INFECTION AND IMMUNITY, Aug. 1995, p. 2840–2845 Vol. 63, No. 8
0019-9567/95/$04.00+0

Characterization of a *Legionella micdadei mip* Mutant

WILLIAM A. O'CONNELL,[1] JETTE M. BANGSBORG,[2] AND NICHOLAS P. CIANCIOTTO[1]*

Department of Microbiology-Immunology, Northwestern University, Chicago, Illinois 60611,[1] and Department of Clinical Microbiology, Statens Seruminstitut, Copenhagen, Denmark[2]

Received 5 December 1994/Returned for modification 19 January 1995/Accepted 3 May 1995

The pathogenesis of *Legionella micdadei* is dependent upon its ability to infect alveolar phagocytes. To better understand the basis of intracellular infection by this organism, we examined the importance of its Mip surface protein. In *Legionella pneumophila*, Mip promotes infection of both human macrophages and freshwater protozoa. Southern hybridization and immunoblot analyses demonstrated that *mip* sequences were present and expressed within a panel of virulent *L. micdadei* strains. Using allelic exchange mutagenesis, we then constructed an *L. micdadei* strain that completely and specifically lacked Mip. Although unimpaired in its ability to grow in bacteriologic media, this Mip mutant was defective in its capacity to infect U937 cells, a human macrophage-like cell line. Most significantly, the Mip$^-$ organism displayed a 24-fold reduction in survivability immediately after its entry into the phagocyte. Similarly, the mutant was less able to parasitize *Hartmannella* amoebae. Taken together, these data argue that Mip specifically potentiates intracellular growth by *L. micdadei*.

Legionella micdadei is the second most common cause of Legionnaires' disease and Pontiac fever (15, 21, 23). In addition, infection by this gram-negative organism can result in abscess formation and cellulitis (29, 34). In contrast to *Legionella pneumophila*, the most common agent of legionellosis, *L. micdadei* infects only immunocompromised hosts (22, 48). This observation alone suggests that *L. micdadei* lacks key virulence determinants and/or has different growth characteristics. Indeed, *L. micdadei* is chemotaxonomically and morphologically fairly distinct from *L. pneumophila* (25, 27, 30). In fact, it has been argued whether or not *L. micdadei* along with *Legionella maceachernii* should be placed within a separate genus (25, 26). However, *L. micdadei*, like *L. pneumophila*, survives within its various environments as an intracellular parasite. Within aquatic habitats, the organisms exist as parasites of amoeboid and ciliated protozoa (47, 51). Similarly, following inhalation, they invade and replicate within alveolar phagocytes (15, 27). In vitro, the legionellae can infect a wide variety of mammalian cells, including human neutrophils, monocytes, and macrophages, guinea pig macrophages, Vero cells, L cells, and HeLa cells (36, 45, 52–54).

As one approach toward understanding *L. micdadei* pathogenesis, investigators have focused on determining whether features first associated with *L. pneumophila* are also manifest by strains of *L. micdadei*. It is becoming clear that although intracellular infections by *L. micdadei* and *L. pneumophila* share significant aspects, they are not identical. Common features of *Legionella* intracellular parasitism include actin-mediated uptake, an inhibition of superoxide anion generation, and a form of intraphagosomal replication which is inhibited by γ interferon (14, 15, 36, 54). However, unlike *L. pneumophila*, *L. micdadei* does not enter monocytes by coiling phagocytosis or complement-mediated opsonophagocytosis, does not inhibit phagosome-lysosome fusion, and does not multiply within a ribosome-studded phagosome (36, 44, 53). In similar fashion, only some of the proteins or factors that may promote *L. pneumophila* pathogenesis are present in *L. micdadei*. Common putative virulence factors include a cytotoxin, a flagellum, an acid phosphatase, and an outer membrane porin (15, 30–32). Factors that are absent from *L. micdadei* include a phospholipase C, a zinc metalloprotease, a gelatinase, and a peroxidase (2, 5, 42, 43). Thus, given both its clinical significance and its apparent uniqueness, *L. micdadei* pathogenesis warrants further analysis.

The 24-kDa Mip surface protein promotes the pathogenesis of *L. pneumophila* (8, 9, 18, 20). Mutants specifically lacking Mip exhibit a reduced ability to both infect alveolar macrophages, lung epithelia, and protozoa and cause pneumonia in experimental animals (8–11, 33). Since these same mutants are unaltered in their capacity to grow within extracellular media, it has been argued that Mip, a peptidyl-prolyl *cis-trans* isomerase, is specifically required for optimal intracellular growth (9, 16, 24, 37). Using probes derived from a cloned *L. pneumophila mip* gene and anti-Mip antibodies, we demonstrated that *mip* sequences are present and expressed in other *Legionella* species, including a strain of *L. micdadei* (7). Sequence analysis confirmed that *L. micdadei* can possess a gene that shares 71% identity with *L. pneumophila mip* (3, 18). However, unlike most *Legionella* Mip proteins, the *L. micdadei* analog migrates as an approximately 30-kDa molecule in denaturing polyacrylamide gels and does not react with an anti-Mip monoclonal antibody (3, 4, 7). To better understand the molecular basis of *L. micdadei* intracellular infection as well as to appreciate the significance of Mip in another pathogen, we constructed and characterized an *L. micdadei mip* mutant. This mutant, which represents the first genetic manipulation of an *L. micdadei* strain, was defective in its ability to infect both human phagocytes and freshwater protozoa.

MATERIALS AND METHODS

Bacterial strains and media. Six clinical isolates of *L. micdadei* were examined (Table 1). Prior to this study, strains X195, 31B, Camilleri, and Rivera had been passaged three times on bacteriologic media. The plate-passage histories for strains ATCC 33218 and Detroit, however, are unknown. The virulent *L. pneumophila* strain 130b (Wadsworth) and its Mip$^-$ derivative, NU203, were described previously (10). Generally, the legionellae were grown on buffered charcoal-yeast extract (BCYE) agar plates for 48 h at 37°C (17). However, to assess extracellular growth rates, bacteria were inoculated into screw-cap tubes containing 2.5 ml of buffered yeast extract (BYE) broth and incubated at 37°C on a rotating platform. When appropriate, 3 μg of chloramphenicol per ml, 25 μg of kanamycin per ml, or 5% (wt/vol) sucrose was added to the medium. *Escherichia*

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Northwestern University, 303 East Chicago Ave., Searle 6-541, Chicago, IL 60611. Phone: (312) 503-0385. Fax: (312) 503-1339. Electronic mail address: ciancio@casbah.acns.nwu.edu.

TABLE 1. Strains of *L. micdadei* used

Strain	Source	Restriction group[a]	ID_{50} for U937 cells[b]
33218	American Type Culture Collection	I	3.98 ± 0.31
Detroit	Michigan Department of Public Health	II	3.94 ± 0.28
X195	California State Health Laboratory	II	3.40 ± 0.30
31B	University of Pittsburgh Hospital	I	2.94 ± 0.30
Camilleri	Stanford University Medical Center	III	2.40 ± 0.36
Rivera	Stanford University Medical Center	III	1.57 ± 0.36

[a] Based upon *Eco*RI digestion patterns.

[b] The ID_{50} is expressed as a log value ± the standard deviation expressed as a log value. In these experiments, *L. pneumophila* 130b had a log ID_{50} of 2.09 ± 0.32.

coli HB101 served as the host for recombinant plasmids (1). It was maintained on Luria-Bertani medium containing either 30 μg of chloramphenicol per ml, 50 μg of kanamycin per ml, or 50 μg of ampicillin per ml.

Plasmids. Recombinant plasmid pBA6004 contains the entire *mip* gene of *L. micdadei* ATCC 33218 (3, 7). The plasmid pBOC102 contains the first 312 bp of that *mip* gene (see below). The vector pNK2794, which was provided by N. Kleckner, served as the source for a kanamycin resistance (Km^r) marker. The high-copy-number plasmid pHXK (from K. Jones) was used as a cloning vehicle. The ColE1 replicon pEA75, obtained from M. Albano and N. C. Engleberg, was used in our allelic exchange mutagenesis protocol. Most importantly, this plasmid contains a selectable chloramphenicol resistance (Cm^r) marker and the counterselectable *sacB*. The *sacB* gene of *Bacillus subtilis* encodes levansucrase and is lethal to *Legionella* organisms grown in the presence of sucrose (12).

Electroporation and allelic exchange mutagenesis. Plasmids were introduced into *L. micdadei* strains by electroporation (10). The efficiency of electroporation into *L. micdadei* was, however, significantly lower than it was for *L. pneumophila*, i.e., ca. 10^2 versus 10^5 transformants per μg of DNA. The procedure for allelic exchange with ColE1 vectors containing counterselectable markers was described previously (12). Using this procedure, we achieved insertional inactivation of *mip* within an *L. micdadei* strain that had been passaged 10 times on BCYE agar plates. Thus, analyses of the *mip* mutant and its isogenic parent utilized bacteria that had been plate passaged between 11 and 14 times.

DNA isolation and molecular genetic techniques. Whole-cell DNA was extracted from *Legionella* strains as described previously (19). Plasmids were isolated from *E. coli* by the standard alkaline lysis procedure and radiolabelled with ^{32}P by use of a random primer labeling kit (Gibco-BRL, Gaithersburg, Md.). Southern hybridizations were performed under high-stringency conditions which permit 10% base pair mismatching (1).

Immunoblot analysis. To assay for the presence of Mip-like proteins, immunoblot analysis was performed with a rabbit polyclonal antiserum raised against Mip purified from recombinant *E. coli* (7). Protein extracts of *Legionella* strains were prepared as described previously and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12.5% acrylamide) (7). Following transfer to filter paper, the proteins were exposed to a 1/1,000 dilution of the anti-Mip antiserum. The immunoblot was completed with a 1/1,000 dilution of horseradish peroxidase-conjugated secondary antibodies (Gibco-BRL) and diaminobenzidine as a colorigenic substrate.

Intracellular infection of U937 cells. U937 cells are a human lymphoma cell line, which, when treated with phorbol esters, differentiate into macrophage-like cells (49). A number of investigators have used these cells for *L. pneumophila* infectivity studies (9, 35, 41, 46). U937 cell monolayers were prepared and infected as described previously (41). Following inoculation, the monolayers were incubated for 2 h to permit bacterial uptake and then rigorously washed to remove unattached, extracellular bacteria. The infected monolayers were incubated at 37°C in RPMI medium supplemented with 10% fetal bovine serum. Since *L. micdadei*, like *L. pneumophila*, did not replicate extracellularly within the tissue culture medium (data not shown), any increases in CFU were the result of intracellular multiplication.

To assess the relative infectivity of *Legionella* strains for U937 cells, 50% infective doses (ID_{50}s) were determined after 72 h of incubation (9). To monitor intracellular growth rates, four replicate monolayers were inoculated with approximately 10^6 bacteria, incubated for various times, and then lysed with 0.1% saponin (Sigma Chemical Co., St. Louis, Mo.) (9). Tenfold serial dilutions of the lysates were plated on BCYE agar, and the resulting CFU were used to calculate the corresponding numbers of bacteria per monolayer. The cytopathic effect of intracellular replication was determined by assaying, at various times, the number of viable U937 cells remaining in the monolayer (41). The vital stain tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was used to distinguish live from dead macrophages.

Infection of amoebae with *L. micdadei*. Intracellular infection of *Hartmannella vermiformis* (strain CDC-19 [ATCC 50237]) was performed as described previously for *L. pneumophila* (10, 35). Briefly, replicate *Hartmannella* cultures containing 10^5 amoebae were infected with approximately 1,000 bacterial CFU, and after various incubation periods at 35°C, the numbers of viable legionellae within the cocultures were determined by plating aliquots on BCYE medium. Since *L. micdadei* did not multiply within the amoebal growth medium (data not shown), any increases in CFU were the result of intracellular infection.

RESULTS

Intracellular infection by strains of *L. micdadei*. Previous in vitro studies reported macrophage and monocyte infection by the EK strain of *L. micdadei* (36, 53). To determine whether intracellular infectivity is a trait conserved among strains of *L. micdadei*, we examined six other clinical isolates for their ability to grow within U937 cells (Table 1). On the basis of the numbers of bacteria required to infect 50% of inoculated monolayers, each strain appeared quite infective for the macrophage-like cell line. Indeed, the two most infective strains, Camilleri and Rivera, exhibited ID_{50}s that were comparable to that of a clinical isolate of *L. pneumophila*. Furthermore, the intracellular growth rate for the Rivera strain was similar to that of *L. pneumophila*, i.e., the numbers of bacteria within an infected monolayer increased approximately 1,000-fold in a 48-h time period (see below) (9, 41).

***mip* expression by strains of *L. micdadei*.** Our earlier studies had identified a functional *mip* gene within *L. micdadei* ATCC 33218 (3, 7). To assess whether *mip* sequences are conserved within the *L. micdadei* species, genomic DNAs from the five other infective strains were hybridized with a probe derived from the cloned gene (Fig. 1, lanes a through f). Each strain contained a single *Eco*RI fragment that hybridized with the *mip* probe. Furthermore, despite restriction fragment length polymorphisms among the strains (Table 1), the size of that hybridizing fragment was always approximately 6.4 kb. To ascertain whether these strains expressed Mip-like proteins, we performed immunoblot analysis with an antiserum raised against the Mip protein of *L. pneumophila* (Fig. 2, lanes a through h). Each *L. micdadei* strain expressed a single, cross-reactive protein which was approximately 30 kDa in size. The conservation of *mip* within a number of clinical isolates suggested that Mip might promote intracellular infection by *L. micdadei.*

Construction of an *L. micdadei mip* mutant. To ultimately determine whether *mip* promotes intracellular infection by *L. micdadei*, we isolated a stable Mip^- derivative of strain Rivera. Specifically, we introduced a DNA insertion into the *mip* gene by allelic exchange; i.e., *mip* was replaced with a mutated, Km^r-tagged gene from a counterselectable vector. The plasmid used for this procedure, pBOC102, was constructed in three stages. First, a 1.8-kb *Bam*HI fragment containing Km^r was introduced into the *Hin*cII site of pBA6004 to yield pBOC100. This DNA insertion, located 44 bp into the coding region of *mip* (3), abolished antigen expression in recombinant *E. coli* (data not shown). Next, a 2.7-kb *Eco*RI fragment of pBOC100 containing the insertionally inactivated *mip* was ligated with *Eco*RI-digested pHXK to yield pBOC101. Finally, the mutated locus was transferred, on a *Not*I fragment, into the Cm^r and *sacB*-containing pEA75.

As the next step toward the construction of our mutant, pBOC102 was electroporated into strain Rivera, and the transformation mixture was plated onto BCYE agar containing kanamycin. A Km^r transformant was then replated onto BCYE agar containing both kanamycin and sucrose. By simulta-

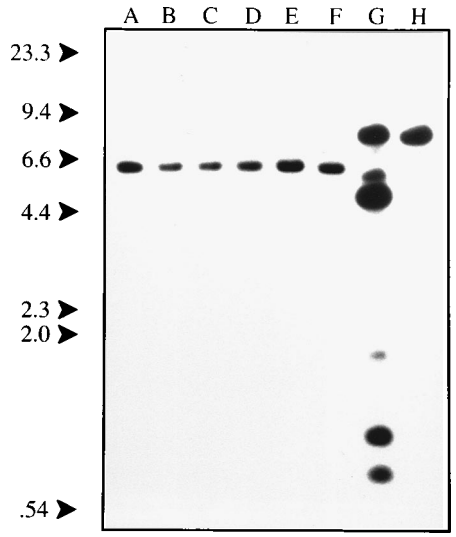

FIG. 1. Hybridization of DNAs from *L. micdadei* strains with a *mip* probe. Whole-cell DNAs were digested to completion with *Eco*RI and electrophoresed through 0.8% agarose. A Southern blot was made, hybridized with ^{32}P-labelled pBOC102 under high-stringency conditions, and autoradiographed. Lanes: a, ATCC 33218; b, Detroit; c, X195; d, 31B; e, Camilleri; f, Rivera; g, Rivera containing an integrated pBOC102; h, Rivera containing only the 1.8-kb Km^r insertion in *mip*. Since pBOC102 contains just the first 312 bp of *L. micdadei mip*, there is only one hybridizing fragment in the ATCC control lane. The slight variation in the intensities of the bands in lanes a to f is a reflection of differences in the amount of DNA loaded into each lane. In lane g, the two faint bands which migrated at ca. 5.2 and 1.8 kb represent partial digestion products. The migration and sizes (in kilobases) of molecular markers are indicated.

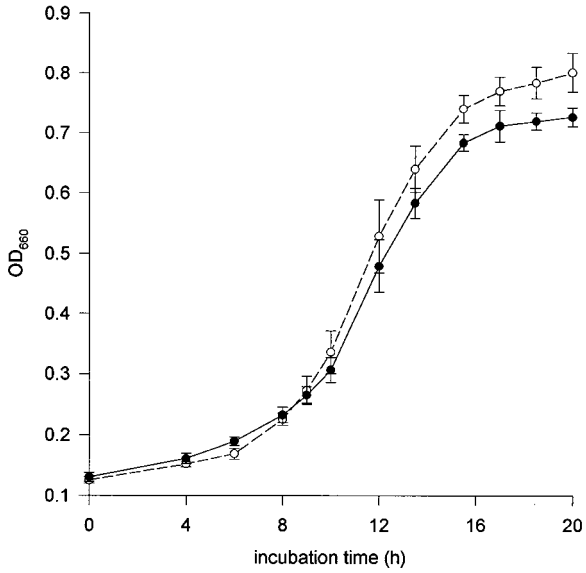

FIG. 3. Extracellular growth of *L. micdadei* strains within BYE broth. Bacteriologic media were inoculated with either the Mip$^+$ Rivera strain (●) or its Mip$^-$ derivative (○), and at various times the extent of bacterial growth was assessed by measuring the optical density at 660 nm (OD_{660}). Each datum point represents the mean optical density for four replicate cultures, and the vertical bars denote the standard deviations. The experiment presented here is representative of two additional experiments.

neously selecting for Km^r within *mip* and counterselecting against *sacB* on the vector, strains can be isolated in which the plasmid is lost and the chromosomal target gene is exchanged, via a double recombination event, for the mutated gene. To identify a strain that had undergone allelic exchange, we performed Southern hybridization analysis on a Km^r, sucrose-resistant clone which had lost its resistance to chloramphenicol. To simultaneously confirm that this strain contained a Km^r insertion while lacking other vector sequences, pBOC102 was used as the probe (Fig. 1). As predicted by the model for allelic exchange, this strain had simply replaced its 6.4-kb *mip*-containing, *Eco*RI fragment with a 7.5-kb hybridizing fragment (Fig. 1, compare lanes f and h). As a control for our infectivity experiments, we also obtained the immediate Suc^r Km^r Cm^r progenitor of the *mip* mutant, a strain which contained pBOC102 integrated into its chromosome (lane g). This Mip$^+$ strain contained both the Km^r-inactivated *mip* gene (on a 7.5-kb hybridizing fragment) and an intact *mip* gene (on a 1.0-kb hybridizing fragment) as well as the vector sequences (on 4.4- and 0.8-kb hybridizing fragments). Southern analysis of genomic DNAs that were digested with *Bam*HI, *Xho*I, *Kpn*I, and *Hin*cII further confirmed the chromosomal structure of the Mip$^-$ and Mip$^{+/-}$ strains (data not shown).

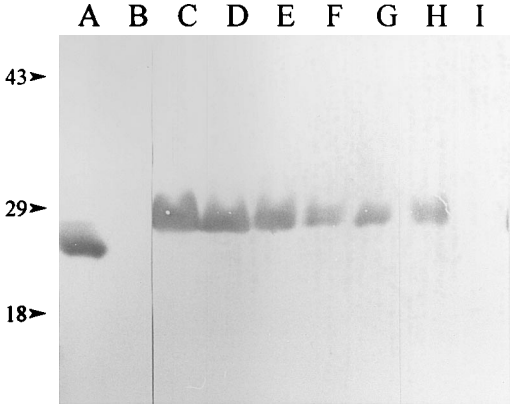

FIG. 2. Immunoblot analysis of *L. pneumophila* (lanes a and b) and *L. micdadei* (lanes c to i) strains with anti-Mip antiserum. Lanes: a, 130b; b, NU203, a Mip$^-$ derivative of 130b; c, ATCC 33218; d, Camilleri; e, Detroit; f, 31B; g, X195; h, Rivera; i, Rivera containing an insertion within *mip*. Although not visible here, the lower portion of this immunoblot did not display any small or truncated Mip-related proteins. The migration and approximate sizes (in kilodaltons) of molecular size markers are indicated.

Immunoblot analysis demonstrated that the allelic exchange event resulted in a complete loss of Mip expression (Fig. 2, compare lanes h and i). Since the *mip* locus is monocistronic (3), it is unlikely that the insertion mutation affects the expression of the genes located downstream of *mip*. On the basis of an examination of Coomassie blue-stained SDS-polyacrylamide gels, the *mip* mutant did not display any gross alterations in protein expression (data not shown). The construction of this mutant demonstrates that the mutagenesis protocols which we first developed for use with *L. pneumophila* can be applied to the study of the other legionellae.

Effect of a *mip* mutation on the extracellular growth of *L. micdadei*. The *mip* mutant grew on BCYE agar plates as well as did its wild-type parent, suggesting that Mip is not critical for extracellular growth by *L. micdadei*. To further explore this notion, we monitored the growth rates of Mip$^+$ and Mip$^-$ strain Rivera within BYE broth (Fig. 3). On three separate occasions, the *mip* mutant multiplied at a rate that was quite comparable to its isogenic parent. While doing these experiments, we also observed that the Km^r marker of the mutant (i.e., insertion mutation) was maintained during growth in the absence of antibiotic selection. Taken together, these data

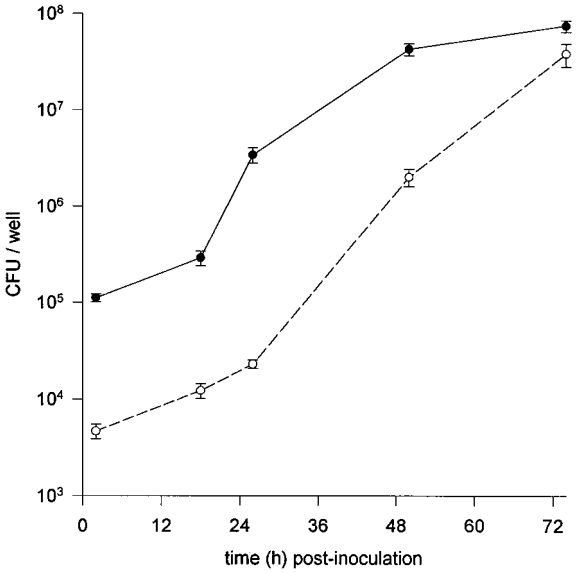

FIG. 4. Intracellular replication of *L. micdadei* strains in U937 cells. Monolayers ($n = 4$) were inoculated with either 1.8×10^6 CFU of Mip$^+$ strain Rivera (●) or 1.4×10^6 CFU of its Mip$^-$ derivative (○), and after various incubation periods, the numbers of viable intracellular bacteria were determined. Since 2 h was allowed for bacterial attachment and entry, the first sample that was collected is presented as a 2-h datum point. Each point represents the mean CFU recovered, and the vertical bars indicate the standard deviations. The differences in recovery of the strains were significant at all time points (P is <0.001 for all time points except 72 h, where P is < 0.005). The experiment presented here is representative of two additional experiments.

indicate that *mip* expression is not required for the growth of *L. micdadei* within bacteriologic media.

Effect of a *mip* mutation on the intracellular infectivity of *L. micdadei*. To determine whether Mip promotes intracellular infection of macrophages by *L. micdadei*, we assessed the relative ability of the Rivera *mip* mutant to infect U937 cells. Specifically, we inoculated U937 cell monolayers with comparable amounts of Mip$^+$ and Mip$^-$ organisms and then examined them for changes in the number of bacteria over a 72-h period (Fig. 4). Throughout the course of the experiment, the monolayers infected with the mutant yielded fewer bacteria than the wild type did. At 2 h postinoculation, there was already an approximately 24-fold difference in recovery, suggesting that the mutant is defective in the earliest stages of intracellular infection. A similar observation was made during studies involving *L. pneumophila mip* mutants (9, 33). Although the reduced recovery of the *L. micdadei* mutant shortly after inoculation could be due to a defect in attachment to and/or uptake into the phagocyte, we suspect, on the basis of earlier work with *L. pneumophila*, that the strain is simply less viable after its entry into the macrophage. Despite a prolonged lag phase, the Mip$^-$ strain, however, increased at rates that were often comparable to those displayed by the Mip$^+$ strain (Fig. 4). By the end of the experiment, the numbers of mutant and wild-type bacteria within the infected monolayers were rather similar (Fig. 4). Likewise, ID_{50} analysis done at 72 h postinoculation did not reveal a significant difference between the two strains (data not shown). These latter two observations are likely due, in part, to the fact that bacterial replication gradually destroys the macrophage monolayer (see below).

To confirm that the *L. micdadei mip* mutant has reduced intracellular infectivity, we assessed its ability to induce cytopathogenicity (9, 45). After inoculating U937 cells with equal numbers of Mip$^+$ and Mip$^-$ bacteria, we assayed the numbers of viable host cells in the monolayers over a 3-day time period (Fig. 5). A reduced cytopathic effect for the mutant was apparent by 42 h postinoculation. This difference in infectivity, although maintained throughout the remainder of the experiment, was most pronounced at 51 h postinoculation (Fig. 5). Taken together, these observations suggest that *mip*, although not necessary for extracellular growth, enhances the ability of *L. micdadei* to infect human macrophage-like cells.

To determine whether *mip* also promotes the ability of *L. micdadei* to infect freshwater protozoa, we assessed the relative ability of the *mip* mutant to infect *H. vermiformis*. Strains of this amoeba have been associated with *Legionella* spp. in water samples implicated in cases of Legionnaires' disease (35, 51). *L. micdadei* Rivera displayed an intra-amoebal growth pattern that was quite reminiscent of that of virulent *L. pneumophila* 130b; i.e., following a 24-h lag phase, the numbers of Mip$^+$ bacteria increased 1,000-fold in a 48-h time period (Fig. 6A) (10). In contrast, the Rivera *mip* mutant exhibited a ca. 10-fold drop in recovery at 24 h postinoculation, supporting the earlier notion that it is more susceptible to intracellular killing (Fig. 6). Over the next 48 h, however, the numbers of mutant bacteria increased at a rate that was comparable to that of the wild type.

To confirm that the reduced intracellular infectivity of the mutant was due to the insertional inactivation of *mip* and not the mutagenesis procedure per se or the Km^r marker, we examined the relative infectivity of a strain which contained both intact and mutated *mip* genes within its chromosome (see above) (Fig. 1). In both the amoebal assay (Fig. 6B) and the U937 cell assays (data not shown), this Mip$^{+/-}$ strain behaved as did the wild type. In summary, these data confirm that *mip* is required for optimal infection of U937 cells and amoebae by

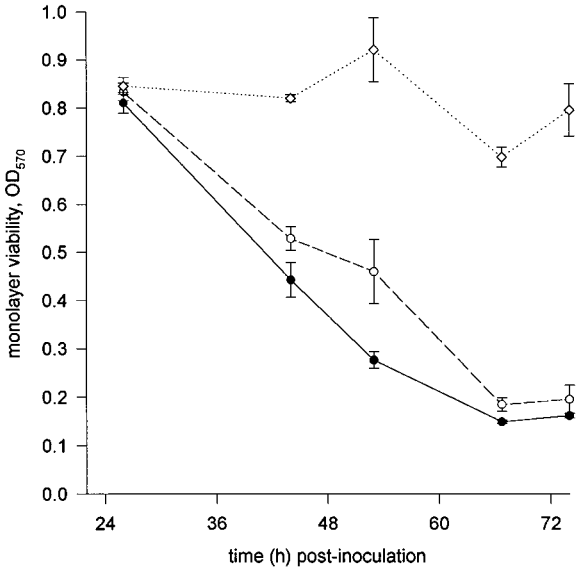

FIG. 5. Cytopathic effect of *L. micdadei* strains on U937 cells. Monolayers ($n = 6$) were inoculated with either no bacteria (◇) or 2.4×10^5 CFU of Mip$^+$ (●) or Mip$^-$ (○) strain Rivera. After various periods of incubation, the number of viable U937 cells was determined by staining with MTT and measuring the optical density at 570 nm (OD_{570}). Each datum point is the mean optical density at 570 nm, and the vertical bars indicate the standard deviations. At and beyond 42 h postinoculation, the differences in cytopathicity were significant (P is <0.001, except at 72 h, where P is <0.025). This experiment is representative of two additional experiments.

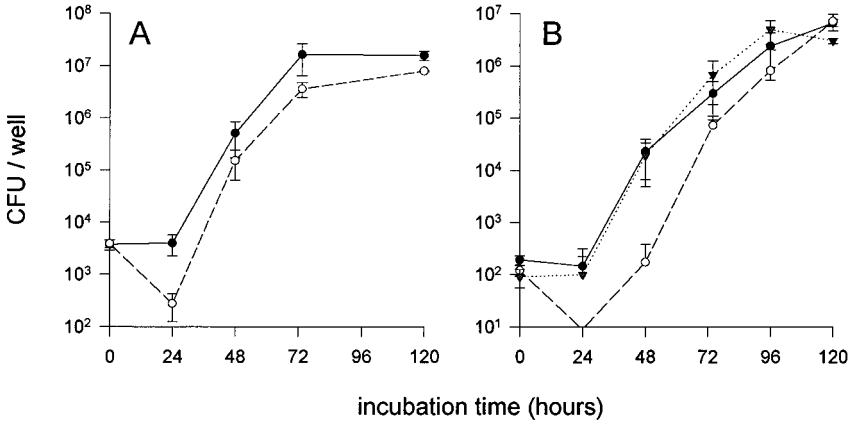

FIG. 6. Intracellular infection of *H. vermiformis* with strains of *L. micdadei*. (A) Amoebal cultures ($n = 4$) were inoculated with either 6.4×10^3 CFU of Mip$^+$ Rivera (●) or 6.6×10^3 CFU of Mip$^-$ Rivera (○), and after various incubation periods, the numbers of viable bacteria were determined. Since increases in the numbers of legionellae ultimately result in reduced monolayer viability, the growth curves converge at the last time point. Each point represents the mean CFU recovered, and the vertical bars indicate the standard deviations. Significant differences in strain recovery were observed at incubation times of 24 h ($P < 0.01$), 72 h ($P < 0.05$), and 120 h ($P < 0.005$). The experiment presented here is representative of one additional experiment. (B) Amoebal cultures were infected as described above with ca. 10^2 CFU of either Mip$^+$ Rivera (●), Mip$^-$ Rivera (○), or Rivera(pBOC102) containing both inactivated and intact *mip* genes (▼). At 24 h postinoculation, no mutant bacteria were recovered; i.e., the infected monolayers contained less than 10 CFU. Significantly fewer Mip$^-$ bacteria were also recovered at 48 h postinoculation ($P < 0.005$). The experiment presented here is representative of one additional experiment.

L. micdadei. They also highlight the similarity between macrophage and protozoan infections (10).

DISCUSSION

Four observations indicate that *L. micdadei* Mip specifically potentiates intracellular infection. First, a mutant lacking Mip was defective in its ability to infect a macrophage-like cell line. Second, that mutant had altered survivability within amoebae. Third, the Mip-negative strain displayed extracellular growth characteristics that were equal to those of the wild type. Fourth, *mip* was expressed within a panel of infective *L. micdadei* strains. Thus, despite their structural and antigenic differences (3, 7), the Mip proteins of *L. pneumophila* and *L. micdadei* appear, at a gross level, to perform similar functions. Indeed, in some ways, the *L. micdadei mip* mutant behaved as did the *L. pneumophila mip* mutant. For example, both strains exhibited an approximately 20-fold-reduced survivability after entry into U937 cells (9, 33). The importance of Mip for the infectivity of these fairly distinct *Legionella* species increases the likelihood that Mip is involved in the pathogenesis of all legionellae (7).

The reduced viability of *Legionella mip* mutants shortly after uptake indicates that Mip may play a role in resistance to immediate intracellular death. Therefore, following our analysis of the *L. pneumophila* mutant, we had suggested that Mip might be linked to the inhibition of phagosome-lysosome fusion (9, 18). However, since *mip* promotes infection by *L. micdadei*, an organism that does not block phagosome-lysosome fusion (44), the potential targets of Mip activity must not be limited to phagosome-lysosome fusion events. Our recent discovery of a 25-kDa Mip-like protein in *Coxiella burnetii*, an organism that grows within a phagolysosome, supports this notion (13, 40). Two additional observations preclude a simple hypothesis for Mip function. First, a related, 27-kDa membrane protein exists in *Chlamydia trachomatis* (38). Second, an *L. pneumophila mip* mutant was impaired in its ability to infect lung epithelia, intimating that the target of Mip activity is not specific to phagocytic cells (11). Although the precise function of the secreted or membrane Mip-like proteins is unclear, the peptidyl-prolyl isomerase activity associated with the *L. pneumophila*, *Coxiella burnetii*, and *Chlamydia trachomatis* proteins may be important (24, 28, 38, 40). For example, Mip may facilitate an adaptation to intracellular environments by altering the conformation of parasite and/or host proteins, including, perhaps, those embedded in newly formed phagosomal membranes. The critical action of Mip might also mimic that of its distant eukaryotic analog, the FK506 binding proteins (3, 28, 50). The FK506 binding proteins can affect the activities of calcium channels as well as the calcium-dependent phosphatase calcineurin (6, 39). Our construction of an additional *mip* mutant should help facilitate the elucidation of the pathogenic activities of Mip.

ACKNOWLEDGMENTS

We thank J. Moffat and L. Tompkins for *L. micdadei* 31B, X195, Camilleri, and Rivera. We thank H. Seifert, M. Albano, N. C. Engleberg, and N. Kleckner for plasmids.

This work was supported by Public Health Service grant AI30064 from the National Institutes of Health (to N.P.C.).

REFERENCES

1. **Ausubel, F. M., R. B. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Baine, W. B.** 1985. Cytolytic and phospholipase C activity in *Legionella* species. J. Gen. Microbiol. **131:**1383–1391.
3. **Bangsborg, J. M., N. P. Cianciotto, and P. Hindersson.** 1991. Nucleotide sequence analysis of the *Legionella micdadei mip* gene, encoding a 30-kilodalton analog of the *Legionella pneumophila* Mip protein. Infect. Immun. **59:**3836–3840.
4. **Bangsborg, J. M., G. Shand, E. Pearlman, and N. Hoiby.** 1991. Cross-reactive *Legionella* antigens and the antibody response during infection. Acta Pathol. Microbiol. Immunol. Scand. **99:**854–865.
5. **Bornstein, N., M. Nowicki, and J. Fleurette.** 1988. Haemolytic activity in the genus *Legionella*. Ann. Inst. Pasteur/Microbiol. **139:**325–329.
6. **Brillantes, A. B., K. Ondrias, A. Scott, E. Kobrinsky, E. Ondriasova, M. C. Moschella, T. Jayaraman, M. Landers, B. E. Ehrlich, and A. R. Marks.** 1994. Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. Cell **77:**513–523.
7. **Cianciotto, N. P., J. M. Bangsborg, B. I. Eisenstein, and N. C. Engleberg.** 1990. Identification of *mip*-like genes in the genus *Legionella*. Infect. Immun. **58:**2912–2918.
8. **Cianciotto, N. P., B. I. Eisenstein, C. H. Mody, and N. C. Engleberg.** 1990. A mutation in the *mip* gene results in an attenuation of *Legionella pneumophila* virulence. J. Infect. Dis. **162:**121–126.

9. **Cianciotto, N. P., B. I. Eisenstein, C. H. Mody, G. B. Toews, and N. C. Engleberg.** 1989. A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. Infect. Immun. **57:**1255–1262.
10. **Cianciotto, N. P., and B. S. Fields.** 1992. *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. Proc. Natl. Acad. Sci. USA **89:**5188–5191.
11. **Cianciotto, N. P., J. Kim Stamos, and D. W. Kamp.** 1995. Infectivity of *Legionella pneumophila mip* mutant for alveolar epithelial cells. Curr. Microbiol. **30:**247–250.
12. **Cianciotto, 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.
13. **Cianciotto, N. P., W. O'Connell, G. A. Dasch, and L. P. Mallavia.** 1995. Detection of *mip*-like sequences and Mip-related proteins within the family *Rickettsiaceae*. Curr. Microbiol. **30:**149–153.
14. **Donowitz, G. R., I. Reardon, J. Dowling, L. Rubin, and D. Focht.** 1990. Ingestion of *Legionella micdadei* inhibits human neutrophil function. Infect. Immun. **58:**3307–3311.
15. **Dowling, J. N., A. K. Saha, and R. H. Glew.** 1992. Virulence factors of the family *Legionellaceae*. Microbiol. Rev. **56:**32–60.
16. **Dumais-Pope, C., W. O'Connell, and N. P. Cianciotto.** 1993. Distribution and regulation of the *Legionella mip* gene, p. 70–72. *In* J. M. Barbaree, R. F. Brieman, and A. P. Dufour (ed.), *Legionella*: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
17. **Edelstein, P. H.** 1982. Comparative study of selective media for isolation of *Legionella pneumophila* from potable water. J. Clin. Microbiol. **16:**697–699.
18. **Engleberg, N. C., C. Carter, D. R. Weber, N. P. Cianciotto, and B. I. Eisenstein.** 1989. DNA sequence of *mip*, a *Legionella pneumophila* gene associated with macrophage infectivity. Infect. Immun. **57:**1263–1270.
19. **Engleberg, N. C., D. J. Drutz, and B. I. Eisenstein.** 1984. Cloning and expression of *Legionella pneumophila* antigens in *Escherichia coli*. Infect. Immun. **44:**222–227.
20. **Engleberg, N. C., and B. I. Eisenstein.** 1991. Progress in the pathogenesis of *Legionella pneumophila*. Microb. Pathog. **10:**11–13.
21. **Fallon, R. J., and T. J. Rowbotham.** 1990. Microbiological investigations into an outbreak of Pontiac fever due to *Legionella micdadei* associated with use of a whirlpool. J. Clin. Pathol. **43:**479–483.
22. **Fang, G. D., V. L. Yu, and R. M. Vickers.** 1987. Infections caused by the Pittsburgh pneumonia agent. Semin. Respir. Infect. **2:**262–266.
23. **Fang, G. D., V. L. Yu, and R. M. Vickers.** 1989. Disease due to the *Legionellaceae* (other than *Legionella pneumophila*). Medicine **68:**116–132.
24. **Fischer, G., H. Bang, B. Ludwig, K. Mann, and J. Hacker.** 1992. Mip protein of *Legionella pneumophila* exhibits peptidyl-prolyl-cis/trans isomerase (PPIase) activity. Mol. Microbiol. **6:**1375–1383.
25. **Fox, K. F., and A. Brown.** 1993. Properties of the genus *Tatlockia*. Differentiation of *Tatlockia* (*Legionella*) *maceachernii* and *micdadei* from each other and from other legionellae. Can. J. Microbiol. **39:**486–491.
26. **Fry, N. K., S. Warwick, N. A. Saunders, and T. M. Embley.** 1991. The use of 16S ribosomal RNA analyses to investigate the phylogeny of the family *Legionellaceae*. J. Gen. Microbiol. **137:**1215–1222.
27. **Gress, F. M., R. L. Myerowitz, A. W. Pasculle, C. R. Rinaldo, Jr., and J. N. Dowling.** 1980. The ultrastructural morphologic features of Pittsburgh pneumonia agent. Am. J. Pathol. **101:**63–78.
28. **Hacker, J., and G. Fischer.** 1993. Immunophilins: structure-function relationships and possible role in microbial pathogenicity. Mol. Microbiol. **10:** 445–456.
29. **Halberstam, M., H. D. Isenberg, and E. Hilton.** 1992. Abscess and empyema caused by *Legionella micdadei*. J. Clin. Microbiol. **30:**512–513.
30. **Hébert, G. A., C. S. Callaway, and E. P. Ewing, Jr.** 1984. Comparison of *Legionella pneumophila*, *L. micdadei*, *L. bozemanii*, and *L. dumoffii* by transmission electron microscopy. J. Clin. Microbiol. **19:**116–121.
31. **Hedlund, K. W.** 1981. *Legionella* toxin. Pharmacol. Ther. **15:**123–130.
32. **Hoffman, P. S., M. Ripley, and R. Weeratna.** 1992. Cloning and nucleotide sequence of a gene (*ompS*) encoding the major outer membrane protein of *Legionella pneumophila*. J. Bacteriol. **174:**914–920.
33. **Hurley, M. C., K. Balazovich, M. Albano, N. C. Engleberg, and B. I. Eisenstein.** 1993. *Legionella pneumophila* Mip inhibits protein kinase C, p. 69–70. *In* J. M. Barbaree, R. F. Brieman, and A. P. Dufour (ed.), *Legionella*: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
34. **Kilborn, J. A., L. A. Manz, M. O'Brien, M. C. Douglass, H. M. Horst, W. Kupin, and E. J. Fisher.** 1992. Necrotizing cellulitis caused by *Legionella micdadei*. Am. J. Med. **92:**104–106.
35. **King, C. H., B. S. Fields, E. B. Shotts, Jr., and E. H. White.** 1991. Effects of cytochalasin D and methylamine on intracellular growth of *Legionella pneumophila* in amoebae and human monocyte-like cells. Infect. Immun. **59:**758–763.
36. **Levi, M. H., A. W. Pasculle, and J. N. Dowling.** 1987. Role of the alveolar macrophage in host defense and immunity to *Legionella micdadei* pneumonia in the guinea pig. Microb. Pathog. **2:**269–282.
37. **Ludwig, B., J. Rahfeld, B. Schmidt, K. Mann, E. Wintermeyer, G. Fischer, and J. Hacker.** 1994. Characterization of Mip proteins of *Legionella pneumophila*. FEMS Microbiol. Lett. **118:**23–30.
38. **Lundemose, A. G., D. A. Rouch, C. W. Penn, and J. H. Pearce.** 1993. The *Chlamydia trachomatis* Mip-like protein is a lipoprotein. J. Bacteriol. **175:** 3669–3671.
39. **Liu, J.** 1993. FK506 and cyclosporin, molecular probes for studying intracellular signal transduction. Immunol. Today **14:**290–295.
40. **Mo, Y.-Y., N. P. Cianciotto, and L. P. Mallavia.** Submitted for publication.
41. **Pearlman, E., A. H. Jiwa, N. C. Engleberg, and B. I. Eisenstein.** 1988. Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line. Microb. Pathog. **5:**87–95.
42. **Pine, L., P. S. Hoffman, G. B. Malcolm, R. F. Benson, and M. G. Keen.** 1984. Determination of catalase, peroxidase, and superoxide dismutase within the genus *Legionella*. J. Clin. Microbiol. **20:**421–429.
43. **Quinn, F. D., M. G. Keen, and L. S. Tompkins.** 1989. Genetic, immunological, and cytotoxic comparisons of *Legionella* proteolytic activities. Infect. Immun. **57:**2719–2725.
44. **Rechnitzer, C., and J. Blom.** 1989. Engulfment of the Philadelphia strain of *Legionella pneumophila* within pseudopod coils in human phagocytes. Comparison with other *Legionella* strains and species. Acta Pathol. Microbiol. Immunol. Scand. **97:**105–114.
45. **Rinaldo, C. R., Jr., A. W. Pasculle, R. L. Myerowitz, F. M. Gress, and J. N. Dowling.** 1981. Growth of the Pittsburgh pneumonia agent in animal cell cultures. Infect. Immun. **33:**939–943.
46. **Rodgers, F. G., and F. C. Gibson III.** 1993. Opsonin-independent adherence and intracellular development of *Legionella pneumophila* within U-937 cells. Can. J. Microbiol. **39:**718–722.
47. **Rowbotham, T. J.** 1986. Current views on the relationships between amoebae, legionellae and man. Isr. J. Med. Sci. **22:**678–689.
48. **Schwebke, J. R., R. Hackman, and R. Bowden.** 1990. Pneumonia due to *Legionella micdadei* in bone marrow transplant recipients. Rev. Infect. Dis. **12:**824–828.
49. **Sundstrom, C., and K. Nilsson.** 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U937). Int. J. Cancer **17:**565–577.
50. **Tropschug, M., E. Wachter, S. Mayer, E. R. Schonbrunner, and F. X. Schmid.** 1990. Isolation and sequence of an FK506-binding protein from *N. crassa* which catalyses protein folding. Nature (London) **346:**674–677.
51. **Wadowsky, R. M., T. M. Wilson, N. J. Kapp, A. J. West, J. M. Kuchta, S. J. States, J. N. Dowling, and R. B. Yee.** 1991. Multiplication of *Legionella* spp. in tap water containing *Hartmannella vermiformis*. Appl. Environ. Microbiol. **57:**1950–1955.
52. **Weinbaum, D. L., J. Bailey, R. R. Benner, A. W. Pasculle, and J. N. Dowling.** 1983. The contribution of human neutrophils and serum to host defense against *Legionella micdadei*. J. Infect. Dis. **148:**510–517.
53. **Weinbaum, D. L., R. R. Benner, J. N. Dowling, A. Alpern, A. W. Pasculle, and G. R. Donowitz.** 1984. Interaction of *Legionella micdadei* with human monocytes. Infect. Immun. **46:**68–73.
54. **Whitaker Dowling, P., J. N. Dowling, L. Liu, and J. S. Youngner.** 1986. Interferon inhibits the growth of *Legionella micdadei* in mouse L cells. J. Interferon Res. **6:**107–114.