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Sequence Analysis of the *mip* Gene of the Soilborne Pathogen *Legionella longbeachae*

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To understand the basis of pathogenesis by *Legionella longbeachae* serogroup 1, the importance of the Mip protein in this species was examined. Amino-terminal analysis of the purified, cloned *L. longbeachae* serogroup 1 ATCC 33462 Mip protein confirmed that the cloned gene protein was expressed and processed in an *Escherichia coli* background. DNA sequence analysis of plasmid pIMVS27, containing the entire *L. longbeachae* serogroup 1 *mip* gene, revealed a high degree of homology to the *mip* gene of *Legionella pneumophila* serogroup 1, 76% homology at the DNA level and 87% identity at the amino acid level. Primer extension analysis determined that the start site of transcription was the same for both species, with some differences observed for the −10 and −35 promoter regions. Primers designed from the *mip* gene sequence obtained for *L. longbeachae* serogroup 1 ATCC 33462 were used to amplify the *mip* genes from *L. longbeachae* serogroup 2 ATCC 33484 and an Australian clinical isolate of *L. longbeachae* serogroup 1 A5H5. The *mip* gene from A5H5 was 100% identical to the type strain sequence. The serogroup 2 strain of *L. longbeachae* differed by 2 base pairs in third-codon positions. Allelic exchange mutagenesis was used to generate an isogenic *mip* mutant in ATCC 33462 and strain A5H5. The ATCC *mip* mutant was unable to infect a strain of *Acanthamoebae* sp. both in liquid and in a potting mix coculture system, while the A5H5 *mip* mutant behaved in a manner siilar to that of *L. pneumophila* serogroup 1, i.e., it displayed a reduced capacity to infect and multiply within *Acanthamoebae*. To determine if this mutation resulted in reduced virulence in the guinea pig animal model, the A5H5 *mip* mutant and its parent strain were assessed for their abilities to establish an infection after aerosol exposure. Unlike the virulent parent strain, the mutant strain did not kill any animals under two different dose regimes. The data indicate that the Mip protein plays an important role in the intracellular life cycle of *L. longbeachae* serogroup 1 species and is required for full virulence.

Legionella longbeachae serogroup 1 was first recognized as a cause of pneumonia in 1981 (30). In May 1987, *L. longbeachae* serogroup 1 was isolated for the first time from a patient in Australia (25). Since then, numerous cases of infection caused by this species have been reported (8, 24), and presently approximately 50% of all pneumonia cases in South Australia are attributable to this species (8, 39a, 45), a statistic which reflects the national trend. Subsequent studies showed that *L. longbeachae* serogroup 1 was present in commercial potting mix and in the soil of potted plants of patients and that it survived for long periods in these environments, indicating that soil, rather than water, may be the natural habitat of this species and a possible source of infection in the community (40). Restriction fragment-length polymorphism and allozyme studies performed to compare *L. longbeachae* serogroup 1 isolates from clinical and environmental origins demonstrated that they were all closely related and similar to isolates from *L. longbeachae* serogroup 1 ATCC 33462, indicating a close relationship between organisms isolated from countries as far apart as Australia and the United States (24).

No virulence studies of *L. longbeachae* serogroup 1 have been done, although *L. longbeachae* serogroup 2 has been examined by intraperitoneal injection into guinea pigs and for the ability to infect and multiply in a protozoan model of infection with *Tetrahymena pyriformis* and *Hartmannella verformis* (17, 44). Recent publications detailing in vitro models for intracellular growth of *L. longbeachae* serogroup 1 have shown that it can replicate in U937 cells (35) but is unable to replicate in Mac 6 cells or in *Acanthamoebae castellanii* (33). Little is known about the intracellular life cycle of this species, the factors which may contribute to pathogenesis, and whether these factors are shared with *Legionella pneumophila* serogroup 1.

L. longbeachae serogroup 1 pathogenesis studies have focused on the Mip protein and have examined the significance of this protein in pathogenesis by the organism. The Mip protein of *L. pneumophila* serogroup 1 has been established as a virulence factor of the organism, playing an important role in the intracellular life cycle, as mutant strains which lack the protein are significantly impaired in their ability to infect alveolar macrophages and protozoa (9, 12). They are also attenuated in their ability to cause disease in experimentally infected guinea pigs (11). The *L. pneumophila* serogroup 1 Mip protein displays homology to the FK506 binding protein (FKBP) class of immunophilins and shows characteristic peptidyl prolyl *cis-trans* isomerase (PPIase) activity (18). A homolog of the Mip protein also occurs in *Legionella micdadei* (2), a species of *Legionella* associated with disease in humans, and a *mip* mutant in this species also shows reduced intracellular infection (34). Mip analogs have been detected in all species of *Legionella* examined so far, including *L. longbeachae* serogroup 1 (10, 37, 38). Mip-like analogs which also display homology to the FKBP class of proteins have been reported in

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
L. pneumophila serogroup 1 (Philadelphia)	ATCC 33152; type strain	CDC[a]
L. longbeachae serogroup 1	ATCC 33462; type strain	CDC
L. longbeachae serogroup 2	ATCC 33484; type strain	CDC
A5H5 (*L. longbeachae* serogroup 1)	Australian clinical isolate	This study
B10	ATCC 33462 *L. longbeachae* serogroup 1 strain with a *mip* deletion mutation	This study
B8	*L. longbeachae* serogroup 1 A5H5 with a *mip* deletion mutation	This study
B8.22	Strain B8 complemented with plasmid SKW27	This study
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_k^- m_k^+) *deoR thi-1 supE44* λ^- *gyrA96 relA1*	20
E. coli S17-1	*recA* derivative of *E. coli* 294 (*hsdR* Pro) with RP4-2Tc::Mu (Ap Km Nm Tc::Mu) Km::Tn*7* in the chromosome	39
Plasmids		
pGEM-7Zf(−)	Ap^r cloning vector	Promega
pUC18K	pUC18 with the *aphA-3* Km^r resistance cassette	P. Sansonetti, reference 31
pCACTUS	Cm^r cloning vector containing *sacB* and a temperature-sensitive replicon	C. A. Clark
pIMVS26	pGEM with ca. 8-kb fragment of *L. longbeachae* serogroup 1 ATCC 33462 genomic DNA	This study
pIMVS27	pGEM carrying a *Sac*I fragment of pIMVS26	This study
pIMVS28	pGEM containing deleted *mip* gene generated in pIMVS27	This study
pCACTUS49	pCACTUS containing deleted *mip* gene fragment from pIMVS28	This study
pCACTUS50	Derivative of pCACTUS49 containing Km^r from pUC18K and *mob* deletion	This study
pWKS130	Km^r cloning, sequencing vector	46
pIMVS29	pWKS130 containing entire *mip* gene on *Sac*I fragment from pIMVS27	This study

[a] CDC, Centers for Disease Control and Prevention, Atlanta, Ga.

other intracellular pathogens such as *Chlamydia trachomatis* (27) and *Coxiella burnetti* (32), with PPIase activity having been demonstrated for both organisms (28, 32). Hence, Mip-like proteins with homology to the FKBP class of immunophilins may play a critical role in the life cycles of these organisms (19).

In this report, we document the cloning and sequence analysis of the *mip* gene from *L. longbeachae* serogroup 1 ATCC 33462 and compare the results with those from *L. pneumophila* serogroup 1 (16), *L. longbeachae* serogroup 2 ATCC 33484, *L. micdadei* (2), and an Australian clinical isolate of *L. longbeachae* serogroup 1, strain A5H5. To understand the significance of Mip in *L. longbeachae* serogroup 1, we constructed and characterized isogenic *mip* mutants in *L. longbeachae* serogroup 1 ATCC 33462 and the Australian clinical isolate of this species, strain A5H5. The mutants, which represent the first reported genetic manipulation of this species, were tested for their abilities to infect a strain of *Acanthamoebae* and to establish infection in guinea pigs. There were apparent differences between the two isolates of *L. longbeachae* serogroup 1 in both of these models.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial isolates of *Legionella*, *E. coli* strains, and plasmids used or constructed in this study are listed in Table 1. *Legionella* strains were routinely cultured on charcoal yeast extract α-ketoglutarate (CYE) plates (24) at 35°C. Legionella broth was used as a liquid growth medium (41). When required, selective agents were used at the following concentrations: chloramphenicol (CM), 5 μg/ml; kanamycin (KM), 25 μg/ml; and aztreonam, 4 μg/ml. For the amoeba coculture experiments, *Legionella* organisms were plated onto CYE plates containing pimafucin (250 mg/liter), polymixin B (80,000 IU/liter), and vancomycin (2 mg/liter) (CYE-VPP). *E. coli* strains were grown in Luria broth or on Columbia agar, and where appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; CM, 25 μg/ml, and KM, 25 μg/ml.

Antisera and antibodies. *L. pneumophila* serogroup 1 polyclonal monospecific anti-Mip antisera, used initially to screen the *L. longbeachae* serogroup 1 plasmid bank, were a kind gift from N. P. Cianciotto (Department of Microbiology and Immunology, Northwestern University, Chicago, Ill.). Polyclonal antiserum was prepared specifically against *L. longbeachae* serogroup 1 Mip, excised from a 15% polyacrylamide gel, and emulsified in phosphate-buffered saline (PBS), pH 7.2. The acrylamide mix was injected subcutaneously into two New Zealand White rabbits. The injection was repeated after 2 weeks, and the serum was harvested at 6 weeks. The antiserum was extensively absorbed with *E. coli* DH5α(pGEM-7Zf[−]) prior to use.

Western immunoblot. Total cell protein extractions were prepared by the method of Pearlman et al. (36). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Lugtenberg et al. (26) with a 15% running gel. Western immunoblot was performed per the procedure of Towbin et al. (43), using the staining procedure of Hawkes et al. (21), with 4-chloro-1-napthol.

Construction and screening of the plasmid bank. Whole chromosomal DNA was extracted from *L. longbeachae* serogroup 1 ATCC 33462, by the method of Manning et al. (29), and digested with *Bam*HI-*Eco*RI. The fragments were cloned into pGEM-7Zf(−) and transformed into DH5α. A clone carrying an 8-kb fragment was identified by colony immunoblot with *L. pneumophila* serogroup 1 anti-Mip serum. This clone, designated DH5α(pIMVS26), expressed a protein of approximately 27 kDa, as demonstrated by Western immunoblot. A subclone expressing the protein was generated by *Sac*I digestion of pIMVS26 and recloning into pGEM-7Zf(−). A clone containing a 1.3-kb *Sac*I fragment was identified, and the plasmid was designated pIMVS27.

DNA sequencing. Sequencing was performed with the Applied Biosystems model 373A DNA sequencer. Plasmid pIMVS27 was sequenced in the forward direction with Dye Primer kits (ABI, Foster City, Calif.). The protocol was applied to templates generated by nested deletion of pIMVS27 with the Erase-a-Base kit (Promega, Madison, Wis.), according to the manufacturer's instructions. The complementary strand of the clone was determined by using the Dye Terminator kit (ABI), with primers designed from the forward-sequence data, with double-stranded pIMVS27 as the template. The entire *mip* gene sequence was analyzed by DNASIS and PROSIS (Hitachi Software). Two primers, 844 (5′-GAGTATGATGAGAAAGAA-3′) and 845 (5′-ACAATTAATCTGATTT AAGG-3′), were designed from the completed sequence to amplify the entire

mip gene from ATCC 33484 and strain A5H5. The expected 850-bp PCR product was purified with the QIAquick PCR purification kit (Qiagen) and sequenced with the Dye Terminator kit (ABI).

Primer extension from total bacterial RNA. Primer extension analysis was used to map the 5′ end of the *mip* mRNA with a synthetic oligonucleotide primer (5′-GGCTGCAACTGATGCTACATCGCTT-3′). Total bacterial RNA was extracted from *L. longbeachae* serogroup 1 ATCC 33462, DH5α(pIMVS27), and DH5α(pGEM-7Zf[−]) by the hot-phenol method of Aiba et al. (1) and treated with RNase-free DNase I (Boehringer Mannheim). The oligonucleotide primer was radioactively labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Boehringer Mannheim). The primer was hybridized to 20 μg of total RNA, and the mix was extended per the method of Williams et al. (47), with Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim). The reaction was loaded onto a 6% acrylamide-urea sequencing gel and visualized by autoradiography. Plasmid pIMVS27 was sequenced with the DNA sequencing kit version 2 (Amersham, Buckinghamshire, United Kingdom).

Allelic exchange mutagenesis, construction, and complementation of *mip* mutants. Allelic exchange was carried out to generate mutations in the *mip* gene with the suicide vector pCACTUS. Vector pCACTUS is a derivative of plasmids containing the *sacB* gene of *Bacillus subtilis* (pIB279) and pIB307, containing a temperature-sensitive pSC101 replicon (6). pCACTUS also contained a *mob* region and a chloramphenicol resistance gene. Plasmid pCACTUS49 was introduced into *L. longbeachae* serogroup 1 ATCC 33462 by conjugation with the modified method from Bradley et al. (7) from a 48-h plate subculture of *Legionella* growth. The mating was incubated for 6 h at 30°C on CYE plates, serially diluted in PBS, and plated onto CYE plates containing 5 μg of CM per ml and 4 μg of aztreonam per ml. The natural resistance of *L. longbeachae* to aztreonam was used to select against the donor. All plates were incubated at 30°C. Electroporation was used to introduce pCACTUS50 into *L. longbeachae* serogroup 1 A5H5. Electrocompetent A5H5 cells were prepared according to the method of Dower et al. (13), except that PBS was used in the initial washes. Glycerol-treated A5H5 cells and plasmid DNA (approximately 1 μg) were subjected to an electric pulse of 2.3 kV in a 0.2-cm cuvette (Bio-Rad) with a Bio-Rad gene pulser at 100 Ω. The cells were incubated in broth at 30°C for 5 to 6 h and plated onto CYE plates containing KM.

The resulting Km^r or Cm^r *L. longbeachae* colony was cultured in broth at 30°C with the appropriate antibiotic. Subsequent culture on CYE plates containing CM or KM at the nonpermissive temperature for pCACTUS replication in *L. longbeachae* (39°C) resulted in the cointegration of the plasmid into the chromosome via homologous recombination. One resultant antibiotic-resistant colony was incubated in broth with antibiotic selection at 30°C and plated onto CYE containing 6% sucrose to select for resolved cointegrates. Colonies from the sucrose plates were patched onto CYE plates and screened by PCR to assess allelic exchange, and potential mutants were confirmed by Southern blot hybridization and immunoblot. Mutant strains were complemented with plasmid pIMVS29, which was introduced by electroporation.

Southern blot hybridization. DNA was transferred to nylon membranes (Hybond-N+; Amersham) by the method of Southern (42) and hybridized with digoxigenin (DIG)-labeled probe at 42°C overnight. Probes were labeled with DIG and hybridized with the filter under conditions described previously (24). The filters were developed according to the manufacturer's protocol (Boehringer Mannheim).

Infection of *Acanthamoebae* with *Legionella* strains. *Acanthamoebae* group 2 spp. used in coculture experiments were originally isolated from potting mix. Their identities were confirmed by Brett Robinson, South Australian Water Corporation, Bolivar, South Australia, Australia, and one strain, designated ACO97, was chosen for all experimental work.

Liquid cocultures of *Acanthamoebae* ACO97 and *Legionella* species were set up essentially as described by other workers (12). Duplicate cocultures containing approximately 10^3 *Legionella* organisms per ml and 10^4 *Acanthamoebae* cysts per ml were set up in 4 ml of amoeba saline (2 mM NaCl, 0.016 mM $MgSO_4$, 0.027 mM $CaCl_2$, 1 mM Na_2HPO_4, 1 mM KH_2PO_4). *Legionella* organisms were prepared by suspending growth from a 72-h CYE plate in sterile tap water to give approximately 10^9 organisms/ml by comparison with a turbidity standard (McFarland standard number 4); this was confirmed spectrophotometrically by using an optical density of 1.0 at 550 nm. These organisms were serially diluted and plated onto CYE agar to determine numbers of viable bacteria. Cocultures were incubated at 30°C. Samples were taken at days 1, 3, and 7, diluted in 0.2 M HCl-KCl buffer (pH 2.2), and plated onto CYE plates. Potting mix coculture samples were set up essentially as for liquid coculture, except that *Legionella* and *Acanthamoebae* were added to presteamed potting mix (Nu-Erth, Meadows, South Australia, Australia). Twenty grams of steamed soil seeded with *Legionella* and amoebae was incubated at 30°C, and samples were taken at days 3, 7, 11, and 15. At each interval, a 1-g aliquot of soil was removed, diluted in sterile tap water, mixed thoroughly, allowed to settle for 15 min, and then diluted in 0.2 M HCl-KCl acid buffer to reduce the number of unwanted soil microorganisms. Aliquots were plated onto CYE-VPP.

Animal studies. (i) Intraperitoneal inoculation. Outbred guinea pigs (IMVS colored stock; Institute of Medical and Veterinary Science-Veterinary Services, Gilles Plains, South Australia, Australia), weighing between 300 and 600 grams, were inoculated intraperitoneally with a suspension of *Legionella* prepared in sterile tap water, as outlined for coculture experiments, and enumerated initially in a counting chamber (Hausser Scientific Partnership, Horsham, Pa.). The actual dose administered was accurately determined retrospectively by serial dilution and plating on CYE plates.

(ii) Aerosol inoculation. Guinea pigs were infected by exposure to an aerosolized dose of *Legionella* within a closed chamber. The test strain dose was prepared as outlined for the amoeba coculture, except that strain B8.22 was grown on CYE plates containing KM and was enumerated retrospectively. The chamber was constructed of Perspex (Lucite) and measured 220 by 220 by 240 mm, with a removable top. A nebulizer pump therapy kit (Ventalair Forte II; Allersearch, Granville, Australia) was used to generate the aerosol, which had an average particle size of 3.9 microns, as specified by the manufacturer. An inlet was constructed on one side of the chamber, through which the nebulizer hose was inserted; this hose was sealed in place. The hose connected the nebulizer bowl on the inside of the chamber to the nebulizer pump unit on the outside. The nebulizer pump generated positive pressure in the chamber which was vented through a small outlet valve on the opposite side of the box. The chamber was placed in a laminar flow hood during aerosolization as a safety measure. Guinea pigs were placed in the chamber, and a 3-ml test suspension (containing approximately 10^9 or 10^{10} *Legionella* organisms total) was aerosolized into the chamber over a 15-min interval. The guinea pigs were held in the chamber for a further 5 min. One animal in each test group was killed immediately after exposure to enumerate the *Legionella* organisms introduced into the lungs. Lungs were homogenized in 100 ml of sterile tap water by using a Waring commercial blender (Waring Products, New Hartford, Conn.), and viable counts were determined by plating the homogenate, in duplicate, onto CYE and CYE-VPP plates.

Animals were checked three times daily for signs of illness, and their weights were recorded. Lungs were taken from animals that died to confirm experimental pneumonia.

Nucleotide sequence accession numbers. The *mip* gene sequence data obtained for *L. longbeachae* serogroup 1 ATCC 33462 and *L. longbeachae* serogroup 2 ATCC 33484 in this study are available under GenBank and EMBL accession numbers X83036 and AF000958, respectively.

RESULTS

Analysis of *L. longbeachae* serogroup 1 Mip. Amino-terminal analysis of clone DH5α(pIMVS26) was performed to ensure the identity of the Mip protein from *L. longbeachae* serogroup 1 and that it was processed in *E. coli*. N-terminal sequencing of the purified Mip protein from clone DH5α(pIMVS26) was performed at Macquarie University Centre for Analytical Biotechnology (Macquarie University, School of Biological Sciences, New South Wales, Australia) on a 470A Applied Biosystems protein sequencer. The protein was homologous to the first 16 amino acids in the processed form of the Mip outer membrane protein from *L. pneumophila* serogroup 1 (16), except for a threonine residue at position 8 in place of an alanine residue (Ala-Thr-Asp-Ala-Thr-Ser-Leu-**Thr**-Thr-Asp-Lys-Asp-Lys-Leu-Ser-Tyr). Subsequent sequencing of plasmid pIMVS27 showed one potential open reading frame (ORF) of 699 bp. A strong ribosome binding site was also found in close proximity to the putative ATG start site for translation. Downstream of the ORF, a stop codon was seen in conjunction with a region of dyad symmetry, corresponding to a putative transcriptional terminator, similar to that seen for the *L. pneumophila* serogroup 1 *mip* gene (16). This most likely represents a factor-independent transcription termination signal and has also been proposed for the *L. micdadei mip* gene (2).

The inferred translated *mip* gene product was a polypeptide of 233 amino acids with a predicted molecular mass of 24,661 Da. The inferred amino acid sequences of the Mip proteins from *L. longbeachae* serogroup 1 A5H5 and the ATCC 33484 serogroup 2 isolate were identical with that of the ATCC 33462 *L. longbeachae* serogroup 1 Mip protein (Fig. 1) and were very similar to that of the *L. pneumophila* serogroup 1 Mip protein (16), displaying approximately 87% identity, and also to that of the *L. micdadei* Mip protein (2) (Fig. 1). The first 20 amino acids suggested a signal sequence and in conjunction with the N-terminal analysis suggest that Mip is processed in *E. coli* in a manner similar to the way it is processed in *L. pneumophila* serogroup 1. The key sites proposed to be involved in the PPIase activity (19) determined for the *L. pneumophila* sero-

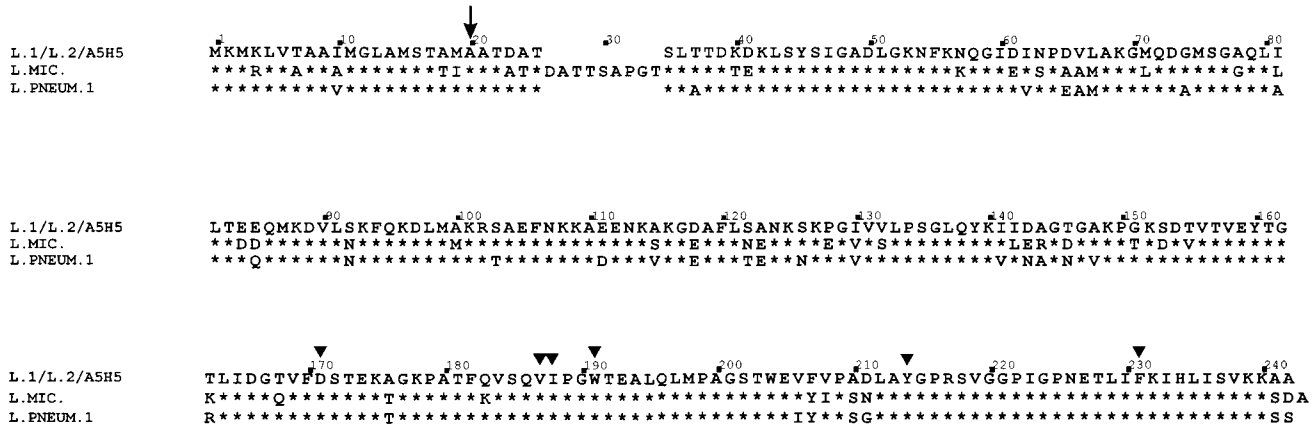

FIG. 1. Amino acid comparison of the Mip proteins of *L. longbeachae* serogroup 1 ATCC 33462 (L.1), *L. longbeachae* serogroup 1 A5H5 (A5H5), *L. longbeachae* serogroup 2 ATCC 33484 (L.2), *L. pneumophila* serogroup 1 (L. PNEUM. 1), and *L. micdadei* (L. MIC.). Asterisks indicate amino acids identical to those of *L. longbeachae*; triangles indicate amino acids predicted to form part of the active site for PPIase activity of Mip. The arrow indicates the site of signal peptidase cleavage.

group 1 (Philadelphia) Mip protein (18) are conserved in *L. longbeachae* serogroup 1 Mip (Fig. 1), suggesting a similar function and role in pathogenesis.

Analysis of *L. longbeachae* serogroup 1 *mip* transcriptional signals. To confirm the start site for transcription of *mip*, and to compare this with the case for *L. pneumophila* serogroup 1, primer extension analysis was performed. Identification of the 5′ ends of the *mip* mRNA isolated from *L. longbeachae* serogroup 1 and the *E. coli* clones was determined by synthesis of cDNA with an oligonucleotide primer that was complementary to a region of DNA 54 bp downstream from the putative ATG start site on the *mip* mRNA. Identical cDNA bands were synthesized from RNA from *L. longbeachae* serogroup 1 ATCC 33462 and DH5α(pIMVS27), with no band detected in the control track where DH5α(pGEM7Zf[−]) was used as a template (data not shown). By comparing these bands with the sequencing reaction of pIMVS27, primed with the same oligonucleotide, the 5′ end of the *mip* mRNA was mapped to the G residue at nucleotide position 473 of the *L. longbeachae* serogroup 1 *mip* gene sequence. This result confirmed that the start sites for transcription in *L. longbeachae* serogroup 1 and *L. pneumophila* serogroup 1 (16) were identical in both species (Fig. 2A). The probable −10 and −35 promoter consensus sequences were identified and compared with those for *L. pneumophila* serogroup 1 (Fig. 2A). The −10 region was the same for *L. longbeachae* serogroup 1 and *L. pneumophila* serogroup 1; however, a −35 region was identified (Fig. 2A) in *L. longbeachae* serogroup 1 that is a more likely part of the promoter sequence than that suggested for *L. pneumophila* serogroup 1 (16). The spacing of the −10 and −35 regions for *L. longbeachae* serogroup 1 is a closer match with the consensus sequences determined for *E. coli*, and the spacing is optimal (17 ± 1 nucleotide).

Construction and complementation of *L. longbeachae* serogroup 1 *mip* mutants. Isogenic *mip* mutants were generated in *L. longbeachae* serogroup 1 ATCC 33462 and strain A5H5 by

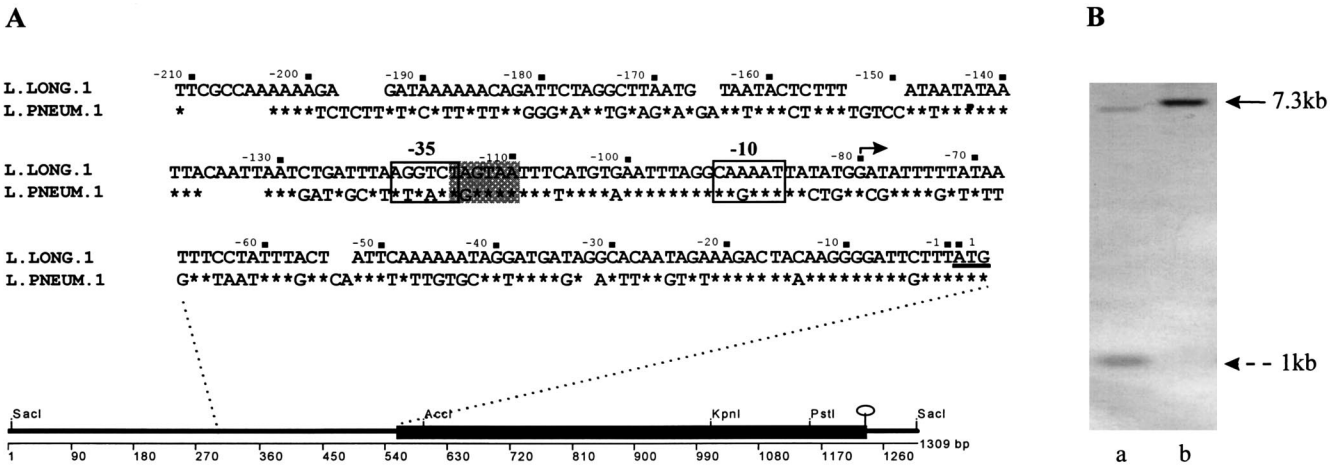

FIG. 2. (A) Line diagram depicts the DNA sequence determined from sequencing pIMVS27. The solid box shows the *mip* gene from *L. longbeachae* serogroup 1 ATCC 33462, selected restriction sites in the *mip* gene, and the stem loop structure at the end of the ORF. The inset sequence is the DNA sequence upstream of the ATG start site for translation in *L. longbeachae* serogroup 1 and *L. pneumophila* serogroup 1, showing the −10 and −35 promoter regions determined by primer extension analysis. The shaded box indicates the −35 promoter region proposed for *L. longbeachae*. The nonshaded box is the −35 region proposed in reference 16. The start site for transcription is shown with a solid arrow. (B) Southern hybridization demonstrating mutagenesis by allelic exchange of the *L. longbeachae* serogroup 1 *mip* gene. DNA was digested with *Kpn*I and probed with DIG-labeled pIMVS27. Lanes: a, *L. longbeachae* serogroup 1 A5H5; b, B8. The solid arrow indicates the 7.3-kb fragment generated in B8 due to the loss of an internal *Kpn*I site which generates 1- and 7-kb fragments in the parent strain. A similar pattern was observed for *L. longbeachae* serogroup 1 ATCC 33462 (data not shown).

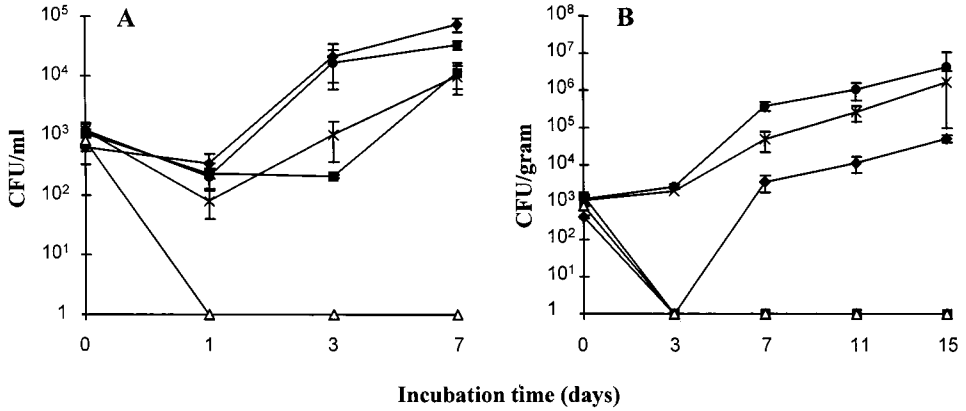

FIG. 3. Coculture of *Acanthomoebae* with strains of *Legionella*. (A) Amoeba liquid cocultures were set up in saline with approximately 10^4 amoebae/ml and 10^3 CFU (each) of *L. pneumophila* serogroup 1 (Philadelphia) (◆), *L. longbeachae* serogroup 1 ATCC 33462 (■), B10 (△), A5H5 (●), and B8 (×) per ml. Samples were taken at various time intervals, and the number of *Legionella* organisms was determined by plating on selective media. Each time point represents the mean number of CFU recovered, and the vertical bars indicate standard deviations. (B) Amoebae were cocultured in an artificial potting mix system with strains of *Legionella* as indicated above. Numbers of viable *Legionella* organisms were determined at various time points by treatment of the soil sample with acid and plating on selective media. The experiments shown are representative of two independent experiments.

allelic exchange with a plasmid construct, pCACTUS49, constructed in several stages. First, the *mip* gene in pIMVS27 was mutated by digestion with *Acc*I and *Pst*I to delete a 650-bp fragment within the coding region (Fig. 2A). The resulting construct, designated pIMVS28, was transformed into DH5α, and transformants were screened by Western blot to confirm the loss of production of Mip (data not shown). The residual 850-bp *Sac*I fragment of pIMVS28 was cloned into pCACTUS to yield pCACTUS49. Plasmid pCACTUS49 was introduced into S17-1, which then served as a donor strain in subsequent conjugation experiments.

Due to difficulties in conjugation with strain A5H5, construct pCACTUS50 was made and delivered by electroporation. Plasmid pCACTUS50 was constructed from pCACTUS49 by removing the *mob* site by restriction digestion and inserting the Km^r marker from pUC18K into the *Sma*I site of the polylinker in order to use KM in addition to CM to select for transformants.

To identify colonies that had undergone complete allelic exchange, PCR analysis was performed with primers 844 and 845. A fragment of approximately 650 bp amplified in the case of the mutant strain was in contrast to an 850-bp fragment for the wild type (data not shown). Chromosomal DNA from the putative *mip* mutants and the parent was then digested with *Kpn*I, a restriction site internal to the *mip* gene sequence (Fig. 2A). Duplicate Southern blots were probed with DIG-labeled pIMVS27 and pCACTUS. The mutant strains had only one hybridizing fragment (Fig. 2B, lane b) lacking the internal *Kpn*I site, which had been removed by restriction deletion, while the parent strain had two hybridizing fragments (Fig. 2B, lane a). No bands were detected with the pCACTUS probe, indicating the vector sequence had been completely resolved from the chromosome (data not shown). In addition, Western immunoblot showed loss of Mip production in the mutant strains (data not shown).

To ensure that the mutation process had not affected genes other than *mip*, complemented mutant strains were constructed with vector pIMVS29. This construct was derived by cloning the *Sac*I fragment from pIMVS27, containing the entire *L. longbeachae* serogroup 1 *mip* gene, into vector pWKS130. Only strain B8 was complemented, as B10 and the parent strain were both avirulent. The complemented *mip* mutant in A5H5 was screened by Western immunoblot to confirm the production of Mip (data not shown).

Effect of *mip* on intracellular infection. To determine whether Mip promotes infection of amoebae in *L. longbeachae* serogroup 1, we assessed the abilities of both *mip* mutants to infect *Acanthamoebae*, a common soil amoeba. Two systems were used to assess the levels of multiplication of *Legionella* strains, with potting mix considered a more natural, nonaquatic environment for *L. longbeachae* serogroup 1. The potting mix was steamed for approximately 1 h to kill any preexisting *Legionella* spp.; however, the steaming process did not sterilize the mix, as spore-forming organisms were not killed. The same multiplicity of infection was used for both systems, and samples were taken frequently during the experiment to determine the level of multiplication of *Legionella* (Fig. 3). The mean number of CFU (± standard deviations) was determined for each time point, and the Student-Newman-Keuls comparison of means ($P < 0.05$) was used to determine statistical significance.

L. pneumophila serogroup 1 (Philadelphia), *L. longbeachae* serogroup 1 ATCC 33462, and strain A5H5 multiplied in liquid coculture similarly to other *Legionella* organisms (12, 34) (Fig. 3A), showing an initial lag period with a steady increase in bacterial numbers during the experiment. The *mip* mutants of the two strains of *L. longbeachae* serogroup 1 showed growth patterns different from that of their parent strain and also from those of each other. Mutant B8 increased in numbers at a lower rate than A5H5, with a statistically significant difference in recovery observed at day 7. Statistically significant differences were not seen at days 1 and 3, most likely due to large variations in the counts and the low sample numbers. However, the expected growth trend was observed, and the end result was similar to that determined for the *L. pneumophila* serogroup 1 *mip* mutant (12). Complemented *mip* mutant B8.22 also grew in amoebae (data not shown) and was recovered at day 7 in numbers that were not statistically different from those of the wild-type strain A5H5. Strain B10 was unable to replicate in this system and in several repeat experiments.

L. longbeachae serogroup 1 A5H5 and B8 were both able to replicate in potting mix, showing similar growth trends, as seen with the liquid coculture (Fig. 3B). Statistically significant differences in strain recovery were observed at days 7 and 11. The numbers of organisms observed at day 15 were not statistically different, most likely due to reasons stated above; however, the expected growth trend was observed. *L. pneumophila* serogroup 1 (Philadelphia) replicated in this system, with an initial

TABLE 2. Intraperitoneal inoculation of *Legionella* strains

Strain	Dose (total CFU)	No. of guinea pigs killed/no. tested	Comments
L. pneumophila serogroup 1 (Philadelphia)	5×10^8	3/3	Spleens contained 10^7 to 10^8 organisms
L. longbeachae serogroup 1 ATCC 33462	2×10^9	0/3	
L. longbeachae serogroup 2 ATCC 33484	5×10^8	0/3	
L. longbeachae serogroup 1 A5H5	1×10^9	1/3	Death at 4 days

lag phase observed at day 3, where numbers dropped to undetectable levels; this may reflect the low number of organisms added initially to the coculture or the inappropriate levels of sampling for that time point. In all experiments, however, *L. pneumophila* serogroup 1 (Philadelphia) multiplied. *L. longbeachae* serogroup 1 ATCC 33462 and mutant B10 were unable to replicate in this system and in several repeat experiments.

Animal model of infection. Two models were established in the laboratory and assessed for their ability to allow a comparison of the virulences of experimental *Legionella* strains. The intraperitoneal model allowed accurate doses to be administered and test strains to be compared (Table 2). *L. pneumophila* serogroup 1 (Philadelphia) was virulent in this model, with death occurring in all animals within 30 h. The *L. longbeachae* serogroup 1 strains, however, rarely caused death by this mode of transmission. The *L. longbeachae* serogroup 1 and serogroup 2 ATCC type strains were completely avirulent in this model, while *L. longbeachae* serogroup 1 A5H5 did produce symptoms and death in 1 of 3 animals after 4 days. The data indicated that this was not a suitable model to assess the mutant strains, given the relative avirulence of *L. longbeachae* in this model.

The aerosol model allowed doses of *Legionella* to be administered by the respiratory route of entry. The symptoms produced and the time course of the disease were similar to those predicted by other workers (11). *L. pneumophila* serogroup 1 and strain A5H5 were both virulent by this model and caused death in 3 of 5 animals tested. Examination of the lungs taken from a guinea pig that died due to exposure to A5H5 revealed that the air spaces of the lung parenchyma were filled with a dense cellular infiltrate consisting of neutrophils and monocyte cells, and the histological appearance was consistent with a severe acute pneumonia similar to that seen with *L. pneumophila* serogroup 1 (3, 4, 15). Animals exposed to aerosols of *L. longbeachae* serogroup 1 ATCC 33462 and *L. longbeachae* serogroup 2 ATCC 33484 developed no symptoms and were avirulent, although the numbers of *Legionella* organisms retained in the lung with each test strain were comparable (Table 3). For this reason, only the A5H5 *mip* mutant (B8) was assessed in the aerosol model, along with the complemented strain B8.22. Guinea pigs were tested with two doses of the mutant strain, and the percentage weight gain or loss was plotted for each animal and compared with that for animals inoculated with the isogenic parent strain as well as with that for animals inoculated with the complemented strain (Fig. 4). The parent strain killed 3 of 5 animals (all symptomatic) within 5 days after exposure (Fig. 4A). Guinea pigs exposed to 10^9 CFU (approximately 10^5 retained organisms) of B8 showed no evidence of the disease (Fig. 4B). Some evidence of disease, predominantly weight loss, was observed in some of the animals exposed to a 10^{10}-CFU dose (approximately 10^6 retained organisms) of the same mutant strain (Fig. 4C). The *mip* mutant in strain A5H5 did not cause death with either dose. Reintroduction of the intact wild-type *mip* gene from *L. longbeachae* serogroup 1 was able to fully complement the mutation in strain B8, leading to restored virulence (Fig. 4D).

DISCUSSION

In this study, the *mip* gene from *L. longbeachae* serogroup 1 was sequenced, and the role played by this protein in facilitating infection of guinea pigs and *Acanthamoebae* was examined. The *mip* gene sequences for *L. longbeachae* ATCC 33462 and A5H5 were identical, while the sequence for *L. longbeachae* ATCC 33484 differed from the former by two bases (positions 517 and 523). The translated protein sequences were identical and highly conserved in comparison to those from *L. pneumophila* serogroup 1 and *L. micdadei*. The start sites of transcription for *L. longbeachae* serogroup 1 and *L. pneumophila* were identical, and this confirms the high degree of conservation of *mip* genes and hence the probability that the proteins have similar functions. PPIase activity was not determined for *L. longbeachae* serogroup 1 Mip, but conserved amino acids critical to this enzymatic activity suggest the protein has a similar mechanism of action.

The role of the Mip protein as a potentiator of intracellular infection in *L. longbeachae* is further suggested by the behavior of the *mip* mutants in the *Acanthamoebae* coculture models. The mutant in strain A5H5 showed a growth pattern similar to those of the *mip* mutants of *L. pneumophila* serogroup 1 and *L. micdadei* (12, 34). The *mip* mutant in *L. longbeachae* serogroup 1 ATCC 33462 was unable to multiply in the amoeba models and warrants further analysis, but these results may simply reflect a greater level of attenuation of the ATCC parent strain. Differences were observed between the two parent

TABLE 3. Aerosol inoculation of *Legionella* strains

Strain	Dose (total CFU)	No. of guinea pigs killed/no. tested	Comments	Retained dose
L. pneumophila serogroup 1 (Philadelphia)	1×10^9	2/3	Death within 5 days	$\sim 2 \times 10^5$
L. longbeachae serogroup 1 ATCC 33462	1×10^9	0/3	No symptoms	2×10^5
L. longbeachae serogroup 2 ATCC 33484	1×10^9	0/3	No symptoms	$\sim 2 \times 10^5$
L. longbeachae serogroup 1 A5H5	1×10^9	3/5	Death within 5 days (days 2 and 4)	3.5×10^5
B8	1×10^9	0/5	No symptoms	1×10^5
B8	1×10^{10}	0/5	Slight symptoms in most animals	1.6×10^6

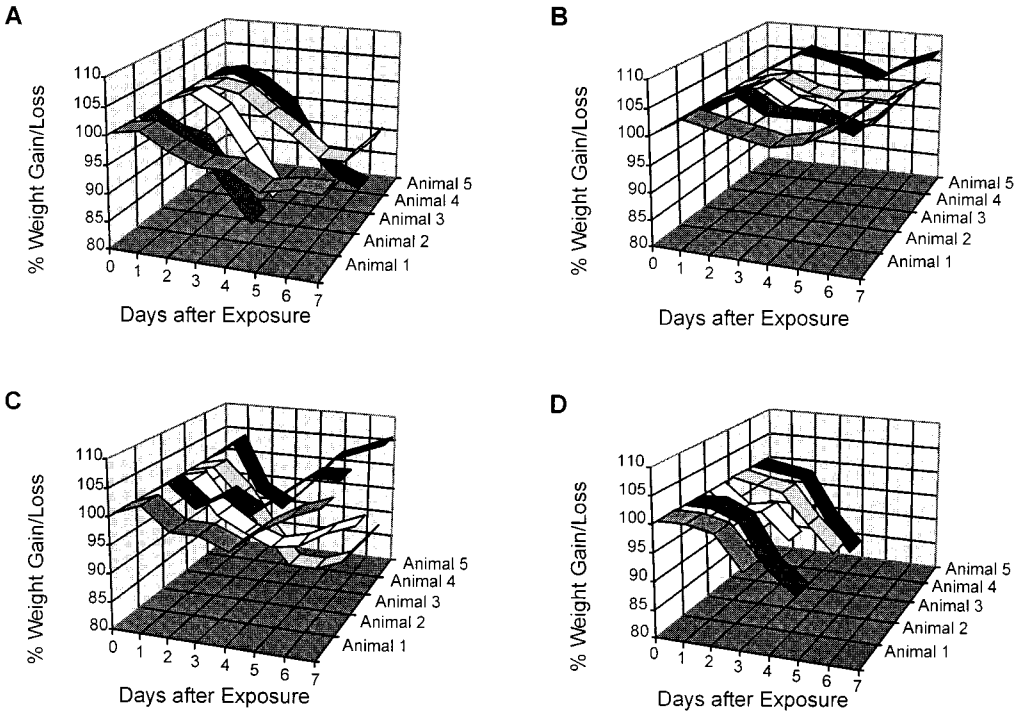

FIG. 4. Percentage weight gain or loss in guinea pigs exposed to an aerosol of different strains of *Legionella longbeachae* serogroup 1. (A) Animals exposed to a dose of 10^9 *L. longbeachae* serogroup 1 A5H5 organisms; (B) animals exposed to a dose of 10^9 B8 organisms; (C) animals exposed to a dose of 10^{10} B8 organisms; (D) animals exposed to a dose of 10^9 B8.22 organisms. Guinea pig death is indicated by the termination of the ribbon graph prior to the end of the experiment on day 7.

strains, in both models, with the type strain ATCC *L. longbeachae* showing a lower level of infectivity in comparison to strain A5H5. This strain difference was most significant in the animal model, where *L. longbeachae* serogroup 1 ATCC 33462 was unable to establish infection in either model, while strain A5H5 was virulent.

The results obtained in the animal model for the *mip* mutant in *L. longbeachae* serogroup 1 A5H5 are of interest, as no other *mip* mutant, other than those of *L. pneumophila* serogroup 1, has been assessed in an aerosol animal model. The results are consistent with those seen for the *mip* mutant of *L. pneumophila* serogroup 1 (11). The mutant was unable to cause death in guinea pigs under two test dose conditions. However, the test doses trialed in this study resulted in lower numbers of bacteria being deposited into the lungs than those achieved by intratracheal inoculation in the study by Cianciotto et al. (11). The aerosol model of infection makes it difficult to achieve higher numbers of deposited bacteria, and hence we cannot say whether the *mip* mutation in *L. longbeachae* serogroup 1 would have yielded different results at higher doses. It is tempting to speculate that this would be the case, as *L. longbeachae* serogroup 1 differs from *L. pneumophila* serogroup 1 in that it does not possess the major outer membrane protein (references 14, 22, and 23 and unpublished observations). The major outer membrane protein is believed to play a role in uptake of *L. pneumophila* serogroup 1 into macrophages through its ability to bind complement component C3b (5). Therefore, *L. longbeachae* serogroup 1 may be more susceptible to changes in outer membrane proteins. The difference between the wild-type parent and the *mip* mutant in *L. longbeachae* serogroup 1 on the severity of the symptoms shown indicates a significant effect on the organism. The Mip protein is likely to have a significant role in pathogenesis of the organism or in survival in protozoa and the environment.

Given the close clonal nature of *L. longbeachae* serogroup 1 (24), why is the American ATCC *L. longbeachae* serogroup 1 isolate less virulent than the Australian clinical isolate? Are there fundamental differences between the two strains that may account for these discrepancies? Work is currently under way to investigate these questions.

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