INFECTION AND IMMUNITY, Dec. 1995, p. 4576–4583 Vol. 63, No. 12
0019-9567/95/$04.00+0

Influence of Site Specifically Altered Mip Proteins on Intracellular Survival of *Legionella pneumophila* in Eukaryotic Cells

EVA WINTERMEYER,[1] BIRGIT LUDWIG,[1]* MICHAEL STEINERT,[1] BETTINA SCHMIDT,[2] GUNTER FISCHER,[2] AND JÖRG HACKER[1]

Institut für Molekulare Infektionsbiologie, Universität Würzburg, D-97070 Würzburg,[1] and Max-Planck-Gesellschaft, Arbeitsgruppe "Enzymologie der Peptidbindung," D-06120 Halle/Saale,[2] Germany

Received 30 May 1995/Returned for modification 1 August 1995/Accepted 5 September 1995

***Legionella pneumophila*, the causative agent of Legionnaires' disease, is able to survive intracellularly in eukaryotic cells such as monocytes, macrophages, and protozoan organisms. The Mip (macrophage infectivity potentiator) protein represents a factor of *L. pneumophila* necessary for optimal intracellular survival. Interestingly, Mip belongs to the substance class of FK 506-binding proteins and exhibits peptidyl-prolyl *cis*/*trans* isomerase (PPIase) activity that can be inhibited by the immunosuppressant FK 506. In order to identify amino acids most likely to be involved in the enzymatic activity of Mip, site-directed mutagenized Mip proteins were constructed and characterized. It was shown that an Asp-142 to Leu-142 mutation and a Tyr-185 to Ala-185 substitution resulted in strongly reduced PPIase activity of the recombinant Mip proteins (5.3 and 0.6% of the activity of the wild-type Mip, respectively). Genes coding for the wild-type and for site-directed-mutagenized Mip proteins were used to complement three different Mip-negative mutants of the *L. pneumophila* Corby, Philadelphia I, and Wadsworth. While Mip protein expression could be restored in the corresponding complementants, significant Mip-specific PPIase activity could be detected only in Mip mutants complemented with wild-type *mip* genes. To investigate the influence of the PPIase activity of Mip on intracellular survival of *L. pneumophila*, invasion assays were performed using the macrophage-like cell line U937, human blood monocytes, and *Acanthamoeba castellanii*. The Mip-negative mutants were approximately 50- to 100-fold less infective for *A. castellanii* and for human mononuclear phagocytes in vitro compared with their isogenic Mip-positive parental strains. The wild-type invasion rate could be restored by introducing an intact copy of the *mip* gene into Mip-negative strains. In addition, no differences in intracellular survival were observed between the wild-type isolates and the *Legionella* strains exhibiting strongly reduced PPIase activity. These data indicated that the enzymatic activity of Mip does not contribute to intracellular survival of *L. pneumophila*.**

Legionella pneumophila, an environmental pathogen, is able to cause severe pneumonia in humans termed Legionnaires' disease (36, 57). Members of the genus *Legionella* are widespread in natural aquatic habitats and in manmade water systems like cooling towers, air conditioners, and fountains (43). In the environment, the bacteria are able to replicate intracellularly in protozoan organisms such as *Acanthamoeba castellanii*, *Hartmannella vermiformis*, and other protozoan species (4, 15, 42, 52). *Legionella* infection occurs after inhalation of aerosolized bacteria. The bacteria enter the human lungs, where they are capable of invading and proliferating in alveolar macrophages and blood monocytes. Legionellae are taken up by alveolar macrophages via complement receptors and coiling phagocytosis. Once inside the eukaryotic cell the bacteria are able to inhibit phagosome-lysosome fusion as well as phagosome acidification and multiply in a membrane-bound, ribosome-studded phagosome (reviewed in reference 25).

Only a few factors have been determined and characterized that contribute to survival of the bacteria in eukaryotic cells. By transcomplementation analysis of *L. pneumophila* mutants which are impaired in their ability to multiply in macrophages, two genetic loci designated *dot* (defect in organelle trafficking) (5, 6) and *icm* (intracellular multiplication) (7, 35) could be identified as contributing to intracellular survival of the bacteria. In addition, the Mip (macrophage infectivity potentiator) protein, a 24-kDa surface-exposed protein, is described as a factor that contributes to intracellular survival of *L. pneumophila* (14). Invasion experiments using isogenic *L. pneumophila* strains with and without an intact *mip* gene revealed that the *mip* gene product is required for early survival processes of *L. pneumophila* Wadsworth in protozoan cells, in the macrophage-like cell line U937, and in human alveolar macrophages (10, 11). In addition, Mip-negative mutants exhibit attenuated virulence in guinea pigs following intratracheal infection (9).

Recently, we were able to demonstrate that Mip exhibits the enzymatic property of a peptidyl-prolyl *cis*/*trans* isomerase (PPIase), catalyzing the slow *cis*/*trans* interconversion of prolyl peptide bonds in oligopeptides (18, 31). The enzymatic activity of Mip is blocked by the potent immunosuppressive drug FK 506. Proteins exhibiting PPIase activity are expressed in various eukaryotic and prokaryotic organisms and are partly designated as immunophilins because they are able to bind immunosuppressive compounds (reviewed in reference 22). Two protein superfamilies, the cyclophilins and the FK 506-binding proteins (FKBPs), constitute the substance class of the immunophilins. The cyclophilins are capable of binding the cyclic undecapeptide cyclosporin A, while FKBPs form complexes with the immunosuppressive macrolide FK 506. Recently, a third class of PPIases, termed parvulins, has been described

* Corresponding author. Mailing address: Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany. Phone: 49-931-312575. Fax: 49-931-571954. Electronic mail address: b.ludwig@rzbox.uni-wuerzburg.de.

TABLE 1. Recombinant DNAs used in this study

Plasmid	Vector	Characteristics	Source or reference
pEWM 101	pBluescript II KS	*mip* on 1.8-kb *Xba*I-*Sac*I DNA fragment (Ap^r Mip^+)	This study
pEWM 102	pBluescript II KS	*mip* on 1.8-kb *Sac*I DNA fragment (Ap^r Mip^+)	This study
pEWMG 101.1	pKNG 101	Δ*mip*::*km* on 3.3-kb *Xba*I-*Sac*I DNA fragment (Sm^r Km^r Mip^-)	This study
pEWMS 101.2	pMSS 704-1	Δ*mip*::*sacB*::*km* on 5.3-kb *Xba*I-*Sac*I DNA fragment (Cm^r Km^r Mip^-)	This study
pBLL 100	pBluescript II KS	*mip* on 1-kb *Sac*I-*Bam*HI DNA fragment (Ap^r Mip^+)	This study
pBLL 142-L	pBluescript II KS	Site-directed-mutagenized *mip* on 1-kb *Sac*I-*Bam*HI DNA fragment (Ap^r Mip^+ [Asp-142 to Leu-142])	31
pBLL 185-A	pBluescript II KS	Site-directed-mutagenized *mip* on 1-kb *Sac*I-*Bam*HI DNA fragment (Ap^r Mip^+ [Tyr-185 to Ala-185])	This study
pEWM 103	pBC KS+	*mip* on 1.8-kb *Sac*I-*Bam*HI DNA fragment (Cm^r Mip^+)	This study
pEWM 142-L	pBC KS+	Site-directed-mutagenized *mip* on 1.8-kb *Sac*I-*Bam*HI DNA fragment (Cm^r Mip^+ [Asp-142 to Leu-142])	This study
pEWM 185-A	pBC KS+	Site-directed-mutagenized *mip* on 1.8-kb *Sac*I-*Bam*HI DNA fragment (Cm^r Mip^+ [Tyr-185 to Ala-185])	This study
pEWMS 102	pMSS 704-1	*mip* on 1.8-kb *Sac*I DNA fragment (Cm^r Mip^+)	This study
pEWMS 142-L	pMSS 704-1	Site-directed-mutagenized *mip* on 1.8-kb *Sac*I DNA fragment (Cm^r Mip^+ [Asp-142 to Leu-142])	This study
pEWMS 185-A	pMSS 704-1	Site-directed-mutagenized *mip* on 1.8-kb *Sac*I DNA fragment (Cm^r Mip^+ [Tyr-185 to Ala-185])	This study

(39, 40, 44). FKBPs as well as cyclophilins seem to assist the cellular folding and maturation of proteins, as shown for rhodopsin in the *Drosophila* eye, for transferrin and collagen in the mammalian endoplasmic reticulum, and for the translocation of mitochondrial precursor proteins (2, 41, 53). In T cells FKBP-FK 506 as well as cyclophilin-cyclosporin A complexes interact with the protein phosphatase calcineurin, thereby inhibiting the nuclear transport of the transcriptional factor NF-AT, a critical step in T-cell activation (50). However, the formation of an immunophilin-immunosuppressant complex seems not to be a prerequisite for calcineurin binding since it was recently shown that immunophilins like FKBP 12 and cyclophilin A, although to a lower extent, interact with calcineurin in the absence of their exogenous ligands FK 506 and cyclosporin A, respectively (8). Interestingly, cytosolic T-cell cyclophilin represents also the target protein of $Pr55^{gag}$ of human immunodeficiency virus type 1 (HIV-1) in infected T cells (20, 30).

The classical FKBPs, proteins of 12 to 13 kDa, are located intracellularly. In addition, bacteria are able to produce FKBPs of 22 to 26 kDa that are located on the bacterial surface. Mip and Mip-like proteins which are produced by a number of pathogenic bacteria such as *L. pneumophila*, *Chlamydia trachomatis*, and various rickettsiae belong to this particular group of FKBPs (12, 18, 33). Recently, it was demonstrated that nonpathogenic bacteria also have the capacity to produce Mip-like proteins (17, 24). The observation that most bacterial FKBPs including Mip and Mip-like proteins exhibit PPIase activity (reviewed in reference 22) gave rise to the question whether full enzymatic activity is required for intracellular survival of bacteria such as *L. pneumophila*. In this study we demonstrated that site specifically altered Mip proteins with strongly reduced PPIase activity in *L. pneumophila* are still able to support the intracellular survival of the bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. pneumophila* Wadsworth NU 201, representing a spontaneous streptomycin-resistant derivative of the clinical isolate 130b, and the isogenic Mip-negative strain NU 203 were recently described (11). Mip-negative mutants were constructed from *L. pneumophila* Philadelphia I JR 32 (45), a restriction-deficient, streptomycin-resistant derivative of strain Philadelphia I, and from *L. pneumophila* Corby (Rif), which is a spontaneous rifampin-resistant isolate of *L. pneumophila* Corby (26). *Escherichia coli* K-12 strain SM10λ*pir* served as a donor strain in bacterial mating (54). The *E. coli* K-12 strain DH5α (23) was used for transformation and propagation of recombinant plasmids derived from the vectors pBluescript II KS and pBC KS+ (Stratagene, La Jolla, Calif.). For the generation of *L. pneumophila* Mip-negative mutants by homologous recombination, inactivated *mip* genes were ligated into the *pir*-dependent R6K plasmids pKNG 101 (27) and pMSS 704-1. Vector pMSS 704-1 represents a derivative of pGP 704 (34) carrying an additional chloramphenicol resistance marker gene derived from Tn*1725* (55). Recombinant plasmids used in this study are listed in Table 1.

Media and growth conditions. *E. coli* strains were cultivated at 37°C in YT broth or on YT agar plates (47). *Legionella* strains were grown on BCYE agar plates supplemented with 0.0025% ferric PP_i and 0.04% L-cysteine (Oxoid, Wesel, Germany) at 37°C in 5% humidified CO_2 for 24 to 48 h. Antibiotics were used at the following concentrations when necessary: ampicillin, 100 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; rifampin, 50 to 100 μg ml^{-1}; kanamycin, 12.5 to 25 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}.

DNA manipulations and sequence determination. Standard methods were used for restriction endonuclease digestion of DNA and other DNA manipulations (47). Transformation of *Legionella* cells was performed by electroporation (35) and by mating procedures (58). DNA sequence determination was carried out as described by the manufacturer with the T7 polymerase sequencing kit from Pharmacia, Freiburg, Germany.

Construction of Mip-negative *L. pneumophila* strains. In order to construct a Mip^- mutant of *L. pneumophila* Corby, the *mip* gene located on a 1.8-kb DNA fragment was amplified by PCR and cloned between the *Xba*I-*Sac*I site of the vector pBluescript II KS, resulting in recombinant plasmid pEWM 101 (Table 1). The insertion of the kanamycin resistance gene derived from Tn*5* (4a) between the *Eco*RI-*Hin*dIII restriction site of the *mip* gene resulted in inactivation of the *mip* gene. Cloning the 3.3-kb *mip*::*km* DNA fragment into the *pir*-dependent suicide vector pKNG 101 resulted in recombinant plasmid pEWMG 101.1 (Table 1). pEWMG 101.1 was transferred into *L. pneumophila* Corby (Rif) by mating procedures to generate the isogenic Mip-negative mutant *L. pneumophila* Corby-1 by homologous recombination using *E. coli* SM10λ*pir* as the donor strain. To construct a Mip^- mutant of *L. pneumophila* JR32, the *sacB* gene of *Bacillus subtilis* was amplified by PCR from the vector pKNG 101 (27). Cloning of the 5.3-kb *mip*::*sac*B::*km* DNA fragment into the suicide vector pMSS 704-1 resulted in pEWMS 101.2 (Table 1). The recombinant plasmid pEWMS 101.2 was transferred into the *L. pneumophila* wild-type strain JR 32 as described above to generate the Mip-negative mutant *L. pneumophila* Philadelphia I JR32-2 (Table 2). The Mip^- mutant NU 203, derived from the wild-type *L. pneumophila* strain Wadsworth NU 201, was kindly provided by N. P. Cianciotto, Northwestern University, Chicago, Ill. The Mip-negative *L. pneumophila* strains Philadelphia I JR 32-2 and Corby-1 grew on BCYE agar plates as well as did their wild-type parent strain, indicating that the *mip* mutations do not influence the extracellular growth behavior of the *L. pneumophila* strains.

PCR. PCRs were carried out according to the method of Saiki et al. (46) using a Thermocycler 60 apparatus from Biomed, Theres, Germany. The 1.8-kb DNA fragment of pEWM 101 (Table 1) containing the wild-type *mip* gene was amplified from the chromosome of *L. pneumophila* Philadelphia I. Primer sequences were as follows: 5′GAATTCCTCTAGAAATTTCTGCTG3′ and 5′CTAAACT TAGAGCTCCCGTCGC3′. To facilitate cloning, an *Xba*I restriction site was

TABLE 2. *E. coli* K-12 and *L. pneumophila* strains used in this study

Strain	Mip protein[a]	PPIase activity[b]
E. coli K-12		
DH5α(pBLL 100)	Mip^+ (wild type)	1.01×10^6 (100%)
DH5α(pBLL 185-A)	Mip^+ (Tyr-185→Ala-185)	6.1×10^3 (0.6%)
DH5α(pBLL 142-L)	Mip^+ (Asp-142→Leu-142)	5.4×10^4 (5.3%)[c]
DH5α(pBluescript II KS)	Mip^-	0
L. pneumophila		
Corby	Mip^+ (wild type)	155
Corby-1	Mip^-	0
Corby-1(pEWM 103)	Mip^+ (wild type)	170
Corby-1(pEWM 185-A)	Mip^+ (Tyr-185→Ala-185)	0
Corby-1(pEWM 142-L)	Mip^+ (Asp-142→Leu-142)	0
Philadelphia I JR 32	Mip^+ (wild type)	226
Philadelphia I JR 32-2	Mip^-	0
Philadelphia I JR 32-2.1	Mip^+ (wild type)	315
Philadelphia I JR 32-2.2	Mip^+ (Tyr-185→Ala-185)	0
Philadelphia I JR 32-2.3	Mip^+ (Asp-142→Leu-142)	0
Wadsworth NU 201	Mip^+ (wild type)	286
Wadsworth NU 203	Mip^-	0
Wadsworth NU 203.1	Mip^+ (wild type)	117
Wadsworth NU 203.2	Mip^+ (Tyr-185→Ala-185)	0
Wadsworth NU 203.3	Mip^+ (Asp-142→Leu-142)	0

[a] As determined by Western blot analysis using polyclonal anti-Mip-specific antiserum.

[b] PPIase activity was measured using Suc-Ala-Pro-Phe-4-nitroanilide as a substrate. For the Mip proteins expressed in recombinant *E. coli* strains the specific constants k_{cat}/K_M ($mM^{-1}\ s^{-1}$) of the purified proteins are indicated. The percentage of the observed PPIase activity is given in parentheses. The activity of the recombinant wild-type Mip protein was set at 100%. For the Mip proteins expressed in the *Legionella* strains, arbitrary units per milligram of protein, as recently described (40), are indicated. Arbitrary units are within the margin of error of the experiment. 0, the arbitrary units per milligram of protein is below the limit of detection (see also Materials and Methods).

[c] Previously determined (31).

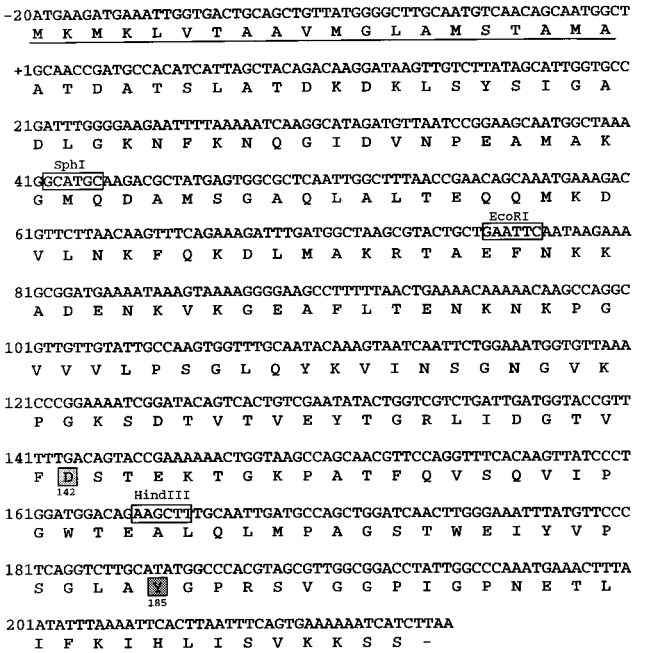

FIG. 1. Nucleotide and deduced amino acid sequences of the *mip* gene of *L. pneumophila* Philadelphia I. The N-terminal signal sequence of the Mip protein is underlined. Site specifically mutagenized amino acids are indicated by stippled boxes. Relevant restriction sites of the DNA sequence of the *mip* gene are boxed.

introduced into the 5′ end and a *Sac*I restriction site was introduced into the 3′ end of the amplified sequence. The 1.8-kb DNA fragment of recombinant plasmid pEWM 102 (Table 1) was amplified by using the primers 5′CCTTTATG AGCTCCTGCTGT3′ and 5′CTAAACTTAGAGCTCCCGTCGC3′, introducing a *Sac*I restriction site into the 5′ and 3′ ends of the amplified sequence. To amplify the 1-kb DNA fragment of pBLL 100 (Table 1), primer 5′GTATGA GCTCTTAAGTGTAAGACTA3′ containing a *Sac*I site and primer 5′TAAGG ATCCCGTCGCAAGCACTGA3′ containing a *Bam*HI site were used. The *sacB* gene of *B. subtilis* was amplified from the vector pKNG 101 by using the primers 5′TACCTGCGGATCCCTATTATTTAG3′ and 5′CATATGGGATCCACCTT TATGTTG3′. DNA amplification conditions were as follows: after an initial denaturation step of 2 min at 94°C, 30 cycles, each at 94°C for 45 s, 55°C for 1 min, and 72°C for 90 s, were completed, with a final elongation step at 72°C for 3 min.

Construction of site-directed-mutagenized *mip* genes. The DNA sequence of the *mip* gene of *L. pneumophila* Philadelphia I and its corresponding amino acid sequence as recently determined (31) are given in Fig. 1. Site-specific mutagenesis of the *mip* gene was performed by PCR as previously described (31) using mutation-directing primer (Tyr-185 to Ala-185, 5′TTGCAGCCGGCCCACGT AGCGT3′). Recombinant plasmid pBLL 100 (Table 1) was used as template DNA. The obtained 1-kb amplification product lacking the promoter region of the *mip* gene was cloned between the *Sac*I-*Bam*HI site of pBluescript II KS adjacent to the T7 promoter resulting in plasmid pBLL 185-A (Table 1). Plasmid pBLL142-L carrying the Asp-142 to Leu-142 mutation (Fig. 1) was constructed as described previously (31). All site specifically mutagenized *mip* genes were sequenced to confirm that only the site-directed mutations were present. The 1-kb DNA fragments of pBLL 100, pBLL 142-L, and pBLL 185-A were further subcloned into the *Sac*I-*Bam*HI site of pBC KS+ following elongation with a 1-kb *Sac*I-*Sph*I DNA fragment of pEWM 102 carrying the promoter region of the *mip* gene (Table 1). This cloning procedure resulted in plasmids pEWM 103, pEWM 142-L, and pEWM 185-A (Table 1).

Complementation of *L. pneumophila* Mip^- strains with genes encoding the wild-type and mutagenized Mip proteins. Plasmids pEWM 103, pEWM 185-A, and pEWM 142-L were used to complement *L. pneumophila* Corby-1 by electroporation, resulting in *L. pneumophila* Corby-1(pEWM 103), *L. pneumophila* Corby-1(pEWM 142-L), and *L. pneumophila* Corby-1(pEWM 185-A), respectively (Table 2). For complementation of the Mip-negative *L. pneumophila* strains JR 32-2 and NU 203, the wild-type and the site-directed-mutagenized *mip* genes were cloned into pMSS 704-1, resulting in plasmids pEWMS 102, pEWMS 142-L, and pEWMS 185-A (Table 1). These plasmids, containing the *pir*-dependent origin of replication R6K, were transferred from *E. coli* K-12 strain SM10λ*pir* into *L. pneumophila* JR 32-2 and *L. pneumophila* NU 203 by bacterial mating and integrated into the chromosomes of the recipients. These experiments resulted in *L. pneumophila* JR 32-2.1 and NU 203-1, expressing the wild-type Mip protein; JR 32-2.2 and NU 203-2, expressing the Tyr-185 to Ala-185 mutated Mip protein; and JR 32-2.3 and NU 203-3, expressing the Asp-142 to Leu-142 mutated Mip protein (see Fig. 2). The transcomplementants are listed in Table 2.

Isolation of Mip proteins and determination of PPIase activity. The wild-type and site specifically altered recombinant Mip proteins were purified to homogeneity as previously described (18, 31). PPIase activities of the purified Mip proteins were determined by a protease-coupled assay as described previously (18), using the synthetic peptide Suc-Ala-Phe-Pro-Phe-4-nitroanilide as a substrate. Mip-specific PPIase activity of the *L. pneumophila* strains listed in Table 2 was analyzed in Mip-specific fractions after gel filtration of crude bacterial cell extracts. *Legionella* cells were disrupted by sonication using a Sonoplus HD 70 sonifier from Bandelin, Berlin, Germany, and centrifuged for 30 min at 11,000 × *g*. One milliliter of the resulting supernatant containing about 10 mg of total protein was loaded onto a Superdex 75 (Hiload 16/60) gel filtration column (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.8, containing 150 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM dithiothreitol. Mip-containing fractions were pooled at retention times according to the size of Mip dimers (49), and their PPIase activities were determined. Because of the low PPIase activity of the site specifically mutagenized Mip proteins (specific activities of 0.6 and 5.3% of that of the wild-type Mip protein), the expected acceleration of the uncatalyzed *cis*/*trans* isomerisation of the substrate calculated from the values measured for wild-type Mip proteins after gel filtration is below the limit of detection in the standard assay; nevertheless the proteins could be detected in Western immunoblot analysis in a concentration comparable to those observed in the *Legionella* wild-type strains.

Preparation of Mip-specific antiserum. Polyclonal Mip-specific antiserum was prepared following intravenous injection of purified, native Mip protein of *L. pneumophila* Philadelphia I into New Zealand white rabbits. The antiserum was diluted 1:500 when used in Western blot analysis.

SDS-PAGE and Western immunoblots. Protein extracts were prepared from recombinant *E. coli* clones and *Legionella* strains as previously described (32) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) (29). Western blotting was performed by semidry electroblotting in a graphite chamber according to the method of Kyhse-Anderson (28).

Infection of U937 cells and *A. castellanii* with *L. pneumophila*. Invasion exper-

iments using the macrophage-like cell line U937 and *A. castellanii* cells were carried out as described previously (52, 58). Briefly, 5×10^5 differentiated macrophage-like U937 cells and 10^5 *A. castellanii* cells were infected with 10^6 legionellae, resulting in a multiplicity of infection of 2 or 10, respectively. After an incubation of 2 h at 37°C, remaining extracellular bacteria were killed by gentamicin (80 μg ml^{-1}) treatment. After 1 h of incubation, the medium was replaced with antibiotic-free medium. This was defined as the zero hour value. Following another 3, 24, and 48 h of incubation, cells were resuspended vigorously in culture supernatants and the number of CFU was determined by plating aliquots on BCYE agar plates.

Isolation and infection of human blood monocytes with *L. pneumophila*. Blood mononuclear cells were isolated from blood of normal adult donors not known to ever have contracted Legionnaires' disease. The blood mononuclear cell fraction was obtained by centrifugation over a double polysucrose sodium diatrizoate gradient (Histopaque 1119 and 1077) according to the manufacturer's instructions (Sigma, Deisenhofen, Germany). Human monocytes were grown at 37°C in RPMI 1640 medium (Gibco/BRL, Eggenstein, Germany) supplemented with 20% inactivated fetal calf serum, 1 mM sodium PP_i, 2 mM L-glutamine, 1× minimal essential medium nonessential amino acids, and 100 U/100 μg of penicillin/streptomycin ml^{-1}. Mononuclear cells were allowed to adhere and to mature for 4 days before they were infected with *L. pneumophila*. Infection experiments were carried out as described above.

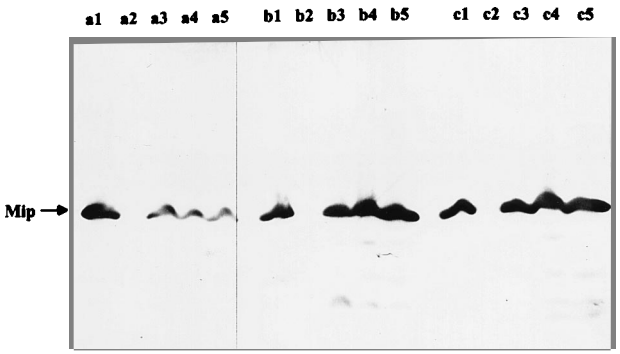

FIG. 2. Western blot analysis of cell extracts of different *L. pneumophila* strains using a polyclonal anti-Mip-specific antiserum. Lanes: a1, Corby (Rif); a2, Corby-1; a3, Corby(pEWM 103); a4, Corby(pEWM 185-A); a5, Corby(pEWM 142-L); b1, JR 32; b2, JR 32-2; b3, JR 32-2.1; b4, JR 32-2.2, b5, JR 32-2.3; c1, NU 201; c2, NU 203; c3, NU 203.1; c4, NU 203.2; c5, NU 203.3.

RESULTS

Characterization of *L. pneumophila* strains with site specifically mutagenized Mip proteins. In order to identify amino acid residues involved in PPIase activity of Mip, site-directed mutagenesis of particular amino acid residues located in the C terminus of the recombinant wild-type Mip protein was performed (31, 48). As indicated in Fig. 1, an aspartyl residue at position 142 of the amino acid sequence of the native Mip protein and a tyrosyl residue at position 185 were converted to a leucyl and an alanyl residue, respectively. The amino acid residues chosen for mutagenesis were highly conserved in the known amino acid sequences of all FKBPs and are thought to be involved in the interaction of human FKBP and FK 506 (reviewed in references 21 and 22). The mutagenized Mip proteins of the recombinant *E. coli* K-12 clones were purified to homogeneity and analyzed for enzymatic activity. As summarized in Table 2 both mutagenized Mip proteins displayed dramatically lower PPIase catalytic efficiency compared with the recombinant wild-type Mip protein. The conversion of Asp-142 to Leu-142 reduced PPIase activity to 5.3% of the wild-type Mip activity (31). The Tyr-185 to Ala-185 substitution had an even more pronounced effect; the activity was decreased to 0.6% of the wild-type enzymatic activity.

Plasmids coding for the wild-type and the site-directed-mutagenized Mip proteins were used to complement the Mip-negative *L. pneumophila* strains Philadelphia I JR 32-2, Corby-1, and Wadsworth NU 203. The complemented *L. pneumophila* strains are given in Table 2. Western blot analysis of the corresponding *L. pneumophila* strains using a polyclonal anti-Mip-specific antiserum revealed that Mip expression was restored in all *Legionella* Mip mutants upon introduction of the wild-type and the mutagenized *mip* genes (Fig. 2). In addition, significant Mip-specific isomerase activity could be detected only in cell extracts from *L. pneumophila* Mip-negative mutants complemented with the wild-type *mip* gene (Table 2).

Intracellular survival of *L. pneumophila* strains in U937 cells. The wild-type *L. pneumophila* strains Philadelphia I JR 32, Corby (Rif), and Wadsworth NU 203, the isogenic Mip-negative derivatives, and the complemented strains expressing the site specifically mutagenized Mip proteins were tested for their ability to initiate infection and to multiply in the phorbol myristate acetate-treated macrophage-like cell line U937. As shown in Fig. 3, all *L. pneumophila* wild-type strains exhibited very similar growth kinetics during infection of differentiated U937 cells. For intracellular multiplication of *L. pneumophila* Philadelphia I JR 32 and Corby (Rif), 5×10^3 bacteria could be detected intracellularly at time point zero and at 3 h postinfection, increasing to 5×10^5 to 10^6 bacteria at 48 h postinfection (Fig. 3A and B). The observed CFU values represent a doubling time of 6 h. Monolayers infected with strain Wadsworth NU 203 yielded approximately 10-fold more intracellular bacteria at 0, 3, and 24 h post infection (Fig. 3C). Only minor differences in the recovery of intracellular bacteria were observed from infected monolayers of U937 cells with wild-type strains and Mip-negative mutants. However, the Mip^- strain JR 32-2 compared with the Mip^+ strain JR 32 exhibited a 10-fold decrease in recovery from the macrophage-like cells 24 and 48 h postinfection (Fig. 3A). In addition, no significant differences were observed for intracellular growth of the wild-type and the *Legionella* strains complemented with genes coding for site specifically mutagenized Mip proteins exhibiting reduced PPIase activity.

Intracellular survival of *Legionella* strains in human blood monocytes. The intracellular survival of the *L. pneumophila* strains listed in Table 2 was also investigated with human blood monocytes. The results of the invasion assays are shown in Fig. 4. *L. pneumophila* wild-type strains were approximately 10-fold less infective for human monocytes 48 h post infection compared with U937 cells. A significant difference (1 to 2 log units) in intracellular growth of Mip^+ and Mip^- strains of *L. pneumophila* Philadelphia I JR 32 and Wadsworth could be detected in human blood monocytes 24 and 48 h postinfection (Fig. 4A and C), while *Legionella* strain Corby and its corresponding Mip mutant exhibited only minor differences (Fig. 4B). The infectivity of Mip-negative mutants for human mononuclear phagocytes could be increased following complementation with genes coding for the wild-type and the site specifically mutagenized Mip proteins. These data suggest that full enzymatic activity of Mip is not a prerequisite for intracellular survival of *L. pneumophila* in human mononuclear phagocytes, at least under the conditions of the assay systems used in these studies.

Intracellular survival of *L. pneumophila* strains in *A. castellanii*. To determine if the PPIase activity of Mip contributes to intracellular survival of legionellae in protozoa, the same infection experiments as described for U937 cells and monocytes were repeated using *A. castellanii* as host cells. The results are shown in Fig. 5. The growth kinetics of *L. pneumophila* wild-type strains within *Acanthamoeba* cells were very similar to those observed within U937 macrophage-like cells and human monocytes. In contrast, 10-fold less bacteria were recovered from *A. castellanii* infected with Mip-negative strains at time

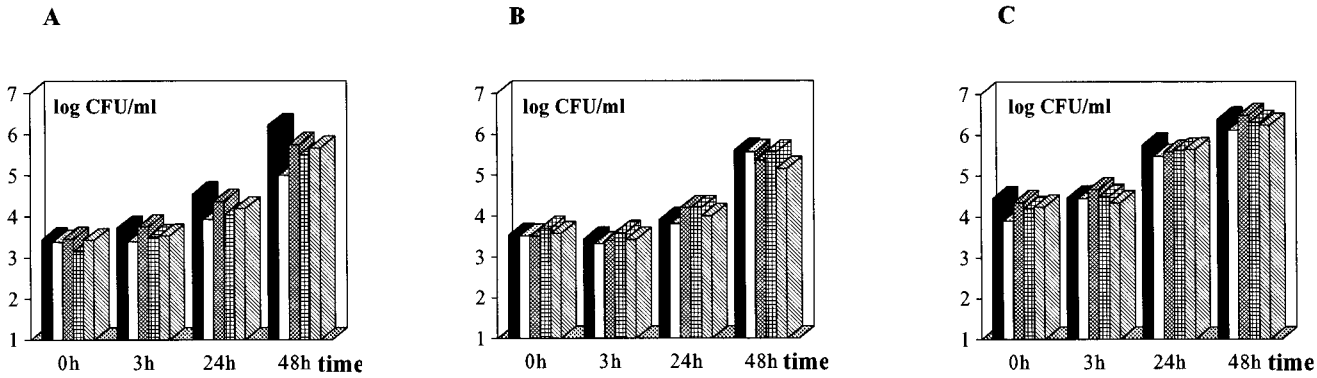

FIG. 3. Intracellular survival of *L. pneumophila* strains in U937-derived macrophage-like cells. Monolayers were infected with *L. pneumophila* strains (Table 2), resulting in a multiplicity of infection of 5. After various incubation periods, the formation of CFU of intracellular bacteria was determined in duplicate for three independent experiments. The standard deviation was below 0.5 log unit. Shown are recombinant strains of *L. pneumophila* Philadelphia I JR 32 (A), Corby (B), and Wadsworth (C). ■, wild-type strain; □, Mip-negative mutant; ▩, Mip-negative mutant complemented with the wild-type *mip* gene; ▦, Mip-negative mutant complemented with the Tyr-185→Ala-185 mutated *mip* gene; ▨, Mip-negative mutant complemented with the Asp-142→Leu-142 mutagenized *mip* gene.

points 0 and 3 h postinfection compared with those recovered from *A. castellanii* infected with an equivalent number of Mip-positive strains. The number of intracellular Mip^- *Legionella* cells increased at a slower rate than the wild-type, resulting in a 2-log-unit difference in recovery between Mip^+ and Mip^- strains 24 and 48 h postinfection. The *Legionella* wild-type phenotype for intracellular survival could be restored in Mip-negative mutants complemented with the wild-type *mip* gene. Similar results were obtained after infection of *A. castellanii* with *Legionella* strains expressing Mip proteins that exhibit reduced PPIase activity. These results indicated that the PPIase activity of the Mip protein does not contribute to intracellular survival of legionellae in *A. castellanii.*

DISCUSSION

Classical FKBPs, cytoplasmic proteins of 12 to 13 kDa, are widely distributed among prokaryotic and eukaryotic organisms (reviewed in references 16 and 21). In addition, many bacteria are able to produce FKBPs of 22 to 26 kDa that are located at the cell surface (reviewed in reference 22). These proteins, termed "Mip" (macrophage infectivity potentiators) or "Mip-like" proteins, were first described for pathogenic bacteria, especially for species which are able to survive intracellularly in eukaryotic cells such as *L. pneumophila* (10), *Legionella micdadei* (3), *C. trachomatis* (33), and members of the rickettsia group (12). Recently, however, Mip-like proteins and *mip*-like genes were also detected in nonpathogenic bacteria, including *E. coli* K-12 (17, 24), supporting the general occurrence of these proteins among prokaryotes. Nevertheless, there is evidence that null mutations in *mip* genes of *L. pneumophila* strongly reduce intracellular survival of the bacteria in eukaryotic cells (10, 11) and also lead to impaired in vivo virulence following intratracheal injection of strains into guinea pigs (9).

In our study we confirmed and extended these observations. Mutations were introduced into *mip* genes of three different *L. pneumophila* strains, Philadelphia I, Corby, and Wadsworth, thereby reducing the intracellular survival of recombinant Mip-negative *L. pneumophila* strains following infection of U937 cells (Fig. 3), human monocytes (Fig. 4), and *A. castellanii* (Fig. 5). Differences, however, exist in the level of reduction of intracellular survival due to the *mip* mutations and depend on the eukaryotic cell type used for the assay. While only minor differences in intracellular survival exist between Mip-positive and Mip-negative strains following uptake by U937 cells, the numbers of intracellular Mip-negative bacteria in monocytes and in *A. castellanii* are reduced by a factor of 50

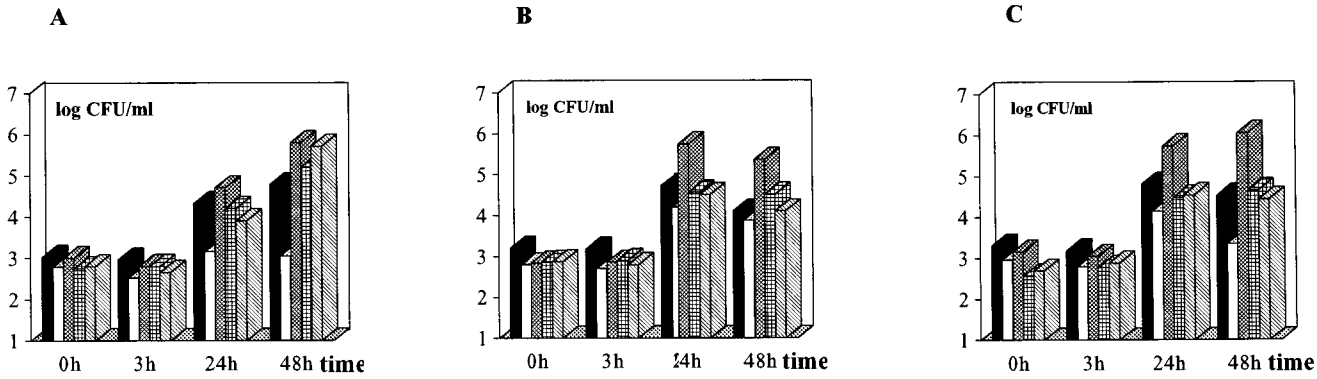

FIG. 4. Intracellular survival of *L. pneumophila* strains in human blood monocytes. Monocytes were infected with *L. pneumophila* strains (Table 2), resulting in a multiplicity of infection of 10. After various incubation periods, the formation of CFU of intracellular bacteria was determined in duplicate for three independent experiments. The standard deviation was below 0.5 log unit. Shown are recombinant strains of *L. pneumophila* Philadelphia I JR 32 (A), Corby (B), and Wadsworth (C). ■, wild-type strain; □, Mip-negative mutant; ▩, Mip-negative mutant complemented with the wild-type *mip* gene; ▦, Mip-negative mutant complemented with the Tyr-185→Ala-185 mutated *mip* gene; ▨, Mip-negative mutant complemented with the Asp-142→Leu-142 mutagenized *mip* gene.

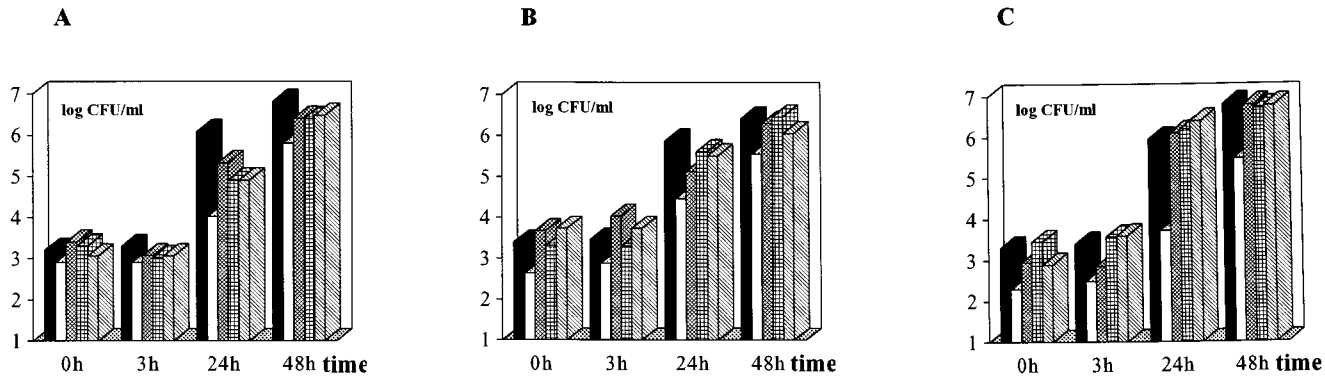

FIG. 5. Intracellular survival of *L. pneumophila* strains in *A. castellanii*. *A. castellanii* cells were infected with *L. pneumophila* strains (Table 2), resulting in a multiplicity of infection of 10. After various incubation periods, the formation of CFU of intracellular bacteria was determined in duplicate for three independent experiments. The standard deviation was below 0.5 log unit. Shown are recombinant strains of *L. pneumophila* Philadelphia I JR 32 (A), Corby (B), and Wadsworth (C). ■, wild-type strain; □, Mip-negative mutant; ▩, Mip-negative mutant complemented with the wild-type *mip* gene; ▦, Mip-negative mutant complemented with the Tyr-185→Ala-185 mutated *mip* gene; ▨, Mip-negative mutant complemented with the Asp-142→Leu-142 mutagenized *mip* gene.

to 100 compared with Mip-positive controls. These host cell type-dependent differences may be caused by the mechanisms of uptake and different kinetics in the establishment of infection. However, our results are not completely comparable to those of Cianciotto and coworkers (10), who observed a more pronounced effect of Mip on intracellular survival of *L. pneumophila* in U937 cells in a different assay system. Nevertheless, our data, as well as those of other groups, clearly demonstrated a contribution of Mip to intracellular survival of *L. pneumophila* in eukaryotic cells. In this study, it is demonstrated for the first time that Mip also contributes to the survival of *L. pneumophila* in human blood monocytes, a cell type frequently used to study the interaction between intracellular parasites and host cells.

FKBPs, as well as cyclophilins, belong to the substance class of immunophilins, possessing the enzymatic activity of PPIases (reviewed in reference 16). Recently, it was shown that Mip also exhibits PPIase activity (18, 31). One aim of our study was to determine whether PPIase activity is necessary for the contribution of Mip to intracellular survival of legionellae. In order to address this question, site-specific mutations were introduced into the *mip* gene. As demonstrated recently (reviewed in references 21 and 22) the amino acid sequence of the C-terminal part of Mip, comprising amino acid residues 107 to 213 of the mature protein (Fig. 1), is similar to the sequences of various FKBPs of other organisms, including those produced by human cells. Crystallographic and nuclear magnetic resonance studies have shown that human FKBP12 contains a central domain of five β-sheets that is interrupted by a small α-helix (37, 38, 56). Ten amino acid residues are thought to be involved in the interaction of the human FKBP and FK506. These structural elements and 8 of the 10 amino acids involved in the interaction of FKBPs and FK506 are conserved among the Mip proteins (22). Consequently, we changed site specifically distinct amino acid residues of the C-terminal part of Mip that are conserved among various FKBPs. These amino acid residues include Ser-115, Glu-130, Tyr-131, Phe-141, Asp-142, Thr-144, Trp-162, Trp-175, Tyr-185, and Phe-202 (31, 48). While some of the amino acid exchanges (e.g., Ser-115 and Glu-130; see references 31 and 48) had no influence on the PPIase activity of Mip, an exchange of Asp at position 142 into Leu and of Tyr at position 185 into Ala reduced PPIase activity in recombinant Mip-positive *E. coli* strains to 5.3 and 0.6%, respectively, compared with that of the wild-type Mip, indicating a strong influence of these amino acids on PPIase activity (Fig. 1; Table 2). Therefore, these mutations, Asp-142 to Leu-142 and Tyr-185 to Ala-185 were introduced into the genomes of the three *L. pneumophila* strains, Philadelphia I, Corby, and Wadsworth. The resulting recombinant *L. pneumophila* clones were tested for intracellular survival in three assay systems: U937 cells, human blood monocytes, and *A. castellanii*. As demonstrated in Fig. 3 to 5, the site-directed mutations in the Mip protein did not influence the ability of the *Legionella* strains to survive in eukaryotic cells; Mip-positive clones with strongly reduced PPIase activity exhibited an identical or similar capability for intracellular survival compared with the PPIase-positive wild-type strains and complemented derivatives. The fact that we used three different *L. pneumophila* isolates in three different assay systems demonstrates the general importance of our data.

How may these findings be explained? First, the mutations introduced into the amino acid sequence of Mip strongly reduced the Mip-specific enzymatic activity of the *Legionella* strains but did not completely abolish PPIase activity. We cannot exclude the possibility that the remaining activity of the site specifically altered Mip proteins (5.3% in case of the Asp-142 to Leu-142 exchange and 0.6% in case of the Tyr-185 to Ala-185 mutation) is still sufficient to induce functional effects in eukaryotic cells. Second, PPIase activity of the wild-type and the site specifically mutagenized Mip proteins was measured using a synthetic peptide as a substrate. It cannot be ruled out that the observed enzymatic activities measured with oligopeptides do not necessarily reflect the efficiency the enzymes exhibit towards natural substrates. Third, *L. pneumophila* is able to produce at least one more enzyme exhibiting PPIase activity, a cyclophilin of 18 kDa (22, 48). Despite the fact that the *Legionella*-specific cyclophilin (Lcy) exhibits a subsite specificity quite different from that of Mip, the loss of Mip-specific PPIase activity in the recombinant *Legionella* strains might be substituted for by the cyclophilin. Fourth, data for human FKBPs have shown that FKBP12 is able to bind to calcineurin in the presence of, and with low affinity in the absence of, FK506 (8, 51). Calcineurin is a protein exhibiting phosphatase activity and contributes to the nuclear transport of one subunit of the transcription factor NF-AT that is involved in T-cell activation (19). We do not have any information about the ligand of Mip in eukaryotic cells. However, it has been shown that certain mutations in FKBP12 strongly influence the PPI ase activity but do not necessarily reduce the capacity of FKBP12 to bind calcineurin and vice versa (1). Therefore, it

seems conceivable that the PPIase activity of Mip may not be associated with the Mip-dependent survival of legionellae in eukaryotes. Fifth, Mip is a cationic protein with a pI of 9.8. Cationic polypeptides are known to induce phagocytosis and may also contribute to the inhibition of the phagosome-lysosome fusion (13), which could be a function of Mip without any involvement of PPIase activity.

The construction of further variants of the Mip protein as well as the analysis of cyclophilin-negative mutants will be one approach to address some of the questions with respect to the contribution of Mip proteins to intracellular survival of *L. pneumophila* in eukaryotic host cells.

ACKNOWLEDGMENTS

We thank M. Horwitz (Los Angeles, Calif.) for discussion, B. Brand and L. Phillips (Würzburg, Germany) for critical reading of the manuscript, and N. P. Cianciotto (Chicago, Ill.) for sending *L. pneumophila* Wadsworth NU 201 and NU 203.

The work was supported by a grant from the BMFT (01 Ki 9210), by personal grants to Eva Wintermeyer (DFG Graduiertenkolleg "Infektiologie") and Michael Steinert (Max Buchner Forschungsstiftung MBFST 1639), and by the Boehringer Ingelheim Stiftung.

REFERENCES

1. **Aldape, R. A., O. Futer, M. T. De Cenzo, B. P. Jarrett, M. A. Murcko, and D. J. Livingston.** 1992. Charged surface residues of FKBP12 participate in formation of the FKBP12-FK506-calcineurin complex. J. Biol. Chem. **267:** 16029–16032.
2. **Baker, E. K., N. J. Colley, and C. S. Zuker.** 1994. The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex *in vivo* with its protein target rhodopsin. EMBO J. **13:**4886–4895.
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. **Barker, J., and M. R. W. Brown.** 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology **140:**1253–1259.

4a. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localisation of the neomycin phosphotransferase gene from transposon Tn5. Gene **19:**327–336.

5. **Berger, K. H., and R. R. Isberg.** 1993. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. Mol. Microbiol. **7:**7–19.
6. **Berger, K. H., J. J. Merriam, and R. R. Isberg.** 1994. Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila dotA* gene. Mol. Microbiol. **14:**809–822.
7. **Brand, B. C., A. B. Sadosky, and H. A. Shuman.** 1994. The *Legionella pneumophila icm* locus: a set of genes required for intracellular multiplication in human macrophages. Mol. Microbiol. **14:**797–808.
8. **Cardenas, M. E., C. Hemenway, R. S. Muir, R. Ye, D. Fiorentino, and J. Heitman.** 1994. Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. EMBO J. **13:**5944–5957.
9. **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.
10. **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.
11. **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.
12. **Cianciotto, N. P., W. O'Connell, G. H. Dasch, and L. P. Mallavia.** 1995. Detection of *mip*-like sequences and Mip-related proteins within the family *Rickettsiaceae*. Curr. Microbiol. **30:**149–153.
13. **Dowling, J. N., A. K. Saha, and R. H. Glew.** 1992. Virulence factors of the family *Legionellaceae*. Microbiol. Rev. **56:**32–60.
14. **Engleberg, N. C., E. Pearlman, and B. I. Eisenstein.** 1989. DNA sequence of *mip*, a *Legionella pneumophila* gene associated with macrophage infectivity. Infect. Immun. **57:**1263–1270.
15. **Fields, B. S.** 1993. *Legionella* and protozoa: interaction of a pathogen and its natural host, p. 70–72. *In* J. M. Barbaree, R. F. Breiman, and A. P. Dufour (ed.), *Legionella*: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
16. **Fischer, G.** 1994. Über peptidyl-prolyl-*cis*/*trans*-Isomerasen und ihre Effektoren. Angew. Chem. **106:**1479–1501.
17. **Fischer, G.** Unpublished data.
18. **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.
19. **Flanagan, W. M., B. Corthésy, R. J. Bram, and G. R. Crabtree.** 1991. Nuclear association of a T-cell transcription factor blocked by FK506 and cyclosporin A. Nature (London) **352:**803–807.
20. **Franke, E. K., H. E. H. Yuan, and J. Luban.** 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature (London) **372:**359–362.
21. **Galat, A.** 1993. Peptidylproline *cis-trans*-isomerases: immunophilins. Eur. J. Biochem. **216:**689–707.
22. **Hacker, J., and G. Fischer.** 1993. Immunophilins: structure-function relationship and possible role in pathogenicity. Mol. Microbiol. **11:**445–456.
23. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
24. **Horne, S. M., and K. D. Young.** 1995. *Escherichia coli* and other species of the Enterobacteriaceae encode a protein similar to the family of Mip-like FK506-binding proteins. Arch. Microbiol. **163:**357–365.
25. **Horwitz, M. A.** 1988. Phagocytosis and intracellular biology of *Legionella pneumophila*. NATO ASI Ser. **H24:**231–237.
26. **Jepras, R. I., R. B. Fitzgeorge, and A. Baskerville.** 1985. A comparison of virulence of two strains of *Legionella pneumophila* based on experimental aerosol infection of guinea pigs. J. Hyg. **95:**29–38.
27. **Kaniga, K., I. Delor, and G. R. Cornelis.** 1991. A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *bla*A gene of *Yersinia enterocolitica*. Gene **109:**137–141.
28. **Kyhse-Andersen, J.** 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods **10:**203–209.
29. **Laemmli, U. K.** 1979. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
30. **Luban, J., K. Leigh Bossolt, E. K. Franke, G. V. Kalpana, and S. P. Goff.** 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell **73:**1067–1078.
31. **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.
32. **Ludwig, B., A. Schmid, R. Marre, and J. Hacker.** 1991. Cloning, genetic analysis, and nucleotide sequence of a determinant coding for a 19-kilodalton peptidoglycan-associated protein (Ppl) of *Legionella pneumophila*. Infect. Immun. **59:**2515–2521.
33. **Lundemose, A. G., J. E. Kay, and J. H. Pearce.** 1993. *Chlamydia trachomatis* Mip-like protein has peptidyl-prolyl *cis*/*trans* isomerase activity that is inhibited by FK 506 and rapamycin and is implicated in initiation of chlamydial infection. Mol. Microbiol. **7:**777–783.
34. **Mahan, M. J., J. M. Stauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.
35. **Marra, A., S. J. Blander, M. A. Horwitz, and H. A. Shuman.** 1992. Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. Proc. Natl. Acad. Sci. USA **89:**9607–9611.
36. **McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, W. R. Dowdle, and the Laboratory Investigation Team.** 1977. Legionnaires' disease. Isolation of the bacterium and demonstration of its role in other respiratory disease. N. Engl. J. Med. **297:**1197–1203.
37. **Michnick, S. W., M. K. Rosen, T. J. Wandless, M. Karplus, and S. L. Schreiber.** 1991. Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. Science **252:**836–839.
38. **Moore, J. M., D. A. Peattie, M. J. Fitzgibbon, and J. A. Thomson.** 1991. Solution structure of the major binding protein for the immunosuppressant FK506. Nature (London) **351:**248–250.
39. **Rahfeld, J. U., K. P. Rücknagel, B. Schelbert, B. Ludwig, J. Hacker, K. Mann, and G. Fischer.** 1994. Confirmation of the existence of a third family among peptidyl-prolyl *cis*/*trans* isomerases. Amino acid sequence and recombinant production of parvulin. FEBS Lett. **352:**180–184.
40. **Rahfeld, J. U., A. Schierhorn, K. Mann, and G. Fischer.** 1994. A novel peptidyl-prolyl *cis*/*trans* isomerase from *Escherichia coli*. FEBS Lett. **343:**65–69.
41. **Rassow, J., K. Mohrs, S. Koidl, I. B. Barthelmess, N. Pfanner, and M. Tropschug.** 1995. Cyclophilin 20 is involved in mitochondrial protein folding in cooperation with molecular chaperones Hsp70 and Hsp60. Mol. Cell. Biol. **15:**2654–2662.
42. **Rowbotham, T. J.** 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amobae. J. Clin. Pathol. **33:**1179–1183.
43. **Rowbotham, T. J.** 1986. Current views on the relationship between amoebae, Legionellae and man. Isr. J. Med. Sci. **22:**678–689.
44. **Rudd, K. E., H. J. Sofia, and E. V. Koonin.** 1995. A new family of peptidyl-prolyl isomerases. Trends Biochem. Sci. **20:**12–14.
45. **Sadosky, A. B., L. A. Wiater, and H. A. Shuman.** 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infect. Immun. **61:**5361–5373.
46. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn,**

K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA-polymerase. Science **239:**487–491.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Schmidt, B.** Unpublished data.
49. **Schmidt, B., J. Rahfeld, A. Schierhorn, B. Ludwig, J. Hacker, and G. Fischer.** 1994. A homodimer represents an active species of the peptidyl-prolyl *cis*/*trans* isomerase FKBP25mem from *Legionella pneumophila*. FEBS Lett. **352:**185–190.
50. **Schreiber, S. L.** 1991. Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science **251:**283–287.
51. **Schreiber, S. L., and G. R. Crabtree.** 1992. The mechanism of action of cyclosporin A and FK506. Immunol. Today **13:**136–142.
52. **Steinert, M., M. Ott, P. C. Lück, and J. Hacker.** 1994. Studies on the uptake and intracellular replication of *Legionella pneumophila* in protozoa and in macrophage-like cells. FEMS Microbiol. Ecol. **15:**299–308.
53. **Steinman, B., P. Bruckner, and A. Superti-Furga.** 1991. Cyclosporin A shows collagen triple-helix formation *in vivo*: indirect evidence for a physiological role of peptidyl-prolyl *cis*/*trans* isomerases. J. Biol. Chem. **266:**1299–1303.
54. **Taylor, R. K., C. Manoil, and J. J. Mekalanos.** 1989. Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. **171:**1870–1878.
55. **Ubben, D., and R. Schmitt.** 1986. Tn 1721 derivatives for transposon mutagenesis, restriction mapping and nucleotide sequence analysis. Gene **41:** 145–152.
56. **Van Duyne, G. D., R. F. Standaert, P. A. Karplus, S. L. Schreiber, and J. Clardy.** 1991. Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex. Science **252:**839–842.
57. **Winn, W. C.** 1988. Legionnaires' disease: historical perspective. Clin. Microbiol. Rev. **1:**60–81.
58. **Wintermeyer, E., M. Flügel, M. Ott, M. Steinert, U. Rdest, K. Mann, and J. Hacker.** 1994. Sequence determination and mutational analysis of the *lly* locus of *Legionella pneumophila*. Infect. Immun. **62:**1109–1117.

Editor: B. I. Eisenstein