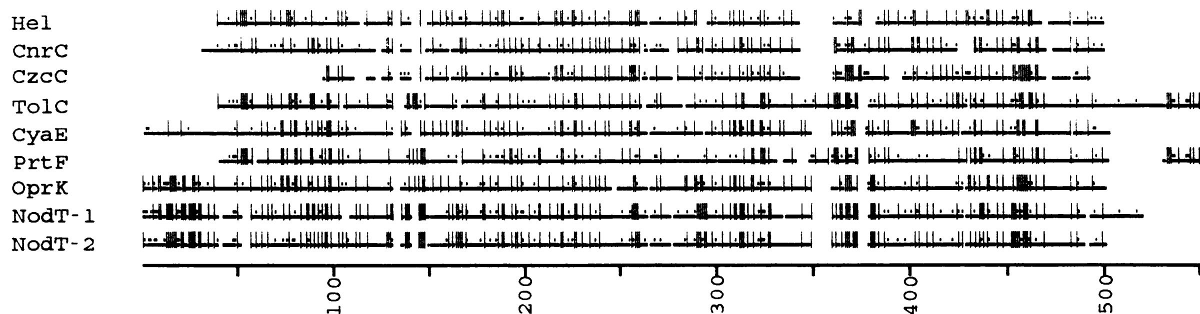
```
        *         *         *         *         *         *         *         *         *       100
AAGCTTCTTGATGATTACATGTATTGCCCTATTTTTGCATAACACACTACTTAATAAAATCCGTTTTTAATTGAAAGCATCTGGTTTTTTACATTGAATT

        *         *         *         *         *         *         *         *         *       200
GGTATCGGTTTTGTTTTATGGATTTCAAGAATTAGGTATTTATAGAGAAAGAAGCATTTACTCATAATTAAAGGCATCAAAACAGTGGTTTAAGTATGGA

        *         *         *         *         *         *         *         *         *       300
TTAAATCCAGTTAGAATGTCAAATCATGGTGTGAACAGATTATCTTGGTTATCTTAATTCAAGGAAAAAGGAATGGGGCTTTTTTTAAGGTTGGTTTCAC
                                                                          M  G  L  F  L  R  L  V  S  L

        *         *         *↓        *         *         *         *         *         *       400
TGGTTTTACTGAGCTTGTCATTCAGTACAAAAGCCAATACAACCTTCACATTCAAACAAGCCTTGGCTATGGCTTATCGCAATAACCCTGAATTGCAAGC
 V  L  L  S  L  S  F  S  T  K  A  N  T  T  F  T  F  K  Q  A  L  A  M  A  Y  R  N  N  P  E  L  Q  A

        *         *         *         *         *         *         *         *         *       500
TGAAATAGATAAAGCGCAAGCCATGAGAGGTGCTTTTATTCAAAGCGGACTTTATCCTAACCCCCAACTTAATTTAACAGCAGAAAATTTTGGTGGTTCT
  E  I  D  K  A  Q  A  M  R  G  A  F  I  Q  S  G  L  Y  P  N  P  Q  L  N  L  T  A  E  N  F  G  G  S

        *         *         *         *         *         *         *         *         *       600
GGTGTCTATTCCAGTTATGAATCAGCAGAAACCACGGCTTCTGTGACACAACCTATTCCTTTAGGGCATCGTCTTCAGTATTTGCAAAAAGCAACCTATG
G  V  Y  S  S  Y  E  S  A  E  T  T  A  S  V  T  Q  P  I  P  L  G  H  R  L  Q  Y  L  Q  K  A  T  Y  A

        *         *         *         *         *         *         *         *         *       700
CGGATTATTTGACTTCTCTTGCGAGTATTAAAGTGCAAAAAACGGTGCTTTATATGGCAGTTGGTAACGCGTACGTGGATGCTTTATATGCAGAACAATG
  D  Y  L  T  S  L  A  S  I  K  V  Q  K  T  V  L  Y  M  A  V  G  N  A  Y  V  D  A  L  Y  A  E  Q  W

        *         *         *         *         *         *         *         *         *       800
GCATAAGGTAACCAAAAAATTAACCAAACTTAATCAAGACATTGTAGTCGCCATTGAACGCCGGGTTAAAGCGGGTGCTGGTGCGGAGTTGGATTTGCGA
 H  K  V  T  K  K  L  T  K  L  N  Q  D  I  V  V  A  I  E  R  R  V  K  A  G  A  G  A  E  L  D  L  R

        *         *         *         *         *         *         *         *         *       900
TTAGCCCAAGTGCGCTTGGGTGAGGCTCGAATTCAGGAAACGAAAGCATCACGTGATGCTTTGCTACAACGAGCAAGGCTCGCCCGTCTATTAGGTTATG
L  A  Q  V  R  L  G  E  A  R  I  Q  E  T  K  A  S  R  D  A  L  L  Q  R  A  R  L  A  R  L  L  G  Y  G

        *         *         *         *         *         *         *         *         *      1000
GGTTAAGAATTGATAAGCCTTTGGTGGATAAAGGATTGCCTGGTCTTACTCTGGATTGGTCCGAATTAATAAAAAAACTACCGCAAAGCCCGCAGCTTGT
  L  R  I  D  K  P  L  V  D  K  G  L  P  G  L  T  L  D  W  S  E  L  I  K  K  L  P  Q  S  P  Q  L  V

        *         *         *         *         *         *         *         *         *      1100
GCAAATGCAATTGCAATTACAAGCCAGGCGAGCCACGATTACTGCCGTTAAAAAATCCGTTTGGCCTGATTTAAATATTCAATTGGGTGGTCGCCATTTT
 Q  M  Q  L  Q  L  Q  A  R  R  A  T  I  T  A  V  K  K  S  V  W  P  D  L  N  I  Q  L  G  G  R  H  F

        *         *         *         *         *         *         *         *         *      1200
TCTGATGATGGCAGTAATGCTGCGGTGATGTCTGCTTTTGCAGAAGTACCTGTTTATAATCGCAATCAAGGGAAAATCATGACGGCTGAAGCGCAATATA
S  D  D  G  S  N  A  A  V  M  S  A  F  A  E  V  P  V  Y  N  R  N  Q  G  K  I  M  T  A  E  A  Q  Y  T

        *         *         *         *         *         *         *         *         *      1300
CTCAAGCGGCTCATGAGTTTCAAAGCACGCGTCTTGAGGTGCGTCAAAATGTCTATGCGGTTTTTTTACAAGCCCAACAAAGTCAATACGAAGCCAATTT
  Q  A  A  H  E  F  Q  S  T  R  L  E  V  R  Q  N  V  Y  A  V  F  L  Q  A  Q  Q  S  Q  Y  E  A  N  L

        *         *         *         *         *         *         *         *         *      1400
AGTCACGGATTCCTTATTGCCCTCCGCGCGTAAGTCCATCCAATTGGCTCAAGAGGGTTATCAAATGGGTCGTTATACTTATGTGGAACTTTATACGGCA
 V  T  D  S  L  L  P  S  A  R  K  S  I  Q  L  A  Q  E  G  Y  Q  M  G  R  Y  T  Y  V  E  L  Y  T  A

        *         *         *         *         *         *         *         *         *      1500
TTAAGTACCTTGTATGAGGAAGAGCGTCATTATCAGCAGGCTCATGCCGATTATCATAAATCTTTAATTCAGATGACAGGGCTTTTAGGATTAGAGCCCA
L  S  T  L  Y  E  E  E  R  H  Y  Q  Q  A  H  A  D  Y  H  K  S  L  I  Q  M  T  G  L  L  G  L  E  P  I

        *         *         *         *         *         *         *         *         *      1600
TTAAGGAGAGTCAATGAAATTTCAAAGGCCATTCATTCCTTATTTTCTATCGCTGATTCTTTTATTGAGCTTTAGTTCGTTTTCCTTAAGAGCTGAAAAC
  K  E  S  Q  *
             M  K  F  Q  R  P  F  I  P  Y  F  L  S  L  I  L  L  L  S  F  S  S  F  S  L  R  A  E  N

        *         *         *         *         *         *         *         *         *      1700
AACCACTCTGAAGAAAGCGAACATCAAAAGACTATGGAAAAAGGTCCACAAGGTGGACGTTTGTTTAAAGAGGGAAATACGGCATTGGAACTTTTAATTT
N  H  S  E  E  S  E  H  Q  K  T  M  E  K  G  P  Q  G  G  R  L  F  K  E  G  N  T  A  L  E  L  L  I  F

        *         *         *         *         *         *         *         *         *      1800
TTGAAAGGGGTATGCCTCCTCGTTTTCGTGCTTACTTGTATCAAAACGGAAAAATGATCTCTCCCTATAAAGCCCATTTAACAGTTGAACTCACTCGGTT
  E  R  G  M  P  P  R  F  R  A  Y  L  Y  Q  N  G  K  M  I  S  P  Y  K  A  H  L  T  V  E  L  T  R  F

        *         *         *         *
TAATGATAAAAAAGAGGTTATTACCTTCATTCCAGTTGAGAATTT
 N  D  K  K  E  V  I  T  F  I  P  V  E  N  >
```

FIG. 3. DNA and predicted amino acid sequences of the *hel* locus. Potential ribosome-binding sites for *hel* and the downstream ORF are underlined. The site of Tn*phoA-oriT* insertion within the *hel* gene is indicated by the arrow between nucleotides 333 and 334. The *hel* gene starts with the ATG codon at nucleotides 273 to 275 and ends with the TGA stop codon (*) at nucleotides 1515 to 1517; the downstream ORF starts with the ATG codon at nucleotides 1514 to 1516.

with products of the *A. eutrophus czc* and *cnr* operons perhaps offers the strongest indication of function. However, it is unclear why an ion export determinant would be required for normal infectivity unless the transported ion is present at high levels in *L. pneumophila*-containing phagosomes. Further studies of the *hel* locus should elucidate the functions of *hel* in vitro and in vivo and may be instructive in characterizing the intracellular environment experienced by *L. pneumophila.*

It also is possible that, like *E. coli* TolC, Hel is involved in many distinct functions; the attenuated phenotype may result from some activity other than protein or ion transport. Additionally, as *hel* appears to be the first gene in an operon, the mutation in AA125a may have a polar effect on a downstream gene whose activity is unrelated to that of *hel*. Indeed, preliminary experimental evidence indicates that the phenotype of AA125a is not corrected by providing only the *hel* gene in *trans*,

A.



B.

	CnrC	CzcC	TolC	CyaE	PrtF	OprK	NodT-1	NodT-2
Hel	44/25	46/25	45/23	38/23	42/22	44/22	41/19	42/17
CnrC		45/31	38/20	50/32	42/24	45/26	41/21	41/21
CzcC			42/25	46/27	47/24	42/23	42/22	46/25
TolC				42/20	46/28	39/21	42/25	41/20
CyaE					44/25	43/25	42/23	44/22
PrtF						40/21	40/19	44/23
OprK							46/26	48/31
NodT-1								72/56

FIG. 4. Similarities between the *hel* amino acid sequence and other bacterial proteins. Hel was aligned with homologous proteins identified by searches of the Swiss-Prot and GenBank/EMBL databases. They are *A. eutrophus* CzcC (24) and CnrC (20), *E. coli* TolC (15, 25), *B. pertussis* CyaE (13), *E. chrysanthemi* PrtF (17), *Pseudomonas aeruginosa* OprK (26), and NodT from two biovars of *Rhizobium leguminosarum* (33). (A) Each amino acid sequence is represented by a horizontal line. Positions having a similar amino acid in half or more of the proteins are indicated with a dot (·) above the line; if half or more of the similar amino acids were identical, the identical amino acids are represented by a vertical bar (|). (B) Each protein was individually aligned with other family members; numbers represent the percent similarity/percent identity.

suggesting that other genes contained on pJAI25 are required for complementation.

Nucleotide sequence accession number. The sequence shown in Fig. 3 has been deposited in GenBank under accession number U11704.

The authors acknowledge helpful discussions with J. Michael Koomey, Garth Jones, and Victor J. DiRita, the technical support of Sue Miller, and the technical advice of Nan Albertson. The authors also thank Paul Hoffman for providing strains.

J.A. was supported by a National Science Foundation predoctoral fellowship. N.C.E. is supported by Public Health Services grants RO1AI26232 and RO1AI24731. Protein homology searches were supported by GCRC grant M01-RR00042.

REFERENCES

1. **Albano, M. A.** Unpublished data.
2. **Albano, M. A., J. Arroyo, B. I. Eisenstein, and N. C. Engleberg.** 1992. PhoA gene fusions in *Legionella pneumophila* generated in vivo using a new transposon, Mud*phoA*. Mol. Microbiol. **6:**1829–1839.
3. **Arber, W., L. Enquist, B. Hohn, N. E. Murray, and K. Murray.** 1983. Experimental methods for use with lambda, p. 433–451. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Arroyo, J.** 1993. Ph.D. thesis. University of Michigan, Ann Arbor.
5. **Arroyo, J., B. I. Eisenstein, and N. C. Engleberg.** 1993. Construction of *phoA* gene fusions in *Legionella pneumophila* that attenuate intracellular infection, p. 85–87. *In* J. M. Barbaree, R. F. Breiman, and A. P. Dufour (ed.), *Legionella*: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1988. Current protocols in molecular biology. John Wiley and Sons, New York.
7. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
8. **Cianciatto, 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.
9. **Cianciatto, N. P., R. Long, B. I. Eisenstein, and N. C. Engleberg.** 1988. Site-specific mutagenesis in *Legionella pneumophila* by allelic exchange using counterselectable ColE1 vectors. FEMS Microbiol. Lett. **56:**203–208.
10. **Dreyfus, L. A., and B. H. Iglewski.** 1986. Purification and characterization of an extracellular protease of *Legionella pneumophila*. Infect. Immun. **51:**736–743.
11. **England, A. C., and D. W. Fraser.** 1981. Sporadic and epidemic nosocomial legionellosis in the United States: epidemiologic features. Am. J. Med. **70:**707–711.
12. **Engleberg, N. C., N. Cianciotto, J. Smith, and B. I. Eisenstein.** 1988. Transfer and maintenance of small, mobilizable plasmids with ColE1 replication origins in *Legionella pneumophila*. Plasmid **20:**83–91.
13. **Glaser, P., H. Sakamoto, J. Bellalou, A. Ullmann, and A. Danchin.** 1988. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. EMBO J. **7:**3997–4004.
14. **Gump, D. W., and M. Keegan.** 1986. Pulmonary infections due to *Legionella* in immunocompromised patients. Semin. Respir. Infect. **1:**151–159.
15. **Hackett, J., and P. Reeves.** 1983. Primary structure of the *tolC* gene that codes for an outer membrane protein of *Escherichia coli* K12. Nucleic Acids Res. **11:**6487–6495.
16. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
17. **Letoffe, S., P. Delepelaire, and C. Wandersman.** 1990. Protease secretion by *Erwinia chrysanthemi*: the specific secretion functions

are analogous to those of *Escherichia coli* alpha-hemolysin. EMBO J. **9**:1375–1382.

18. **Levin, A. S., H. H. Caiafa Filho, S. I. Sinto, E. Sabbaga, A. A. Barone, and C. M. Mendes.** 1991. An outbreak of nosocomial Legionnaires' disease in a renal transplant unit in Sao Paulo, Brazil. Legionellosis Study Team. J. Hosp. Infect. **18**:243–248.
19. **Lewis, K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem. Sci. **19**:119–123.
20. **Liesgegang, H., K. Lemke, R. A. Siddiqui, and H. G. Schlegel.** 1993. Characterization of the inducible nickel and cobalt resistance determinant *cnr* from pMOL28 of *Alcaligenes eutrophus* CH34. J. Bacteriol. **175**:767–778.
21. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.
22. **McClain, M. S.** Unpublished data.
23. **Murray, N. E., W. J. Brammer, and K. Murray.** 1977. Lambdoid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. **150**:1016–1023.
24. **Nies, D. H., A. Nies, L. Chu, and S. Silver.** 1989. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA **86**:7351–7355.
25. **Niki, H., R. Imamura, T. Ogura, and S. Hiraga.** 1990. Nucleotide sequence of the *tolC* gene of *Escherichia coli*. Nucleic Acids Res. **18**:5547.
26. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175**:7363–7372.
27. **Reeves, M. W., L. Pine, S. H. Hunter, J. R. George, and W. K. Harrell.** 1981. Metal requirements of *Legionella pneumophila*. J. Clin. Microbiol. **13**:688–695.
28. **Rogers, J., G. W. Jones, and N. C. Engleberg.** 1993. Growth and phenotypic characterization of *Legionella* species on semisolid media made with washed agar. J. Clin. Microbiol. **31**:149–151.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Seifert, H. S., R. S. Ajioka, D. Paruchuri, F. Heffron, and M. So.** 1990. Shuttle mutagenesis of *Neisseria gonorrhoeae*: pilin null mutations lower DNA transformation competence. J. Bacteriol. **172**:40–46.
31. **Seifert, H. S., E. Y. Chen, M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83**:735–739.
32. **Stibitz, S., W. Black, and S. Falkow.** 1986. The construction of a cloning vector designed for gene replacement in *Bordetella pertussis*. Gene **50**:133–140.
33. **Surin, B. P., J. M. Watson, W. D. O. Hamilton, A. Economou, and J. A. Downie.** 1990. Molecular characterization of the nodulation gene, *nodT*, from two biovars of *Rhizobium leguminosarum*. Mol. Microbiol. **4**:245–252.
34. **Wandersman, C., and P. Delepelaire.** 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl. Acad. Sci. USA **87**:4776–4780.
35. **Yakobson, E. A., and D. G. Guiney, Jr.** 1984. Conjugal transfer of bacterial chromosomes mediated by the RK2 plasmid transfer origin cloned into transposon Tn*5*. J. Bacteriol. **160**:451–453.