

Nucleotide Sequence Analysis of the *Legionella micdadei* mip Gene, Encoding a 30-Kilodalton Analog of the *Legionella pneumophila* Mip Protein

JETTE M. BANGSBORG,^{1*} NICHOLAS P. CIANCOTTO,² AND PETER HINDERSSON¹

Department of Bacteriology, Institute of Medical Microbiology, University of Copenhagen, DK-2100 Copenhagen, Denmark,¹ and Department of Microbiology and Immunology, Northwestern University, Chicago, Illinois 60611²

Received 10 April 1991/Accepted 8 July 1991

After the demonstration of analogs of the *Legionella pneumophila* macrophage infectivity potentiator (Mip) protein in other *Legionella* species, the *Legionella micdadei* mip gene was cloned and expressed in *Escherichia coli*. DNA sequence analysis of the *L. micdadei* mip gene contained in the plasmid pBA6004 revealed a high degree of homology (71%) to the *L. pneumophila* mip gene, with the predicted secondary structures of the two Mip proteins following the same pattern. Southern hybridization experiments, with the plasmid pBA6004 as the probe, suggested that the mip gene of *L. micdadei* has extensive homology with the mip-like genes of several *Legionella* species. Furthermore, amino acid sequence comparisons revealed significant homology to two eukaryotic proteins with isomerase activity (FK506-binding proteins).

The intracellular life-style of at least two *Legionella* species encountered in human infection, *Legionella pneumophila* and *L. micdadei*, is well established (13, 15, 16, 22, 25). When a human is the host organism, the bacteria are phagocytized by alveolar macrophages. Once inside, the bacteria prevent lysosome-phagosome fusion by a yet-unknown mechanism, thus evading one possible route of eradication. The infectivity of *L. pneumophila* for phagocytic cells was recently shown to be enhanced by a 24-kDa protein designated macrophage infectivity potentiator (Mip) (6). Experiments in the macrophage-like U937 cell line with a mutant *L. pneumophila* strain deficient in Mip expression indicated that the protein exerts its effect in the earliest stages of infection and that the intracellular growth rate of the mutant is similar to that of the wild type. The mutant strain also was found to be less virulent than the parent strain in an animal model (5).

An analog of the *L. pneumophila* Mip has been identified in *L. micdadei*. The *L. micdadei* Mip analog, with a molecular mass of 30 kDa, was cloned in *Escherichia coli*, and the corresponding gene was isolated on a 6-kb plasmid (4). We now report the sequence analysis of the *L. micdadei* mip gene as well as results from homology studies of this gene to other, both pathogenic and nonpathogenic, *Legionella* species.

Recent research data have demonstrated the presence of Mip analogs in other *Legionella* species (4) and lately in another intracellular pathogen outside the family *Legionellaceae*, i.e., *Chlamydia trachomatis* (14). These findings lead to the hypothesis that the Mip protein and its equivalents might represent a virulence factor common to several intracellular microorganisms.

A genomic library of *L. micdadei* DNA in *E. coli* SC181 (11) was constructed by using the cosmid vector pHC79 (12) as described previously (1). Recombinants expressing *Legionella* proteins were identified by screening individual clones for reactivity with a purified, *E. coli* SC181-absorbed

rabbit anti-*L. micdadei* antibody in a Western blot (immunoblot) (1, 7).

For all subcloning procedures, *E. coli* JM109 (26) was used as the host. Restriction enzyme digestion, subcloning in the vector pBGS18⁺ (20), and other DNA manipulations were performed as described previously (19).

Both the original recombinant SC181(pBA20) and the subclone JM109(pBA6004) expressed a 30-kDa *L. micdadei* protein. This protein also reacted with a monospecific antibody raised against the recombinant *L. pneumophila* Mip of 24 kDa (4) (data not shown).

DNA sequencing of deletion mutants of pBA6004 was done by using the commercially available DNA sequencing kit Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Plasmid DNA to be sequenced was purified on an Elutip-D column (Schleicher and Schuell, Dassel, Germany) and sequenced by the protocol for sequencing double-stranded DNA given in the instructions of the manufacturer. Sequence reactions were run on an LKB MacroPhor electrophoresis unit (LKB, Bromma, Sweden). The oligonucleotide primers used were either commercially available standard primers or synthesized on a PCR-mate model 381 DNA synthesizer (Applied Biosystems, Foster City, Calif.). DNA sequence results were analyzed by using the Pustell Sequence Analysis Programs, version 2.02 (International Biotechnologies, Inc., New Haven, Conn.). The Chou-Fasman (3) and Robson-Garnier (10) algorithms were used in combination to predict secondary structure, i.e., alpha helices, beta sheets, and turns.

The nucleotide sequence of 1,536 bp of the insert in pBA6004 is shown in Fig. 1. An open reading frame of 243 amino acid residues from nucleotides 686 to 1414 was identified. Deletion of DNA downstream of the *Hind*III site at nucleotide coordinate 1038 resulted in the production of a 12-kDa truncated peptide, indicating that the direction of transcription is from left to right (Fig. 1).

Upstream of the initial methionine start codon, -35 and -10 expression signals similar to the experimentally determined *L. pneumophila* signals (8) are found. The initial 22 amino acid residues are hydrophobic, and a typical alpha helix structure is predicted (Fig. 2). This probably represents

* Corresponding author.

```

100
GCGGACGAGCGTTGTCGAAGGTTGTCAAATTCCTCTTCTGTCGTAATAAAACCACCGCGATCGAATAAACTTGACTGATTTGAATTGCACCTGCG

200
TTATCATAAGCCAAAAATCGCATGCCGTTGCTGTGTTTTGGTAATAATTCCTCTGATAAAGTAAAAATCATTCTGTTTCATGGAAAGGAATGATTTTT

300
TTCCCTGACAGGTTAAGGGGCAAGAAGAAATAATTTCTGCATGCGAGGAACCATACTTTAGGGCGCAACGGTCTCAGGGCATTACCTCCAAACCA

400
TAAGATGGCTTTGTCGGTACCATGTCTTTACCTGTTAAAGCTAGGGATCCATAAAGTCAATTTAATTCGAGCTACTTCTCAAAATATTCTTTTTT

500
TCAAGCAGATCCAAAAACGCATTGGCTGCAAGCATAGGACCAACTGTATGCGAACTCGATGGGCCGATGCCGATGGAAAAAAGTGAACACACTGATAC

600
TCATGGTACAAAACTCTATGATTAATAATGTAAGTTTAGAAGAAAACAATCGATTAATAACAGGCAACATTAAGTTTCTATTAATAAAGTTTACC

-35          -10          RBS          700
TTACTGGTAATTTTACTCAAATTTAGGCAGAAITGGTGGAGCATTTTCGCAAAGTTCTATTCTGAAAAGACAAAAGGGGATTGTTTATGAAGATGAGATTG
M K M R L

800
GTCGCTGCAGCTGCCATGGGTTTGGCAATGTCAACGACAATAGCTGCAACCGCTACAACCTGATGCGACAACCTCTGCACCAGGAACATCATTGACTACAG
V A A A A M G L A M S T T I A A T A T T D A T T S A P G T S L T T

900
ACACAGAAAAGCTCTCATACAGCATTGGTGTGATTTGGGTAAGAAATTTAAAAAGCAGGGAATAGAAATAGTCCTGCTGCTATGGCAAAAGGTTTACA
D T E K L S Y S I G A D L G K N F K K Q G I E I S P A A M A K G L Q

1000
AGATGGAATGAGCGGCGCAATTTGCTGACCGACGACCATGAAAGGATGTGCTAAATAAGTTTCAAAAAGATCTAATGATGAAACGCAGCGCAGAA
D G M S G G Q L L L T D D Q M K D V L N K F Q K D L M M K R S A E

HindIII          1100
TTCAATAAGAAAGCTGAAGAGAATAAGTCGAAAGGAGAAAGCTTTCCCTTAACGAAAAATAATCAAAAAGAGGTTGTTAGTTTACCTAGCGGTTGCAGT
F N K K A E E N K S K G E A F L N E N K S K E G V V S L P S G L Q

1200
ATAAGATCCTGAACGAGGCGATGGTGTAAACCGACCAAGGATGACGTGCTTACTGTGGAATACACCGGCAAGCTGATTGACGGTCAGGTTTTTCGACAG
Y N I L E R G D G A K P T K D D V V T V E Y T G K L I D G Q V F D S

1300
TACTGAAAAGACAGGCAACCTGCAACCTTTAAAGTTTCTCAAGTTATTCAGGTTGGACTGAAGCACTGCAATTAATGCCAGCAGGTTCTACTTGGGAA
T E K T G K P A T F K V S Q V I P G W T E A L Q L M P A G S T W E

1400
GTGTATATCCCTTCAATCTGGCTTATGGCCACGTAGCGTTGGCGGCCAATTGGACCTAATGAACTTTAATTTTCAAAATTCATCTGATTTCAAGTGA
V Y I P S N L A Y G P R S V G G P I G P N E T L I F K I H L I S V

1500
AGAAATCTGACGCGTAAATGCGTTTTATCCAGTCTGCTCAGGAGGCTGGGATAAATACCAATTTAAATTTCTCGAGTATTCCAAGCATGAACAAACGCC
K K S D A

1536
TATTGATTGTTTTTGTGGGCTTTTCTCAGGGC

```

FIG. 1. Nucleotide sequence of the 1,536-bp fragment from pBA6004 encoding the *L. micdadei* Mip protein. The open reading frame corresponding to the Mip protein is given below the DNA sequence. The putative -10 and -35 signals and the ribosomal binding site (RBS) are indicated upstream of the translation starting point. An inverted repeat with features of a rho-independent transcription termination signal is underlined downstream of the open reading frame. The *Hind*III site within the Mip-encoding region is indicated.

a secretory signal, but no typical signal sequence cleavage site can be identified by using the prediction algorithm of von Heijne (24); in contrast, according to this prediction algorithm, the *L. pneumophila mip* gene has an easily distinguishable leader sequence with a typical cleavage site. Residues 55 to 120 are predicted to form an uninterrupted alpha helix. The remaining carboxy-terminal part of the open reading frame forms beta sheets and turns, interrupted only

by a short segment of alpha helix from residues 184 to 196. The extended alpha helix of the *L. micdadei* Mip from residues 55 to 120 could represent an elongated structure projecting from the surface of the bacterium, as suggested previously (8). A similar, extended alpha helix structure can be identified in the corresponding proteins of *L. pneumophila* and *C. trachomatis* (in the latter organism, predicted from the sequenced 525 bp of the 3' end of the *C. tracho-*

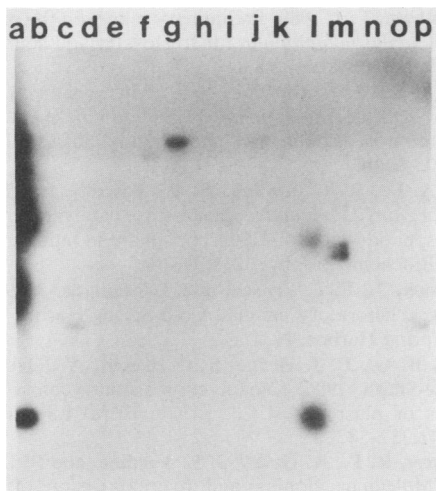


FIG. 4. Southern hybridization of DNA preparations from *Legionella* species with an *L. micdadei mip* probe. Genomic DNA was purified from each strain, digested with *Eco*RI, and electrophoresed in 0.8% agarose. A Southern blot was made, hybridized with ³²P-labeled pBA6004 under high-stringency conditions, and autoradiographed. Lanes: a, *L. micdadei*; b, *L. bozemanii*; c, *L. dumoffii*; d, *L. gormanii*; e, *L. longbeachae*; f, *L. jordanis*; g, *L. wadsworthii*; h, *L. hackeliae*; i, *L. cincinnatiensis*; j, *L. birminghamensis*; k, *L. maceachernii*; l, *L. feeleii*; m, *L. oakridgensis*; n, *L. tucsonensis*; o, *L. anisa*; p, *L. pneumophila*. Also tested, but not shown, were *L. cherrii*, *L. sainthelensi*, *L. jamestowniensis*, *L. rubrilucens*, *L. erythra*, *L. spiritensis*, *L. parisiensis*, *L. steigerwaltii*, *L. santicrucis*, *L. israelensis*, *L. moravica*, *L. brunensis*, *L. quinlivanii*, and *L. gratiana*.

Under reduced-stringency conditions, DNA from every strain hybridized with pBA6004, indicating that each species contains a *mip*-like gene which is at least as homologous to its counterpart in *L. micdadei* as it is to the *mip* gene of *L. pneumophila* (data not shown). In contrast to results obtained with the *L. pneumophila mip* gene as the probe (4), pBA6004 also hybridized strongly with DNA from a number of species under high-stringency conditions (examples shown in Fig. 4, lanes g, l, and m). These species do not necessarily express a Mip-related protein which has the same size as that produced by *L. micdadei* (4); some represent species not yet associated with human disease but isolated from environmental samples only. Taken together, these results suggest that the *mip* gene of *L. micdadei* has extensive homology ($\geq 90\%$) with the *mip*-like genes of several *Legionella* species and moderate homology (ca. 70 to 90%) with the *mip*-like genes of the remaining species. The *mip* gene family in strains of legionellae may thus be divided into the following three homology groups: (i) the *mip* gene of *L. pneumophila* which has moderate homology to its analogs in the other *Legionella* species (4); (ii) the *mip*-like genes of *L. micdadei*, *L. feeleii*, *L. jamestowniensis*, *L. oakridgensis*, *L. quinlivanii*, *L. sainthelensi*, *L. spiritensis*, and *L. wadsworthii*, which have moderate homology to the *L. pneumophila mip* but extensive homology to the *L. micdadei mip*; and (iii) the *mip*-like genes of the remaining *Legionella* species which have moderate homology to their counterparts in both *L. pneumophila* and *L. micdadei*. Whether this genetic heterogeneity has any functional implications remains to be shown.

Our present results show that there is a high degree of similarity between the *L. pneumophila* Mip and the *L.*

micdadei Mip and that the secondary structure of the two Mip proteins is very conserved. Until now, there have been few studies of virulence factors and of other phenotypic characteristics in *L. micdadei*, in contrast to the massive interest in *L. pneumophila*. Both species, however, are involved in the classical fulminant presentation of Legionnaires' disease. The relative rarity of *L. micdadei* infections could, at least in part, be ascribed to differences in growth kinetics in the environment (2) rather than a genuine difference in virulence between the two species. Certain cell components of *L. micdadei* possibly involved in pathogenesis have been identified, i.e., a phosphatase blocking superoxide anion production (18) and a protein kinase catalyzing phosphorylation of (and thereby inhibiting) neutrophil proteins, such as tubulin (17). *L. micdadei*, like *L. pneumophila*, is taken up and multiplies well within phagosomes in human monocytes but with somewhat different electron microscopy features (25).

The striking similarity between the carboxy-terminal end of the two *Legionella mip* genes and the recently described FKBP of *N. crassa* (NcFKBP) (23), the human analog FKBP (HsFKBP), and a cryptic *N. meningitidis* sequence (C114) (21) deserves further comment. Both the NcFKBP and the HsFKBP are peptidyl-prolyl-*cis-trans*-isomerases that are strongly and specifically inhibited by the immunosuppressant FK506. The prolyl-isomerases function as slow foldases, and it has been suggested that they act in vivo on proline-containing chains of other peptides, serving as a switch between alternative conformations of a target protein. Given this theory and the fact that the *L. pneumophila* and *L. micdadei* Mip proteins are surface exposed (4, 6) (unpublished observations), it is a logical assumption that the extended alpha helix predicted in the N-terminal part of the sequenced Mip proteins forms a rodlike spacer arm, bringing the enzymatically active part (the carboxy-terminal half) close to a target membrane structure. The possible enzymatic function of the beta sheet-dominated carboxy-terminal region of the *Legionella* Mip proteins and the putative function upon the macrophage membrane are important matters for future research in this area.

This work was supported by grants from the Danish Medical Research Council (12-8594), The NOVO Foundation, and Fonden til Lægevidenskabens Fremme. N.P.C. was supported in part by Public Health Service grants (RR-05370 and R29 AI30064) from the National Institutes of Health. P.H. was supported by Weimanns legat, Foersoms legat, and Sygekassernes Helsefond.

Lene Michelsen and Pia Frøslev provided expert technical assistance. We thank A. Lundemose for providing unpublished sequence data.

REFERENCES

- Bangsberg, J. M., M. T. Collins, N. Høiby, and P. Hindersson. 1989. Cloning and expression of the *Legionella micdadei* "common antigen" in *Escherichia coli*. *APMIS* 97:14-22.
- Best, M. G., J. E. Stout, V. L. Yu, and R. R. Muder. 1985. *Tatlockia micdadei* (Pittsburgh pneumonia agent) growth kinetics may explain its infrequent isolation from water and the low prevalence of Pittsburgh pneumonia. *Appl. Environ. Microbiol.* 49:1521-1522.
- Chou, P. Y., and G. D. Fasman. 1978. Prediction of secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* 47:45-148.
- Cianciotto, N. P., J. M. Bangsberg, B. I. Eisenstein, and N. C. Engleberg. 1990. Identification of *mip*-like genes in the genus *Legionella*. *Infect. Immun.* 58:2912-2918.
- 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.
6. 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.
 7. Collins, M. T., J. M. Bangsberg, and N. Høiby. 1987. Antigenic heterogeneity among *Legionella*, *Fluoribacter*, and *Tatlockia* species analyzed by crossed immunoelectrophoresis. *Int. J. Syst. Bacteriol.* 37:351-356.
 8. 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.
 9. 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.
 10. Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* 120:97-120.
 11. Hansen, E. B., P. E. Pedersen, L. M. Schouls, E. Severin, and J. D. A. van Embden. 1985. Genetic characterization and partial sequence determination of a *Treponema pallidum* operon expressing two immunogenic membrane proteins in *Escherichia coli*. *J. Bacteriol.* 162:1227-1237.
 12. Hohn, B., and J. Collins. 1980. A small cosmid for efficient cloning of large DNA fragments. *Gene* 11:291-298.
 13. Horwitz, M. A., and S. C. Silverstein. 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *J. Clin. Invest.* 66:441-450.
 - 13a. Lundemose, A. Personal communication.
 14. Lundemose, A. G., S. Birkelund, S. J. Fey, P. M. Larsen, and G. Christiansen. 1991. *Chlamydia trachomatis* contains a protein similar to the *Legionella pneumophila mip* gene product. *Mol. Microbiol.* 5:109-117.
 15. Oldham, L. J., and F. G. Rodgers. 1985. Adhesion, penetration and intracellular replication of *Legionella pneumophila*: an in vitro model of pathogenesis. *J. Gen. Microbiol.* 131:697-706.
 16. Rowbotham, T. J. 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J. Clin. Pathol.* 33:1179-1183.
 17. Saha, A. K., J. N. Dowling, N. K. Mukhopadhyay, and R. H. Glew. 1989. *Legionella micdadei* protein kinase catalyzes phosphorylation of tubulin and phosphatidylinositol. *J. Bacteriol.* 171:5103-5110.
 18. Saha, A. K., J. N. Dowling, A. W. Pasculle, and R. H. Glew. 1988. *Legionella micdadei* phosphatase catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate in human neutrophils. *Arch. Biochem. Biophys.* 265:94-104.
 19. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 20. Spratt, B. G., P. J. Hedge, S. te Heesen, A. Edelman, and J. Broome-Smith. 1986. Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. *Gene* 41:337-342.
 21. Standaert, R. F., A. Galat, G. L. Verdine, and S. L. Schreiber. 1990. Molecular cloning and overexpression of the human FK506-binding protein FKBP. *Nature (London)* 346:671-674.
 22. Summersgill, J. T., M. J. Raff, and R. D. Miller. 1990. Interactions of virulent and avirulent *Legionella pneumophila* with human monocytes. *J. Leukocyte Biol.* 47:31-38.
 23. Tropschug, M., E. Wachter, S. Mayer, E. R. Schönbrunner, and F. X. Schmidt. 1990. Isolation and sequence of an FK506-binding protein from *N. crassa* which catalyzes protein folding. *Nature (London)* 346:674-677.
 24. von Heijne, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* 133:17-21.
 25. Weinbaum, D. L., R. R. Benner, J. H. Dowling, A. Alpern, A. W. Pasculle, and G. R. Donowitz. 1984. Interaction of *Legionella micdadei* with human monocytes. *Infect. Immun.* 46:48-73.
 26. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103-119.