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# The *Legionella pneumophila hel* Locus Encodes Intracellularly Induced Homologs of Heavy-Metal Ion Transporters of *Alcaligenes* spp.

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**We continued characterization of the *Legionella pneumophila hel* locus. Mutagenesis and DNA sequencing identified three genes similar to the *czc* and *cnr* loci of *Alcaligenes eutrophus* and the *ncc* locus of *Alcaligenes xylosoxidans*. On the basis of their similarity to these loci, we designated the *L. pneumophila* genes *helC*, *helB*, and *helA*. Mutations in the *hel* genes led to reduced cytopathicity towards U937 cells, although the mutant strains did not appear defective in other assays of virulence. Transcription of the *hel* locus was induced by the intracellular environment but was not induced by any of a variety of in vitro stress conditions. The function of the *hel* gene products remains to be determined.**

We recently identified a locus, *hel*, associated with *Legionella pneumophila* cytopathicity (4). We cloned and sequenced the mutated gene and found the predicted product to be similar to a family of transport proteins of bacteria, including *Alcaligenes eutrophus* CzcC and CnrC and *Alcaligenes xylosoxidans* NccC (4, 18, 22, 26, 35). The *czc*, *cnr*, and *ncc* loci each encode three proteins that function together in the resistance to heavy metal ions (18, 26, 35). The CzcA, CnrA, and NccA proteins are members of the RND family of gram-negative bacterial transport proteins (17, 33, 34). RND proteins are believed to be proton antiporters located in the cytoplasmic membrane and to transport their substrates from the cytoplasm to the periplasm. The CzcB, CnrB, and NccB proteins are members of the MFP family of gram-negative bacterial transport proteins (11, 17, 34). MFP proteins are believed to span the cytoplasmic membrane, extend into the periplasm, and mediate localized fusion between the cytoplasmic and outer membranes. The locations of CzcC, CnrC, and NccC are uncertain (26). It has been suggested that RND and MFP proteins function together with an outer membrane protein to transport substrate directly from the cytoplasm of gram-negative bacteria to the extracellular space (11, 33). Consistent with this model, we showed that CzcC, CnrC, and NccC are members of a family of outer membrane transport proteins from gram-negative bacteria (4, 22).

The current study continues our characterization of *hel* (4). We examined the genetic organization of the locus with mutagenesis and DNA sequencing. We studied the function of the *hel* gene products by examining the effect of various ions on the growth of mutants and on the expression of gene fusions in *L. pneumophila*. We also continued our study of the role of the *hel* gene products in the virulence of *L. pneumophila*, including a comparison of *hel* expression intracellularly and in vitro.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains are listed, with genotypes and sources, in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Bacto Tryptone and Bacto Yeast extract were purchased from Difco Laboratories, Detroit, Mich.) at 37°C with aeration for the purposes of plasmid construction and propagation. For plate medium, agar (BBL, Becton Dickinson, Cockeysville, Md.) was added to 1.5%. Media were supplemented with ampicillin (40 μg/ml), chloramphenicol (20 μg/ml), kanamycin (25 μg/ml), or diaminopimelic acid (40 μg/ml) where appropriate (Sigma Chemical Co., St. Louis, Mo.).

*L. pneumophila* strains were grown in BYE medium or chemically defined medium at 37°C with aeration for propagation (31); BCYE medium or washed-agar chemically defined medium was used for the plate medium (32). Media were supplemented with chloramphenicol (5 μg/ml), kanamycin (25 μg/ml), or streptomycin (300 μg/ml) where appropriate (Sigma).

**Recombinant DNA techniques.** Restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; or New England Biolabs, Beverly, Mass.) and T4 DNA ligase (Bethesda Research Laboratories) were used as described in the manufacturers' recommendations. Oligonucleotides were synthesized at the University of Michigan's Biomedical Research Core Facilities. DNA sequencing was performed with Sequenase II (U.S. Biochemical Corporation, Cleveland, Ohio). DNA labelling was performed with the ECL Random Prime Labelling System purchased from Amersham Corp. (Arlington Heights, Ill.). All other molecular genetic procedures were performed essentially as described previously (5).

**Shuttle mutagenesis.** Shuttle mutagenesis of *L. pneumophila* was performed with Tn*phoA-oriT* as described previously (4). Briefly, *E. coli* CC118 carrying cosmid pJA3 (including the *hel* locus) was transduced with λ::Tn*phoA-oriT*. Colonies that contained $PhoA^+$ insertions in pJA3 were identified. The sites of insertion were identified by restriction enzyme analysis and confirmed by DNA sequencing of the sites of insertion. Cosmids of interest were introduced into *E. coli* EA105 by electroporation, and the cosmids were subsequently introduced into *L. pneumophila* AA200 by triparental mating (12). For allelic exchange, AA200 clones resistant to kanamycin were identified, cultured in broth containing kanamycin, and plated on BCYE plates supplemented with streptomycin to select for loss of the cosmid. Colonies resistant to streptomycin were rescreened for resistance to kanamycin. Southern hybridization analysis confirmed the allelic exchange.

**Antimicrobial sensitivity testing.** The sensitivities of *L. pneumophila* strains to a variety of compounds was determined. The compounds tested included cations ($Ca^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Mg^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Na^+$, $Hg^{2+}$, $Pb^{2+}$, $Mn^{2+}$, $Cr^{3+}$, $Ag^+$, $Fe^{2+}$, and $Fe^{3+}$) antimicrobial drugs (acridine orange, gentamicin, tetracycline, crystal violet, ciprofloxacin, 2,2′-dipyridyl), and fatty acids (caproate and caprate). Isogenic wild-type and mutant strains were grown on BCYE plates for 48 h and then resuspended to similar optical densities in BYE broth or chemically defined broth.

For determination of the MIC, the bacterial suspensions were used to inoculate BYE broth or chemically defined broth containing twofold serial dilutions of a given compound to be tested. These cultures were grown for 16 to 24 h at 37°C with aeration, at which time the optical density of each culture was determined. The MIC was defined as the lowest concentration of compound that inhibited the growth of *L. pneumophila.*

For determination of zones of inhibition, the bacterial suspensions were used to inoculate washed-agar chemically defined medium such that a lawn of bacterial growth would develop following incubation. Filter paper discs soaked in concentrated solutions of the test compounds were aseptically placed on top of the bacterial inoculum. Plates were incubated at 37°C for 48 to 72 h. The zone of inhibition was determined as the diameter of the region around the filter paper discs in which bacterial growth had been inhibited.

**Determinations of alkaline phosphatase and β-galactosidase activity.** Qualitative determinations of alkaline phosphatase expression in *L. pneumophila* were

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TABLE 1. Bacterial strains, plasmids, and phages used

| Strain, plasmid, or phage | Characteristics | Reference or source |
|---|---|---|
| *L. pneumophila* | | |
| AA100 | *L. pneumophila* SG1 130b | 9 |
| AA200 | $Str^r$ derivative of AA100 | M. A. Albano |
| AA201 | AA200 *helC*::Tn*phoA-oriT* | This study |
| AA205 | AA200 *helB*::Tn*phoA-oriT* | This study |
| AA209 | AA200 *orf2*::Tn*phoA-oriT* | This study |
| AA213 | AA200 Δ(*helBA-orf2*)-*lacZ*-$Kan^r$ | This study |
| *E. coli* | | |
| EA105 | $F^-$ $\lambda^-$ Δ41(*proAB-lacYZ*) $T3^r$ Δ*asdA4* Δ*zhf2*::Tn*10 cycA1 recA56 srl3000*::Tn*10* | M. A. Albano |
| CC118 | *araD139* Δ(*ara-leu*)*7696* Δ*lacX74 phoA* Δ20 *galE galK thi rpsE rpoB argE*(Am) *recA1* | 21 |
| XL1 Blue | | Stratagene |
| Plasmids and bacteriophage | | |
| pJA3 | ~14-kb *Sac*I-*Bam*HI *hel*-containing fragment cloned into pTLP5 | J. Arroyo |
| pJAI25::Tn*phoA-oriT* | pJA3 with Tn*phoA-oriT* insertion into *helC* | 4 |
| pJA3::Tn*phoA-oriT* #9 | pJA3 with Tn*phoA-oriT* insertion into *helB* | This study |
| pJA3::Tn*phoA-oriT* #7 | pJA3 with Tn*phoA-oriT* insertion into *helA* | This study |
| pJA3::Tn*phoA-oriT* #12 | pJA3 with Tn*phoA-oriT* insertion into *orf2* | This study |
| pMM202 | ~1.8-kb *Sac*I-*Bsp*EI *orf2* fragment cloned into *Sac*I-*Xma*I-cut pBluescript $SK^-$ | This study |
| pMM205 | ~2.2-kb *Hin*dIII *helCB* fragment inserted into *Hin*dIII site of pMM202; *helCB* is in the same orientation as *orf2* | This study |
| pMM206 | ~4.7-kb *Pst*I fragment carrying *lacZ*-$Kan^r$ cassette from pKOK6 cloned into *Pst*I site of pMM205; *lacZ* is transcriptionally fused to *helB* | This study |
| pMM207 | ~9-kb *Apa*I-*Xba*I fragment from pMM206 cloned into *Apa*I-*Xba*I-cut pTLP6; used to generate AA213 | This study |
| pTLP5 | $Chl^r$ $Str^s$ | 4 |
| pTLP6 | Mobilizable (*oriT*) derivative of pTLP5 | J. Arroyo |
| pKOK6 | *lacZ* $Kan^r$ | 15 |
| pRK212.1 | $Amp^r$ $Tet^r$ $Tra^+$ | D. Figurski |
| pBluescript $SK^-$ | | Stratagene |
| λ::Tn*phoA-oriT* | | 4 |

made as described previously (3). Quantitative determinations of alkaline phosphatase or β-galactosidase expression in *L. pneumophila* were made essentially as described previously (23, 24). For alkaline phosphatase assays, the reactions were carried out in 0.1 M CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer (pH 11).

To examine induction of the reporter genes, bacteria were grown on BCYE plates for 48 h, resuspended to similar optical densities in BYE broth or BYE broth containing subinhibitory concentrations (i.e., those concentrations used in determining the MIC which did not inhibit growth) of various ions (including $Cd^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Zn^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Ca^+$, $K^+$, and $Na^+$), and incubated at 37°C with aeration. Enzyme assays were performed after 16 to 24 h of growth, and β-galactosidase activity was calculated in terms of enzyme units per optical density at 550 nm rather than 600 nm (24).

In addition, β-galactosidase expression was monitored under various growth conditions in vitro. Culture aliquots from the various growth conditions were plated on BCYE medium to determine the number of CFU per milliliter; β-galactosidase activity could thus be expressed as units per CFU (24). Bacteria were grown on BCYE plates for 48 h and used to inoculate BYE medium. Bacteria were then grown at 37°C with aeration for 16 h. Bacteria from the overnight culture were then diluted into prewarmed BYE medium to an optical density of 0.01 to 0.03. Cultures were incubated with aeration at 30, 37, or 42°C with aeration; growth and β-galactosidase activity were monitored at the mid-log phase (optical density at 550 nm of 0.1 to 0.6), the late log phase (optical density at 550 nm of 0.7 to 1.0), or the stationary phase (optical density at 550 nm of 1.1 to 1.5). To assess the effect of a heat shock on β-galactosidase expression, cultures incubated at 30°C in the mid-log phase were rapidly shifted to 42°C; β-galactosidase activity was measured 1 h following the temperature shift. To assess the effect of osmotic shock on β-galactosidase expression, NaCl was added to a concentration of 0.5 M to cultures incubated at 37°C at the mid-log phase; β-galactosidase activity was measured 1 h following the addition of NaCl. To assess the effect of oxidative stress on β-galactosidase expression, $H_2O_2$ was added to a concentration of 0.05 mM to cultures incubated at 37°C at the mid-log phase; β-galactosidase activity was measured 1 h following the addition of $H_2O_2$. To assess the affect of acid shock on β-galactosidase expression, HCl was added to cultures incubated at 37°C at the mid-log phase to adjust the pH of the medium from 7 to between 5 and 6; β-galactosidase activity was measured 1 h following the addition of HCl. To assess the effect of tissue culture medium on β-galactosidase expression, bacteria were grown on BCYE plates for 48 h at 37°C, resuspended in RPMI 1640 with 10% fetal bovine serum, and incubated at 37°C for 4 h.

To determine β-galactosidase activity from intracellularly grown bacteria, bacteria cultured on BCYE plates for 48 h were used to inoculate differentiated U937 cells in six-well tissue culture dishes at a multiplicity of infection of approximately 100 *L. pneumophila* bacteria per cell in RPMI 1640 with 10% fetal bovine serum (28). Infection was allowed to proceed for 2 h at 37°C in 7% $CO_2$. Following this incubation, monolayers were washed once with medium and then overlaid with medium containing 50 μg of gentamicin per ml. Monolayers were incubated for 2 h at 37°C in 7% $CO_2$. Following this incubation, monolayers were washed three times in medium, overlaid with fresh medium ($t$ = 0 h), and incubated at 37°C in 7% $CO_2$. At various times (ranging from $t$ = 15 to $t$ = 24 h), dishes were washed three times in phosphate-buffered saline, and monolayers were lysed osmotically by the addition of 0.2 ml of $H_2O$ to each well; cell lysis was monitored microscopically. The cell lysates from each well of a six-well dish were combined. Aliquots of the lysate were diluted and plated on BCYE medium to determine the number of viable bacteria per milliliter of lysate. Additionally, β-galactosidase activity was determined from the lysate and calculated in terms of enzyme units per CFU (24).

**Assays of *L. pneumophila* virulence.** Cytopathicity of *L. pneumophila* strains towards differentiated U937 cells was monitored as described previously, with minor modifications (4). *L. pneumophila* strains were cultured on BCYE plates at 37°C for 48 h and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum. U937 cell monolayers were infected at a multiplicity of infection of approximately 20 *L. pneumophila* bacteria per U937 cell. Infection was allowed to proceed for 2 h at 37°C, at which time monolayers were washed three times with medium. Following the third wash, medium containing 50 μg of gentamicin per ml was added to kill extracellular bacteria. Infected cells were incubated for 48 h at 37°C, at which time Alamar blue (Alamar, Sacramento, Calif.) was added. Cytopathicity was determined by comparing the amount of Alamar blue dye reduced in infected samples with that reduced in uninfected samples and was expressed in terms of the percent monolayer survival.

The ability of *L. pneumophila* strains to enter and grow within U937 cells was determined as described previously (28). Briefly, monolayers were infected as described above. Following 2 h of infection and washing, infected monolayers were incubated in the presence of 50 μg of gentamicin per ml for 2 h and then washed three times with medium; fresh medium was then added, and the monolayers were incubated at 37°C. At various times (0, 4, and 24 h) after the addition of antibiotic-free medium, monolayers were washed three times with phosphate-buffered saline and then osmotically lysed by the addition of $H_2O$. Tenfold serial dilutions of the lysates were spotted onto BCYE plates to determine the number of bacterial CFU.

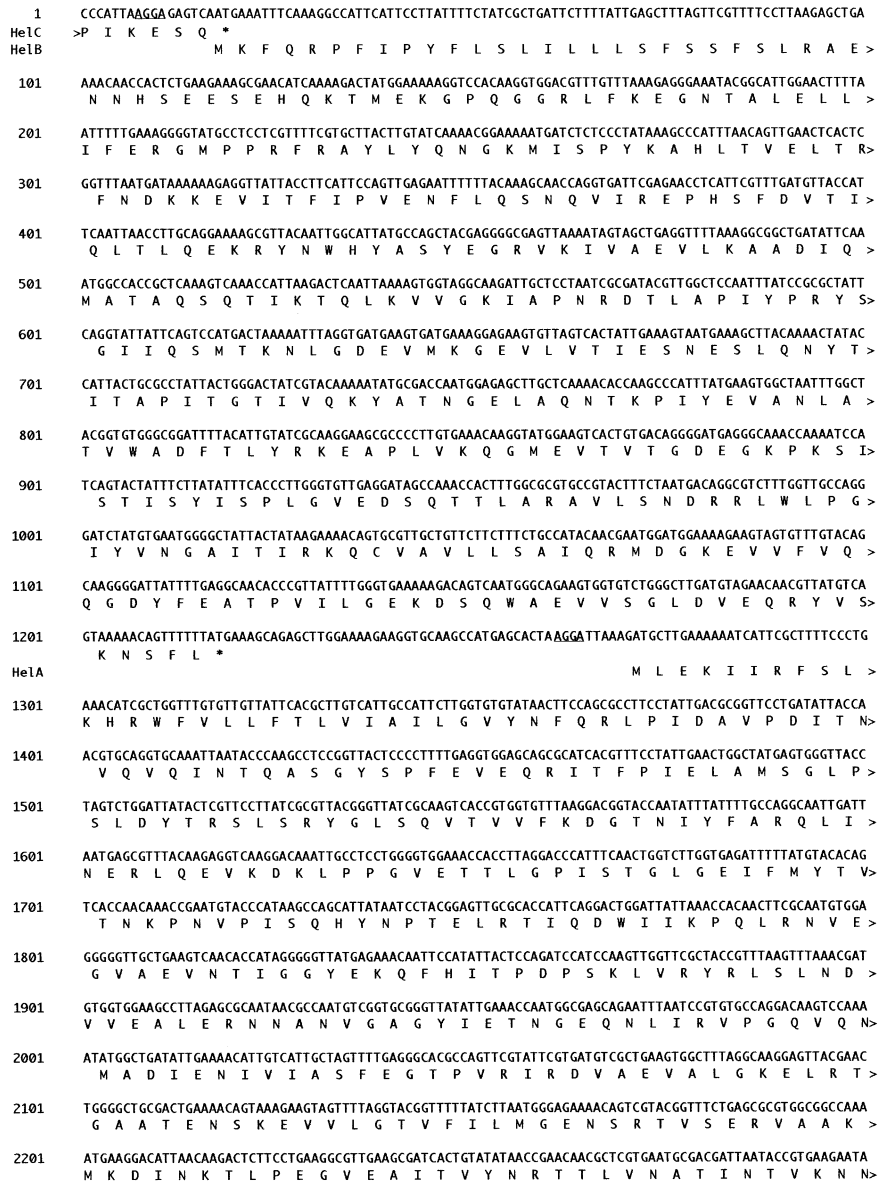


FIG. 1. DNA and predicted amino acid sequences of the region including the 3′ end of *helC*, *helB*, *helA*, and *orf2*. Potential ribosome binding sites are underlined.

The dose of *L. pneumophila* required to infect 50% of U937 cell monolayers (50% infective dose) was determined as described previously (8, 30). Briefly, six to eight replicate U937 cell monolayers were infected separately with each of a series of 10-fold serial dilutions of *L. pneumophila* suspensions. Monolayers were incubated for 2 h at 37°C and washed three times as described above. Monolayers were incubated in the presence of 50 μg of gentamicin per ml for 2 h and then washed three times with medium. Fresh medium without antibiotics was added, and the monolayers were incubated for 24 h at 37°C. Aliquots of the medium from each monolayer as well as lysates (prepared as described above) of each monolayer were spotted onto BCYE plates. The 50% infective dose was defined as the minimum inoculum size that yielded *L. pneumophila* in 50% of the monolayers (8).

The abilities of *L. pneumophila* strains to infect guinea pigs were determined as described previously, with minor modifications (7, 36). *L. pneumophila* strains (AA200, AA201, and AA205) were cultured on BCYE plates at 37°C for 48 h and resuspended in water to ~$2.5 \times 10^7$ bacteria per ml. The animals were anesthetized by intraperitoneal injection of 5 mg of xylazine per kg and 30 mg of ketamine per kg. The tracheas of anesthetized animals were exposed, and 200 μl of bacterial suspension (~$5 \times 10^6$ bacteria) was injected into the trachea. Following intratracheal inoculation, the incision was closed with a single surgical staple. Each of the bacterial suspensions was used to infect eight animals. The animals were monitored daily for weight changes and survival.

The abilities of *L. pneumophila* to infect the amoeba *Hartmannella vermiformis* were determined as described previously (13). Amoebas were infected at a multiplicity of infection of 1 *L. pneumophila* bacterium per 10 *H. vermiformis* amoebas. The number of bacterial CFU recovered from the coculture supernatants was monitored daily over the first 4 days.

**Nucleotide sequence accession numbers.** The complete nucleotide sequences reported in this communication have been deposited in GenBank under accession numbers U49498 (Fig. 1) and U49497 (Fig. 3).

## RESULTS

**Sequence analysis of *hel*.** We subjected the *hel*-containing cosmid pJA3 to Tn*phoA-oriT* mutagenesis (4). DNA sequence analysis of the region downstream of the mutated *hel* gene, aided by sequencing from sites of Tn*phoA-oriT* insertion and exonuclease III-generated deletions of subclones (Erase-a-Base; Promega), indicated two genes that are likely to be part of an operon with the mutated *hel* gene (Fig. 1). The complete sequence of the previously identified open reading frame

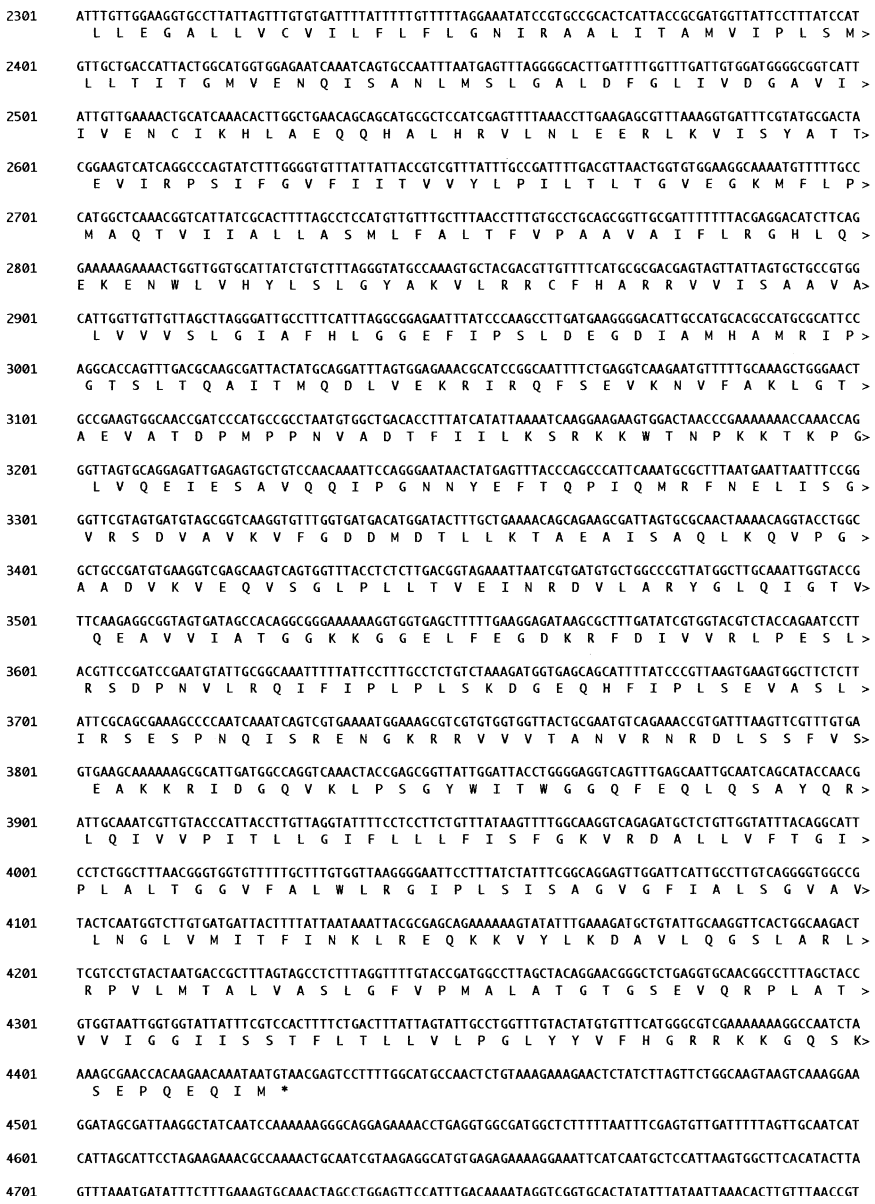


FIG. 1—*Continued.*

downstream of the mutated *hel* gene is predicted to encode a protein of 400 amino acids (45 kDa, pI 8.4). The newly identified third open reading frame is preceded by a potential ribosome binding site and begins 50 nucleotides downstream of the second open reading frame. The predicted protein consists of 1,052 amino acids (116 kDa, pI 9.1).

Searches of the Swiss protein and GenBank-EMBL nucleotide sequence databases revealed that the predicted product of the second open reading frame is homologous to members of the MFP family of proteins, including CzcB, CnrB, and NccB from *Alcaligenes* species; the third open reading frame is homologous to members of the RND family of proteins, including CzcA, CnrA, and NccA from *Alcaligenes* species (11, 17, 18, 26, 33–35). Because the previously mutated *hel* gene and the two downstream open reading frames are similar in arrangement and predicted protein sequences to genes of the *czc*, *cnr*, and *ncc* loci of *Alcaligenes* species, we have named the *hel* genes *helC*, *helB*, and *helA* (Fig. 2) (18, 26, 35).

The *Alcaligenes* loci are each regulated by products of several genes that map nearby, upstream of the *helC* homolog and/or downstream of the *helA* homolog (10, 18, 25, 35). These regulatory genes may include members of a newly recognized subfamily of RNA polymerase σ-factors involved in the regulation of extracytoplasmic functions (19). To determine whether such genes map near the *hel* locus, we continued to determine the DNA sequence both upstream of *helC* (Fig. 3) and downstream of *helA* (Fig. 1). The predicted amino acid sequence of an open reading frame upstream of *helC*, *orf1* (corresponding to the C terminus of the predicted protein), exhibited significant similarity to the catalytic domains of a diverse family of NAD-binding dehydrogenases (data not shown). One of the most similar proteins (44.8% amino acid


4801 TTAATTAACTATGTTAACAGAAGACCAAATTATCACATTAAGTGACATCTTACATTTAATGGCGAAGCCAATCGGTTAAAGCTCCTCTTGAGGTGTCTGG
4901 AAGGTCCCAAATCCGTATCTGAGCTTGCTGAGCAATTACAGCTTTCTGTGCCTTTAACCAGCCATCATCTGAGTCTCGTCCGCTCAGCACGTCTTTTAAT
5001 AGCCACTCGAGAAGGCAAACACATTTACTACAGTATTTATGACAGGCATGTGCGCTGCATCTTAGAGGACATGCTCAAACACTTTACTGAGGAAATGGAG
5101 AATTAACATGGGCTTTTTAAAAAGAATTTTTGGCAATGGCTTGAGCGATCATCATGGCGGTTACCGTAACTCTCATCATGGTCAAAGAAATGATTATCGA
5201 GAGGACTCAGCTAATCTTTCACGAGGCAAAATGAATTGCCCACGATGTCAAACTTCTCTTACTCCTGGGATGCGGTTTTGCAGTGAATGTGGCAGTAGCC
5301 TGGAAAGTGCACTATGCATTTGTGGTGCAGTGATTGCCATAGGTGCGAAATTTTGTGGCCAATGTGGGAAATCTTTATGACAACGCCTTTAAGGTTAACA
Orf2 M H L W C S D C H R C E I L W P M W E I F M T T P L R L T >
5401 ACGTTTTTTGTGGCAGGACTGGATTGTCCTGCAGAAGAGCAACTGATTCGACGACAATTACAGGGCATTCCTGAAATTGAACATTTGGATTTTAATTTCA
T F F V A G L D C P A E E Q L I R R Q L Q G I P E I E H L D F N F I>
5501 TTGCTGAAGAAGTGACTATTCACCATCGTCTTTCTTCCATAGATTTGTTGCAACAACGTATTGCAGCATTAGGAATGAGTGTGCGTTCTAAAAGCGGCAT
A E E V T I H H R L S S I D L L Q Q R I A A L G M S V R S K S G I >
5601 CCATCCAGCAAAAGAAGGAGGGAGGTCTCTTTCTGATTCGTCTTGGCTACTCATTATTTTAGCTGGTGTTTTCGCTCTTTTTTCCGAATTGGCGGCTTAT
H P A K E G G R S L S D S S W L L I I L A G V F A L F S E L A A Y >
5701 TTTTTAGGCACAGAGCAATCCACCTGGAGTATTATCCCTGCCTTATTGGCTATGGTCTTAAGTGGTTCACCTACTTTCAAAAAAGGTTGGCTTGCTTTGC
F L G T E Q S T W S I I P A L L A M V L S G S P T F K K G W L A L R>
5801 GCACCAAAGCCATGAATATTAACAGTTGATGTTGATTGCAATCAGTGGTGCTGTATTGATAGGCGAGTGGCCTGAGGCTGCGATGGTTACCGTGTTGTT
T K A M N I N S L M L I A I S G A V L I G E W P E A A M V T V L F >
5901 TGCTTTGGCTGAGCGTATCGAGCGTTATTCGTTAGATAAAGCGCGATTAGCTATTCGAAGTTTGATGCAAATTGCTCCGGAAGTGGCTCGAGTTAAAATG
A L A E R I E R Y S L D K A R L A I R S L M Q I A P E V A R V K M >
6001 GACAATGGTCAATGGCAAACCATGCCGGTAGAAGAGGTACCACTAGAGGCTGTTTTCAGGGTAAGGCCGGGTGAGCGAATTCCCTTGGATGGAGTAGTGA
D N G Q W Q T M P V E E V P L E A V F R V R P G E R I P L D G V V I>
6101 TTTCGGGGCAAAGTACCGTCAATCAAGCGCCGATTACAGGTGAGTCCATGCCAGTAGTCAACAGAGGGGATGAGGTTTTTGCAGGTAGTTTAAATGAGCT
S G Q S T V N Q A P I T G E S M P V V N R G D E V F A G S L N E L >
6201 AGGTGCTTTTGAGGTCCAGGTCACAAAAGCATCAGGAGATACGCTGCTTGCTAAAATTGGTAAGGCCATTGAACAAGCGCAGGCTGAACGGCGCCAACTC
G A F E V Q V T K A S G D T L L A K I G K A I E Q A Q A E R R Q L >
6301 AACGTTTTGTGGACCAATTTGCCAAGTATTACACACCCATCATGGTCTTATAGAATTCTTATTGCACTGTTCCCTCCATTGGCATTGGGTTATCCTTTTT
N V L W T N L P S I T H P S W S Y R I L I A L F P P L A L G Y P F Y>
6401 ATGACTGGCTCTATAAGGCGTTAACTTTGCTGGTGATTGCTTGCCCTTGTGCTTTAGTCATTTCAACGCCGGTGACCGTT
D W L Y K A L T L L V I A C P C A L V I S T P V T V >

FIG. 1—*Continued.*

identity over 87 amino acids) was the EnvM (FabI), NADH-dependent enoyl-acyl carrier protein reductase from *E. coli* (6). The predicted amino acid sequence of the first significant open reading frame downstream of *helA*, *orf2* (corresponding to the N terminus of the predicted protein), is homologous to members of the P-type ATPase family of proteins and is most similar to the CadA, $Cd^{2+}$-transporting proteins from *Listeria monocytogenes* (37% amino acid identity over 183 amino acids), *Staphylococcus aureus* (32% amino acid identity over 199 amino acids), and *Bacillus firmus* (32% amino acid identity over 197 amino acids) (14, 16, 27). Thus, the *hel* locus does not appear to be regulated by the products of adjacent upstream or downstream genes.

**Function of the *hel* gene products in vitro.** To aid in determining the function of the *hel* gene products, we constructed *helC*, *helB*, and *orf2* mutant derivatives of strain AA200 (AA201, AA205, and AA209, respectively) by allelic exchange with cosmids carrying Tn*phoA-oriT* insertions (Fig. 4 and data not shown). Under conditions that inhibit the endogenous alkaline phosphatase of *L. pneumophila*, strains AA201 and AA205 demonstrated alkaline phosphatase activity in the presence of 5-bromo-4-chloro-3-indolyl-phosphate (data not shown). This indicated that the *hel* gene products HelC and HelB are expressed in the cytoplasmic membrane or beyond in *L. pneumophila*. We did not detect alkaline phosphatase activity from the fusion in strain AA209, although cosmid pJA3::Tn*phoA-oriT* #12 was phosphatase positive in *E. coli* (data not shown). We were unable to introduce cosmid pJA3::Tn*phoA-oriT* #7 carrying an insertion in *helA* into AA200, although this cosmid was also phosphatase positive in *E. coli* (data not shown).

Reasoning that perhaps the *hel* gene products and the product of *orf2* served similar (i.e., redundant) functions, we constructed strain AA213 that carries a deletion from *helB* through *orf2* (Fig. 4). The intervening DNA in strain AA213 was replaced with a promoterless *lacZ* gene followed by a $Kan^r$ determinant, generating a transcriptional fusion between *helB* and *lacZ* (15).

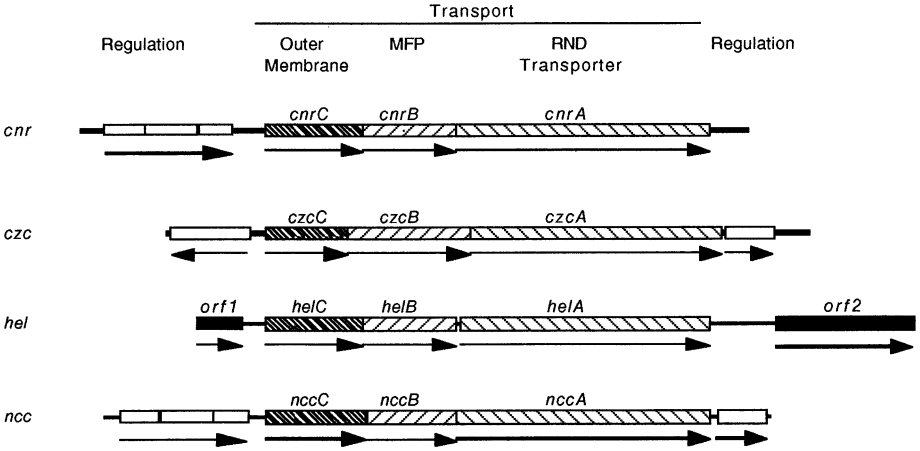
On the basis of the similarities between *hel* and the *czc*, *cnr*, and *ncc* loci, we examined the effects of various ions, including $Cd^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Zn^{2+}$, on the growth of strains AA200, AA201, AA205, AA209, and AA213. We examined growth in liquid medium (determining the MIC) and on solid medium (measuring zones of inhibition). Under no condition did we detect a difference in the sensitivities or resistances of the bacterial strains examined (Table 2 and data not shown).

Since transcription of *cnr*, *czc*, and *ncc* is induced by the presence of a relevant substrate ion, we examined the effect of various ions on expression of the *phoA* fusions in AA201, AA205, and AA209 as well as *lacZ* expression in AA213. Bacteria were cultured in the presence of various ions, and alkaline phosphatase assays or β-galactosidase assays were performed (3, 24). We found no ion that led to significant induction of any of the gene fusions examined (data not shown).

Some members of the RND and MFP families of proteins mediate resistance to a variety of compounds, including antimicrobial agents and fatty acids (17, 20, 29, 33, 34). We therefore examined the effects of various antimicrobial agents, including acridine orange, as well as the fatty acids caproate and caprate on the growth of strains AA200, AA201, AA205, AA209, and AA213. We examined growth in liquid medium (determining the MICs) and/or on solid medium (measuring zones of inhibition). Under no condition did we detect a difference in the sensitivities or resistances of the bacterial strains examined (Table 2 and data not shown).

**Transcription of the *hel* locus is induced intracellularly.** We previously reported that a *helC* mutant exhibited reduced cy-

A




B

| | HelC | CnrC | CzcC | NccC |
|---|---|---|---|---|
| HelC | | 25 | 25 | 24 |
| CnrC | 10 | | 31 | 75 |
| CzcC | 15 | 12 | | 29 |
| NccC | 10 | 71 | 14 | |

| | HelB | CnrB | CzcB | NccB |
|---|---|---|---|---|
| HelB | | 22 | 30 | 21 |
| CnrB | 8 | | 28 | 75 |
| CzcB | 27 | 16 | | 31 |
| NccB | 6 | 83 | 20 | |

| | HelA | CnrA | CzcA | NccA |
|---|---|---|---|---|
| HelA | | 48 | 60 | 48 |
| CnrA | 84 | | 48 | 88 |
| CzcA | 125 | 65 | | 49 |
| NccA | 110 | 153 | 105 | |

FIG. 2. Comparisons between *hel*, *cnr*, *czc*, and *ncc*. (A) The genes in each of the indicated loci are represented by boxes: genes encoding regulatory functions are indicated by open boxes, genes encoding transport functions are represented by hatched boxes, and genes of unknown function are represented by filled boxes. The names of relevant, individual genes are indicated. Arrows indicate the direction of transcription of each gene. (B) Matrices indicate the similarities between the indicated gene products. Numbers above the diagonal in each matrix show the percent amino acid identity shared by the indicated proteins. Numbers below the diagonal in each matrix show a measure of the statistical significance of each alignment calculated as follows. The second of two sequences being aligned was repeatedly randomized, maintaining its length and composition, and then realigned to the first sequence. The mean alignment score, plus or minus the standard deviation, following 100 (10 in the case of aligning HelA, CnrA, CzcA, and NccA) randomizations and alignments of the second sequence was determined. The values in the matrices represent the number of standard deviations between the actual alignment score and the mean alignment score from the randomizations. Alignments were performed with the Bestfit program of the Genetics Computer Group version 7.3 VAX system.

topathicity towards human macrophage-like U937 cells although the mutant did not exhibit a general growth defect in standard laboratory media (4). These observations suggested that the function of the *hel* gene products is unnecessary in vitro, although the function is important in the intracellular environment. It would not be unexpected, therefore, if expression of the *hel* gene products was differentially regulated under these conditions.

```
     1   TCGTTATCTTGCTATGGAACTTGGGAGTAAAAAGATTAGGGTCAATGCCATTTCTCCGGGGCCAATCAGTACGCGCGCGGCATCAGGGTTGGCCGATTTT
Orf1     > R  Y  L  A  M  E  L  G  S  K  K  I  R  V  N  A  I  S  P  G  P  I  S  T  R  A  A  S  G  L  A  D  F  >

   101   GATAAACTGATGGAGAAGGCAGCCAATGAGGCACCCCTTCATCAGCTGGTGACCATAGAGGCTATAGGTGAGATGGCGGCGTTTTTGGTGTCTGATAAAG
           D  K  L  M  E  K  A  A  N  E  A  P  L  H  Q  L  V  T  I  E  A  I  G  E  M  A  A  F  L  V  S  D  K  A>

   201   CCGTTTCTATTACGGGGCAAATTCTCTATGTGGATGCCGGTTATAATATCAAAGGCTAACAAGCTTCTTGATGATTACATGTATTGCCCTATTTTTGCAT
             V  S  I  T  G  Q  I  L  Y  V  D  A  G  Y  N  I  K  G  *

   301   AACACACTACTTAATAAAATCCGTTTTTAATTGAAAGCATCTGGTTTTTTACATTGAATTGGTATCGGTTTTGTTTTATGGATTTCAAGAATTAGGTATT

   401   TATAGAGAAAGAAGCATTTACTCATAATTAAAGGCATCAAAACAGTGGTTTAAGTATGGATTAAATCCAGTTAGAATGTCAAATCATGGTGTGAACAGAT

   501   TATCTTGGTTATCTTAATTCAAGGAAAAAGGAATGGGGCTTTTTTTAAGG
HelC                          M  G  L  F  L  R  >
```

FIG. 3. DNA and predicted amino acid sequences of the region including the 3′ end of *orf1* and the 5′ end of *helC*. A potential ribosome binding site for *helC* is underlined.

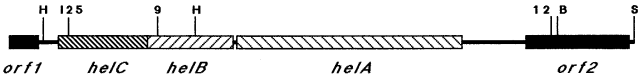



FIG. 4. Locations of relevant restriction enzyme cleavage sites and transposon insertions within and around the *hel* locus. Open reading frames are indicated by boxes. Restriction enzyme cleavage sites for *Bsp*EI, *Hin*dIII, and *Sac*I are indicated by B, H, and S respectively; the deletion in strain AA213 extends from the *Hin*dIII site in *helB* through the *Bsp*EI site in *orf2*. The locations of Tn*phoA-oriT* insertions in AA201 (from cosmid pJAI25::Tn*phoA-oriT*), AA205 (from cosmid pJA3::Tn*phoA-oriT* #9), and AA209 (from cosmid pJA3::Tn*phoA-oriT* #12) are indicated by I25, 9, and 12, respectively.

To investigate this possibility, we measured β-galactosidase activity from strain AA213 grown in U937 cells and in standard laboratory media (Fig. 5) (24). Strain AA213 grown inside U937 cells expressed approximately six times the amount of β-galactosidase as mid-log phase cells grown at 37°C in BYE broth. Consistent with this result, an approximately 10-fold induction in *helC* mRNA levels was detected from *L. pneumophila* grown inside *H. vermiformis* compared with those of in vitro-grown bacteria (1).

In an effort to reproduce the intracellular environment in vitro, we measured β-galactosidase expression from strain AA213 under various growth conditions, including heat shock, osmotic shock, acid shock, and oxidative stress. None of the conditions tested induced significant expression of the *helB-lacZ* fusion (Fig. 5).

**Function of the *hel* gene products in vivo.** We previously showed that reduced cytopathicity towards U937 cells exhibited by a *helC* mutant could be complemented in *trans* by a *hel*-containing cosmid but not by the cloned *helC* gene alone (4). Consistent with these previous findings, AA205, AA209, and AA213 exhibited reduced cytopathicity compared with AA200 towards U937 cells (Fig. 6); the levels of reduction in cytopathicity observed with these mutants was similar to the reduction observed previously for a *helC* mutant (4). However, the mutant strains showed no defect in their abilities to enter and/or grow within U937 cells nor in the infectious dose required to infect 50% of U937 cell monolayers (data not shown).

The mutants AA201 and AA205 also were indistinguishable from AA200 in virulence towards guinea pigs. The severity of illness as indicated by the day at which peak weight loss was observed and the percent of initial body weight lost was similar for each group of infected animals (peak weight loss occurred, on average, between day 4 and day 5 within each group of infected animals, and the average weight loss within each group was between 21 and 25% of initial body weight). In addition, there was no significant difference in the numbers of animals that died within each group of infected animals (comparing AA200 and AA201, $\chi^2 = 1.33$, $0.25 > P > 0.1$; comparing AA200 and AA205, $\chi^2 = 2.62$, $0.25 > P > 0.1$).

TABLE 2. Effect of ions, acridine orange, and fatty acids on growth of strains

| Condition | MIC of strain: | | | |
|---|---|---|---|---|
| | AA200 | AA201 | AA209 | AA213 |
| $Cd^{2+}$ | 62.5 μM | 62.5 μM | 62.5 μM | 62.5 μM |
| $Co^{2+}$ | 31.25 μM | 31.25 μM | 31.25 μM | 31.25 μM |
| $Ni^{2+}$ | 31.25 μM | 31.25 μM | 31.25 μM | 31.25 μM |
| $Zn^{2+}$ | 1 mM | 1 mM | 1 mM | 1 mM |
| Acridine orange | 235 μM | 235 μM | 235 μM | 235 μM |
| Caprate | 15.6 μM | 15.6 μM | 15.6 μM | 15.6 μM |
| Caproate | 10 mM | 10 mM | 10 mM | 10 mM |

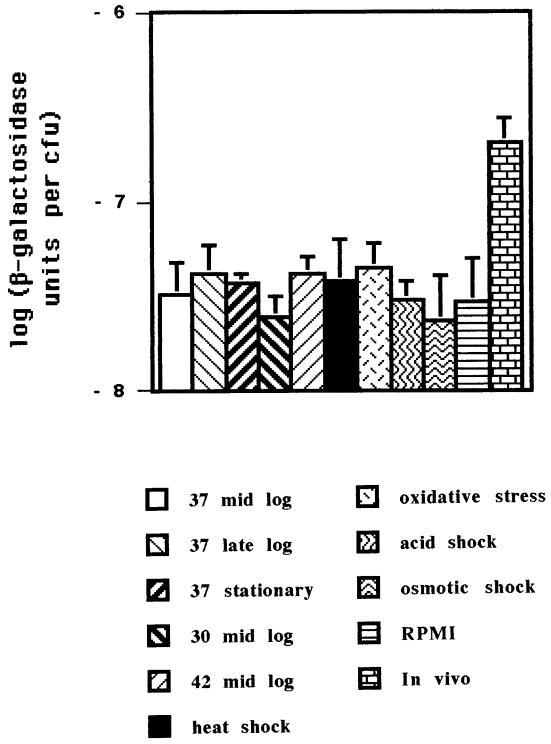



FIG. 5. β-Galactosidase expression from *L. pneumophila* AA213 in log(β-galactosidase units per CFU) (24). In vivo data were obtained for *L. pneumophila* grown inside U937 cells as described in Materials and Methods. Data for the other growth conditions were obtained with *L. pneumophila* grown as described in Materials and Methods. Some conditions include temperature (°C) and growth phase. Values represent the mean and standard deviation of at least three replicate experiments. The amount of β-galactosidase produced in vivo was determined to be significantly greater than the amounts produced under each of the other conditions tested ($P \leq 0.00076$; *t* test for two samples with different variances; Microsoft Excel 4.0); none of the other differences was found to be significant.

Finally, the mutants AA201 and AA209 showed no defect compared with AA200 in their abilities to multiply in coculture with *H. vermiformis*. Log increases in recoverable CFU on day 3 postinfection from representative, duplicate experiments for AA200, AA201, and AA209 averaged 3.9, 3.7, and 3.8, respectively.

## DISCUSSION

On the basis of homologies to other proteins, we suspect that the *hel* gene products serve as a transporter, although the identity of the putative substrate is unclear. The *hel* gene products are most similar to the cation transporters encoded by the *czc*, *cnr*, and *ncc* loci although we have not identified any ion to which *hel* mutant *L. pneumophila* strains appear hypersensitive (4, 18, 26, 35). It has been suggested that transporters that function with an MFP protein (e.g., HelB) transport molecules that are too large or hydrophobic to escape through the outer membrane porins (33). Consistent with this model, other RND proteins are known to transport larger substrates, including antibiotics and fatty acids (17, 20, 29, 33, 34). However, *hel* mutants did not appear hypersensitive to any of the antibiotics or fatty acids tested. Normal cytopathicity towards U937 cells likely requires transport of the substrate(s); however, loss of

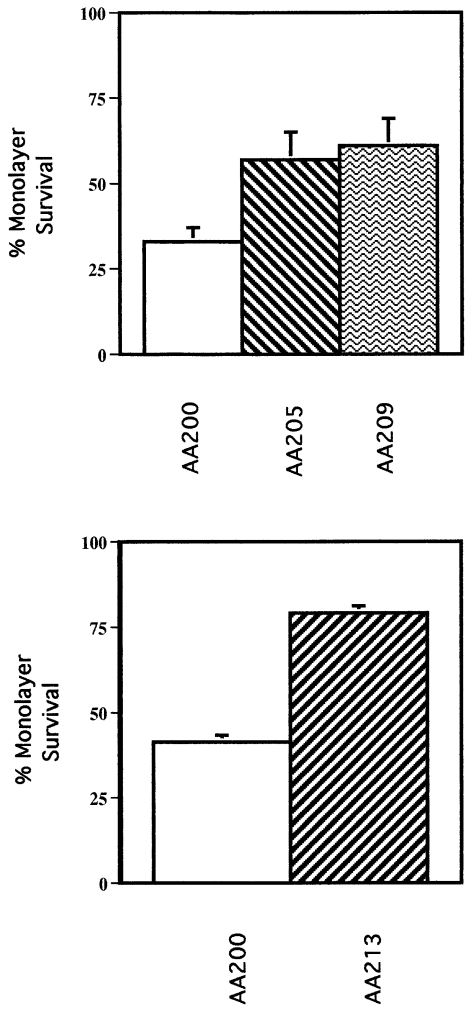



FIG. 6. U937 cell cytopathicity assay of *L. pneumophila* strains. Cytopathicity assays were performed as described in Materials and Methods. Values represent the mean and standard deviation of three or more replicate experiments. A significantly greater proportion of the infected monolayers survived infection by the mutant strains AA205, AA209, and AA213 compared with monolayers infected with AA200 ($P = 0.004$, 0.001, and $3.5 \times 10^{-7}$, respectively, by *t* test for two samples with equal variances; Microsoft Excel 4.0).

the *hel* transport function does not appear to significantly impact *L. pneumophila* virulence as determined by other assays. Identification of the substrate is expected to help explain this anomaly.

Transcription of the *hel* locus is significantly induced by the intracellular environment experienced by *L. pneumophila* in both U937 cells and *H. vermiformis*. The reason *L. pneumophila* induces expression of the *hel* locus intracellularly although the products of the locus do not seem to be required for virulence is not understood. Perhaps the product(s) of another *L. pneumophila* gene(s) carries out a similar or redundant function. This would be consistent with our inability to identify a substrate in vitro. In addition, *hel* may have evolved to be part of a multigene regulon in which some of the other gene products are required for virulence. The phenotypic modulation *L. pneumophila* undergoes to adapt to the intracellular environment is complex and may involve multiple regulons (2). Consistent with this complexity, transcription of the *hel* locus was not induced by heat shock, osmotic shock, acid shock, or oxidative stress; these stresses previously have been shown to be similar to portions of the adaptive response of *L. pneumophila* to the intracellular environment (2). Determination of the conditions necessary to induce transcription of the *hel* locus is expected to help identify the substrate as well as provide valuable insight into the growth environment inside phagocytic cells.

Transcription of the *hel* locus does not appear to be regulated by the products of genes mapping immediately upstream or downstream from the locus. The predicted gene product of *orf1* is perhaps an *L. pneumophila* homolog of the fatty acid-synthesizing enzyme EnvM. The predicted gene product of *orf2* is homologous to $Cd^{2+}$-transporting proteins from gram-positive bacteria. What association, if any, the products of these open reading frames have with the *hel* gene products is unknown. The identification of the gene(s) whose product(s) regulates *hel* transcription might help determine the function of the *hel* gene products. Additionally, and perhaps more significantly, elucidation of what factors regulate the *hel* locus might lead to the identification of a multigene regulon associated with *L. pneumophila* virulence.

Our current belief is that the phenotype associated with a mutation in *helC*, *helB*, or *helA* is due to loss of Hel function rather than to a polar effect on the expression of *orf2*. The gene designated *orf2* is located at the end of the insert in cosmids pJA3 and pJAI25 (data not shown), a cosmid that was able to complement the defect in a *helC* mutant (4). It would be very surprising, on the basis of the distance between the end of *helA* and the start of *orf2*, if *orf2* and *helA* were cotranscribed. Thus, the phenotypes associated with transposon insertions into any of the *hel* genes are likely due to loss of Hel function. Like *hel*, the role of *orf2* in the cytopathicity of *L. pneumophila* is unclear. It is curious that the products of both *hel* and *orf2* are homologous to ion transporters.

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