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# *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages

(intracellular parasitism/evolution/*Hartmannella*/*Tetrahymena*/FK506-binding proteins)

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ABSTRACT **_Legionella pneumophila_ is an intracellular parasite of freshwater protozoa and human macrophages. Recent studies determined that the macrophage infectivity potentiator (Mip) surface protein, a prokaryotic homolog of the FK506-binding proteins, is required for optimal infection of macrophages. To determine whether Mip is also involved in _L. pneumophila_ infection of protozoa, we examined the ability of a strain lacking Mip to parasitize _Hartmannella_ amoebae and _Tetrahymena_ ciliates. After 3 days of incubation, ≈1000-fold fewer bacteria were recovered from protozoan cocultures infected with the Mip⁻ strain than from those cocultures infected with an isogenic Mip⁺ strain. The _mip_ mutant was, however, not impaired in its ability to bind to amoebae cell surfaces, indicating that Mip is involved in bacterial resistance to intracellular killing and/or intracellular multiplication. These data suggest that _L. pneumophila_ employs similar genes and mechanisms to infect human cells and protozoa. Furthermore, they support the hypothesis that the ability of _L. pneumophila_ to parasitize macrophages and hence to cause human disease is a consequence of its prior adaptation to intracellular growth within protozoa.**

*Legionella pneumophila*, the etiologic agent of Legionnaires' disease, represents a major environmental pathogen (1, 2). Strains of this bacterium are ubiquitous within aquatic environments and can infect humans following the inhalation of contaminated aerosols generated by air-conditioners, fountains, and other man-made devices (1, 3). Within the lower respiratory tract, *L. pneumophila* invades and proliferates to large numbers within alveolar macrophages (1). In the absence of an adequate cell-mediated immune response, the rapid intracellular replication of legionellae and the release of tissue-destructive substances from either the bacteria, the host, or both result in acute bronchopneumonia (2, 4). *L. pneumophila* infection of macrophages is characterized by an evasion of the bactericidal oxidative burst, an inhibition of phagosome–lysosome fusion, and lysis of the host cell (2, 4). Despite our increased understanding of the pathogenesis of legionellosis, it is still not clear how *L. pneumophila*, an organism that neither possesses a mammalian reservoir nor exhibits a "natural" route of infection, evolved the facility to parasitize human professional phagocytes. The answers to this paradox may lie within studies that identify commonalities between the human host and the seemingly disparate aquatic environment.

Although the legionellae are capable of extracellular growth, much evidence supports the notion that *L. pneumophila* flourishes within aquatic environments as an intracellular parasite of protozoa (5). *L. pneumophila* can grow within a variety of amoebae, including strains of *Acanthamoeba*, *Echinamoeba*, *Hartmannella*, *Naegleria*, and *Valkampfia*, as well as within the ciliate protozoa *Tetrahymena* (5–16). These protozoa are present in water samples that have been implicated as the sources for cases of legionellosis and, more important, the capacity of such water samples to support the growth of *L. pneumophila* is dependent upon the presence of the protozoa (7, 14, 15, 17). We share the belief that adaptation to intracellular niches within protozoa engendered in *L. pneumophila* the ability to infect mammalian cells. However, support for this hypothesis requires the demonstration that *Legionella* employs similar mechanisms (genes) to infect its various host cells.

Recent studies demonstrated that a *L. pneumophila* mutant lacking the 24-kDa macrophage infectivity potentiator (Mip) surface protein is impaired in its ability to infect human alveolar macrophages *in vitro* and to cause disease in experimental animals following intratracheal inoculation (18–20). With the identification of *mip*, a recently described genetically defined virulence factor of *Legionella* (21), we can begin to ascertain whether infection of macrophages and infection of protozoa have a common molecular basis. This report demonstrates that a *mip* mutant is also defective in its ability to parasitize two different types of freshwater protozoa, *Hartmannella vermiformis* and *Tetrahymena pyriformis*, suggesting that intracellular parasites of mammalian cells may have evolved from free-living forms that first adapted to growth within lower eukaryotes.

## MATERIALS AND METHODS

**Bacterial Strains and Medium.** The Mip⁺ *L. pneumophila* strain derived for this study, NU201, is a spontaneous streptomycin-resistant derivative of the virulent, clinical isolate strain 130b (18). Strain NU203 is an isogenic Mip⁻ derivative of strain NU201. To construct NU203, a DNA insertion was introduced into the *mip* gene of strain NU201 by allelic exchange—i.e., *mip* was replaced with a mutated, kanamycin-resistance (Km$^r$)-tagged gene from a counterselectable plasmid vector (22). The mutagenesis procedure was performed as before (18) but with one significant modification; electroporation (see below), rather than conjugation, was used to introduce the mutated *mip* gene into *L. pneumophila*. As a result of this modification, we achieved the insertional inactivation of *mip* within a virulent strain that had been passaged less than 10 times on artificial medium. Strain NU203 exhibited the same defect in macrophage infection as the original *mip* mutant AA105 (18), indicating that *mip*'s role in intracellular infection is not strain dependent. Although they still differ in their ability to infect macrophages, strain

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Abbreviations: Mip (or *mip*), macrophage infectivity potentiator; Km$^r$, kanamycin-resistance; BCYE, buffered charcoal yeast extract; cfu, colony forming unit(s).

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AA105 and its Mip$^+$ parent AA103 have diminished infectivity due to prolonged passage on artificial medium.

*Legionella* strains were grown on buffered charcoal yeast extract (BCYE) medium for 48–72 hr at 37°C (23).

**Electroporation of *L. pneumophila*.** Electroporation was performed using the "Cell-Porator electroporation system and voltage booster" according to the manufacturer's specifications (BRL). To prepare *L. pneumophila* for electroporation, bacteria from fresh BCYE plates were suspended in 20 ml of double-distilled water to an $OD_{660}$ of ≈1.8 and centrifuged. The bacterial pellet was dispersed in 500 ml of sterile, ice-cold 10% glycerol/90% double-distilled water, and the cell suspension was centrifuged for 25 min at 4300 × *g* (6000 rpm, Beckman JA-20 rotor). The bacteria were then subjected to a second glycerol wash and recentrifuged. Finally, the bacteria were resuspended in 500 μl of 10% glycerol, resulting in a suspension of ≈$10^{11}$ colony-forming units (cfu)/ml. The cells either were used immediately or were stored as 100-μl samples at −70°C.

In the presence of plasmid DNA, glycerol-treated legionellae were exposed to an electric pulse of 2.4 kV. The pulsed cells were inoculated into 1 ml of buffered yeast extract broth, incubated at 37°C for 60–90 min, and then plated on the appropriate antibiotic-containing BCYE medium. The 15-kilobase (kb) pNC31.3 plasmid (18) used for allelic exchange of *mip* was transferred into strain NU201 at a frequency of about $10^{-3}$ Km$^r$ cfu per recipient or 4 × $10^5$ Km$^r$ cfu/μg of DNA. In comparison, electroporation of strain 130b with the 8.6-kb Km$^r$ plasmid pEYDG1 (24) yielded $10^{-2}$ Km$^r$ cfu per recipient and resulted in ≈1 × $10^6$ Km$^r$ cfu/μg of DNA.

**Protozoan Strains and Media.** *H. vermiformis* strain CDC-19 [registered with American Type Culture Collection (ATCC) as strain 50237] was isolated from a water sample obtained during a case of nosocomial legionellosis (25). The amoebae were grown in ATCC medium 1034 at 35°C as before (26). *T. pyriformis* cultures were obtained from a University of Georgia stock strain (no. 500, Midwest Cultures Services) and were maintained in Elliot medium no. 2 at 25°C as outlined previously (8).

**Infection of Protozoa with *L. pneumophila* Strains.** The detailed protocols for infection of *Hartmannella* and *Tetrahymena* have been reported (8, 25–27). Briefly, replicate protozoan cultures were infected separately with ≈$10^3$ bacterial cfu, and after various incubation periods at 35°C the numbers of viable *L. pneumophila* within the cocultures were determined by plating, in triplicate, aliquots on BCYE medium. Since *L. pneumophila* does not replicate extracellularly within the growth medium either in the presence or in the absence of protozoa, any increases in cfu are the result of intracellular multiplication (8, 25–27). The growth kinetics of the Mip$^+$ strain NU201 within the two types of protozoa was similar to that of other strains of *L. pneumophila* (Figs. 1 and 3) (7, 26–28).

**Binding of *L. pneumophila* to *Hartmannella*.** The details of this assay will be described elsewhere (B.S.F., unpublished data). Briefly, [$^{35}$S]cysteine-labeled bacteria were allowed to adhere to amoebae for various lengths of time at 35°C. Monolayers were then washed extensively to remove unattached bacteria, and then cell-associated cpm were determined. The cell-associated cpm represent bacteria that are adherent to the amoeba cell surface as well as bacteria that have penetrated into the amoeba. Since radiolabeled NU201 and NU203 cultures exhibited comparable cpm per cfu, any differences in cell-associated cpm would reflect differences in the numbers of bacteria associated with the amoebae.

## RESULTS

To determine if *mip* has a role in infection of protozoa, we compared the abilities of the *mip* mutant NU203 and its isogenic parent NU201 to replicate within the amoeba *H. vermiformis*. Strains of *H. vermiformis* have been associated with *L. pneumophila* in water samples implicated in cases of legionnaires' disease (25, 28). Within *Hartmannella* cultures, the numbers of Mip$^-$ bacteria did not increase until 48 hr after inoculation, indicating that the mutant is defective in its ability to initiate intracellular multiplication within amoebae (Fig. 1). Following this prolonged lag period, the numbers of NU203 bacteria increased at a slower rate than did the numbers of NU201 bacteria such that by 72 hr after inoculation there was a 1000-fold difference in recovery between the Mip$^+$ and Mip$^-$ strains. These data confirm that *mip* is required for optimal intracellular infection of amoebae as well as human macrophages. Interestingly, the mutant's growth kinetics within amoeba cultures were similar to those observed within alveolar macrophage cultures (18), suggesting that *mip* has a common role in infection in both cell types.

To begin to understand the function of Mip in the initiation of amoeba infection, we quantitated the relative rate at which Mip$^-$ bacteria associate with *Hartmannella* cells (Fig. 2). Over the first 12 hr of incubation, comparable numbers of Mip$^+$ and Mip$^-$ bacteria became associated with amoebae, suggesting that Mip is not critical for the initial interactions between *L. pneumophila* and its amoebic host. Similarly, Mip does not appear to have a role in bacterial uptake into macrophages (18). Consequently, Mip must be involved in bacterial resistance to intracellular killing and/or bacterial intracellular replication. Regardless of Mip's precise function, these observations provide genetic evidence that intracellular infection of human phagocytes and freshwater protozoa can share a common molecular basis.

Since strains of *L. pneumophila* can exhibit broad host ranges, it is important to determine whether similar genes are required for infection of physiologically distinct protozoan hosts. Consequently, we examined the relative infectivity of the *mip* mutant for the ciliate *T. pyriformis*, a species that, like *H. vermiformis*, is naturally associated with legionellae bacteria (7, 8, 27). Within this ciliate host, strain NU203 exhibited a 10-fold drop in recovery at 24 hr, supporting the notion that it is more susceptible to intracellular killing (Fig. 3). Although the mutant bacteria increased in number at a rate comparable to that of the parent bacteria, there were 100- to 1000-fold differences in recovery between strains over the next 4 days. Despite some differences in the growth kinetics of the *mip* mutant within *Hartmannella* versus *Tetrahymena*, Mip was required for optimal intracellular infection of amoe-

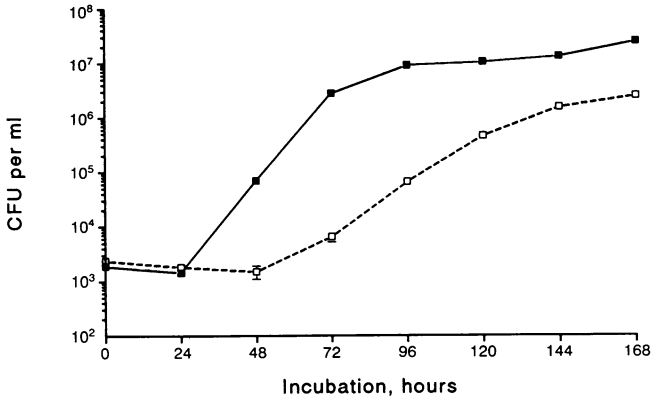


FIG. 1. Intracellular infection of the amoeba *H. vermiformis* with strains of *L. pneumophila*. Bacterial strains shown are Mip$^+$ NU201 (■) and Mip$^-$ NU203 (□). Each point represents the mean cfu recovered. Vertical bars indicate the standard errors, but, in most instances, they do not extend beyond the symbol. Since increases in the numbers of bacteria eventually result in loss of monolayer viability, the growth curves converge at the later time points.

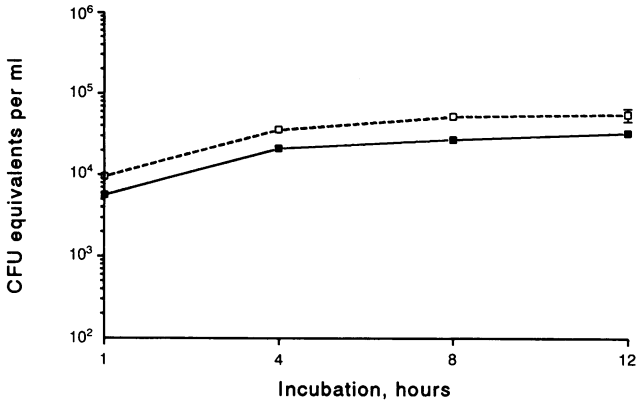


FIG. 2. Association of *L. pneumophila* strains with *H. vermiformis*. Bacterial strains are Mip$^+$ NU201 (■) and Mip$^-$ NU203 (□). Each point represents the mean and standard error from three infected monolayers.

bae, ciliates, and macrophages. These results confirm that similarities exist between these three forms of intracellular infection.

## DISCUSSION

Our findings provide genetic evidence that *Legionella* infection of protozoa is related to *Legionella* infection of human cells. Earlier observations support the notion that the requirements for parasitic growth within a protozoa mimic those within macrophages. (*i*) *Legionella* parasites replicate in amoebae, ciliates, and macrophages within ribosome-studded vesicles (9, 27, 29). (*ii*) Bacteria grown in amoebae maintain their ability to infect macrophages and to cause disease (13). (*iii*) Strains of *Legionella* that had been rendered avirulent by prolonged passage on artificial medium lose their ability to infect protozoa and macrophages (27). (*iv*) Amoebae possess a respiratory burst apparatus that is quite similar to that of human phagocytes (30). However, a recent study indicates that *L. pneumophila* infection of amoebae and macrophages may differ at the stage of bacterial invasion. Whereas *L. pneumophila* enters into a human macrophage cell line by microfilament-dependent and -independent processes, it enters into *Hartmannella* solely by a microfilament-independent mechanism akin to adsorptive pinocytosis (26). Although *Legionella* infections of amoebae, ciliates, and macrophages are likely to differ in additional ways, they remain notably similar.

Given that *Legionella* entry into the human host is essentially an adventitious event, the ability of *L. pneumophila* to

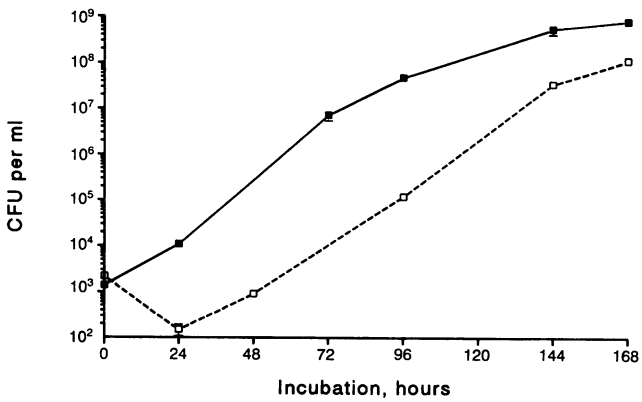


FIG. 3. Intracellular infection of the ciliate *T. pyriformis* with strains of *L. pneumophila*. Bacterial strains and markers are as described in the legend to Fig. 1.

infect human cells is likely a result of its natural adaptation to growth within freshwater protozoa. Consequently, we argue that *mip*, as well as many other "virulence factor" genes, evolved in response to selective pressures within the protozoan environment and is interacting with conserved cellular component(s). The phenotype of the *mip* mutant within protozoa and macrophages suggests that Mip is interacting with an intracellular target to disable bactericidal function and/or promote intracellular multiplication. Perhaps, it is not surprising that Mip was not critical for bacterial attachment and/or entry since, as noted above, *Legionella* entry into amoebae may differ significantly from entry into macrophages. Protein sequence comparisons have provided further clues to Mip's possible function and cellular target. Mip has homology with cytosolic FK506-binding proteins (FKBP), a newly identified class of protein isomerases or rotamases (31–33). FKBP, in the presence of the immunosuppressive drug FK506, can inhibit calcineurin, a calcium- and calmodulin-dependent phosphatase (34). Interestingly, FKBP and calcineurin exist within the entire range of eukaryotic cells (35, 36). The ability to alter the activity of a host cell regulatory protein such as calcineurin would represent a potent mechanism for deregulating host cell function.

Presently, the legionellae are considered unique among bacteria as parasites of protozoa and mammalian cells. However, a wide variety of other bacteria flourish within protozoa as either commensals, symbionts, or parasites (37, 38). Given the example of *Legionella*, it is possible that these microbes, by virtue of their adaptation to growth within protozoa, acquire the ability to infect mammalian cells. Alternatively, bacteria that are known to infect mammalian cells may be related to these inhabitants of protozoa or may themselves possess the ability to infect protozoa. Through the use of PCR technology, it was recently determined that *Holospora*, an endosymbiont of amoebae, is most closely related to *Rickettsia*, a pathogenic intracellular parasite of mammalian cells (39). We submit that primitive host–parasite interactions have broad significance for the evolution and natural history of infectious diseases.

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