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

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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 *Hin*dIII 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

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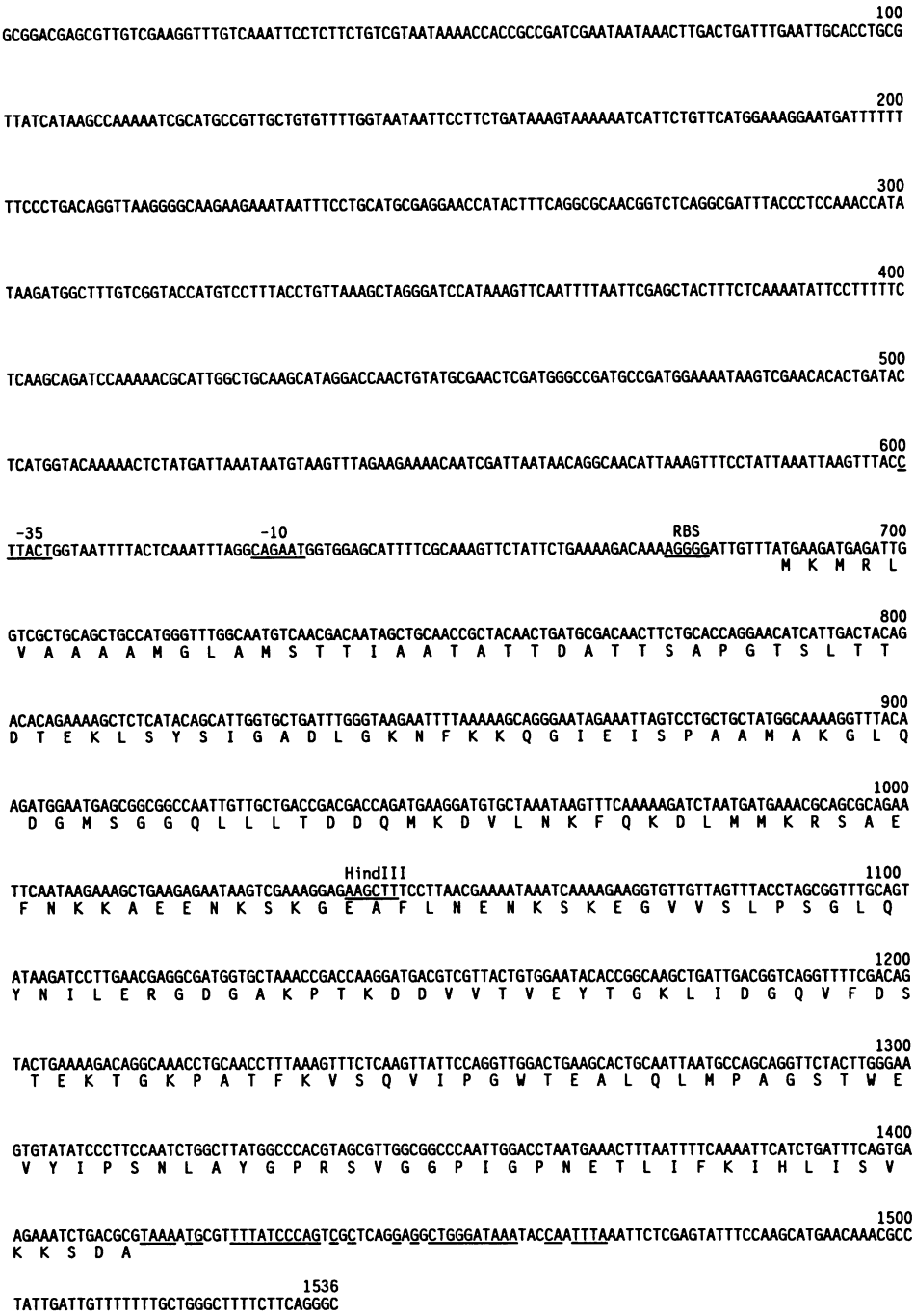

FIG. 1. Nucleotide sequence of the 1,536-bp fragment from pBA6004 encoding the *L. micdadei* Mip protein. The open reading frame corresponding to the Mip protein is given below the DNA sequence. The putative −10 and −35 signals and the ribosomal binding site (RBS) are indicated upstream of the translation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

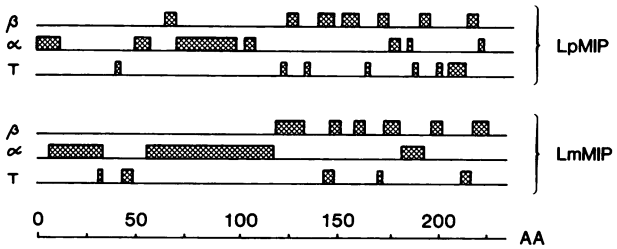

FIG. 2. Secondary structure prediction of *L. micdadei* and *L. pneumophila mip* gene products (LmMIP and LpMIP, respectively). The plot was constructed by using a combination of the Chou-Fasman and the Robson-Garnier algorithms. Abbreviations: α, alpha helix; β, beta sheet; T, turns. The scale unit is amino acids (AA).

matis mip gene [14]). Likewise, the short alpha helix segment (residues 184 to 196) in the carboxy-terminal half of *L. micdadei* Mip is conserved in both Mip proteins of *L. pneumophila* and *C. trachomatis*. The carboxy-terminal part of all three proteins is predicted to form beta sheets and turns. It is tempting to speculate that the function of the Mip is the same in the two (and, possibly, in other) *Legionella* species and that the Mip is involved in a more universal infectivity mechanism also found in other intracellular microorganisms. In *L. pneumophila*, the Mip is necessary for optimal macrophage infectivity, exerting its role possibly by a bacterium-host membrane interaction (6). The nucleotide sequences immediately upstream of the *L. micdadei* and *L. pneumophila mip* genes are very conserved and the expression signals are almost identical, evidence that the expression of the Mip proteins is similar in the two species. The upstream regulatory sequences of the *C. trachomatis mip* gene have not yet been determined, but this work is in progress (13a). The inverted repeat found downstream of the *L. pneumophila mip* gene (8), also present in the *mip* genes of *L. micdadei* (Fig. 1) and *C. trachomatis*, most likely represents a factor-independent transcription termination signal (8).

The calculated molecular mass of the *L. micdadei mip* gene product is 25.927 kDa (slightly different from the experimentally determined value of 30 kDa), and the pI value is 8.1. The calculated pI value of the *L. micdadei* Mip differs from the pI value of 9.0 of the *L. pneumophila* Mip but is closer than that of the *C. trachomatis* Mip (pI, 3.8), as determined by two-dimensional gel electrophoresis (14). In spite of the widely variable pI values, all of the proteins seem to have an uncommon abundance of charged residues. Thus, the amino acid sequence of the *L. micdadei* Mip, with a slightly basic pI, contains approximately 10% lysines, as does the sequence of the *C. trachomatis* analog with a pI of 3.8.

The DNA sequence of the *L. micdadei mip* gene is very similar to the corresponding sequence of the *L. pneumophila mip*, with a homology of 71%; the predicted secondary structures of the two Mip proteins are also closely related, as stated above. The homology between the *L. micdadei mip* sequence and the partial sequence data of the *C. trachomatis mip* (14) amounts to 57%. Interestingly, two newly sequenced isomerases, the FK506-binding protein (FKBP) of *Neurospora crassa* reported by Tropschug et al. (23) and the corresponding human FKBP (21) show a high degree of homology to the 120 carboxy-terminal residues of the *Legionella* Mip proteins (Fig. 3) and to the sequenced part of the *C. trachomatis* Mip. A hypothetical protein derived from a cryptic *Neisseria meningitidis* sequence with homology to the human FKBP (21) has been included in Fig. 3. The extended alpha helix structure from residues 55 to 120 in both of the *Legionella* Mip proteins and in the *Chlamydia* Mip is missing in the FKBPs.

The extent of homology between the *mip* gene of *L. micdadei* and that of *Legionella* species other than *L. pneumophila* was investigated by Southern hybridization. Chromosomal DNA from all genetically characterized *Legionella* species was isolated as previously described (9). Hybridizations were performed under high- and reduced-stringency conditions as previously described (4). The high- and low-stringency conditions used permit hybridization with approximately 10 and 30% base pair mismatching, respectively. The plasmid pBA6004 was radiolabeled with ^{32}P by using a random primer labeling kit (Bethesda Research Laboratories, Gaithersburg, Md.).

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LmMIP     NENKSKEGVVSLPSGLQYNILERGDGAKPTKDDVVTVEYTGKLIDGQVFDSTEKTGKPATFKVSQVIPGWTEALQLMPAGSTWEVYIPSNLAYGPRSVGGPIGPNETLIFKIHLISVKKSDA
          *** * *** ******* **  *** ** * * ************ ************** *************************** ****************************

LpMIP     TENKNKPGVVVLPSGLQYKVINAGNGVKPGKSDTVTVEYTGRLIDGTVFDSTEKTGKPATFQVSQVIPGWTEALQLMPAGSTWEIYVPSGLAYGPRSVGGPIGPNETLIFKIHLISVKK>
                *  *  ***  * * *** * ** * * * * * *  *  **     * *  * * *** ** * *  *  *  * * *  ***** * *** *  * ****   ** **
                                        R                        GQ
NcFKBP          MTIPQL-DGLQIEVQQEGQGTRETRGDNVDVHYKGVLTSGKKFDASYDRGEPLNFTVGQVIKGWDEGLLGMKIGEKRKLTIAPHLAYGNRAVGGIIPANSTLIFETELVGIK>
          *** * *** **  **  **  * *          * *** **** **** **   *  * * *  ****  * ** *  *  * * ***  *****     * * **  *** * **
                    E
CtMIP     KENKEKAGVIELPNKLHDRVVKEGTGRVLSGKPTALLHYTGSFIDGKVFDSSEKNKEPILLPLTKVIPGFSQGMQGMKEGEVRVLYIHPDLAYG--T-AGQLPPNSLLIFEVKLIEAN>
                       *   **   *    *   *** *** * **  ***      * *  * *** ** * *  *  *  * * *** * **    ** * ** **** * ** *
                                                                 LG
C114              MGSLIIEDLQESFGKEAVKGKEITVHYTGWLEDGTKFDSSLDRRQPLTITVGQVIKGWDEGFGGMKEGGKRKLTIPSEMGYGATRRGGVIPPHATLIFEVELLKV>
                     ***   *  ***  * * *   * *** * **  ***     **  * * *** ** * *  *  *    * *  * ***     * * ** **** * ** *
                               F                                 KQ
HsFKBP              MGVQVETISPGDGRTPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKL>
```

FIG. 3. Comparison of the carboxy-terminal half of the *L. micdadei* Mip protein to the carboxy-terminal residues of the *L. pneumophila* and *C. trachomatis* Mip proteins (LmMIP, LpMIP, and CtMIP, respectively) and to the *N. crassa* FKBP (NcFKBP), the human FKBP (HsFKBP), and a putative gene product deduced from an *N. meningitidis* cryptic sequence (C114). The *L. micdadei* Mip sequence is used as the reference for all of the amino acid sequences shown below it. Identical or functionally identical amino acids (score value ≥2 according to Dayhoff's PAM 250 scoring matrix, Pustell Sequence Analysis Programs, version 2.02) are indicated (*).

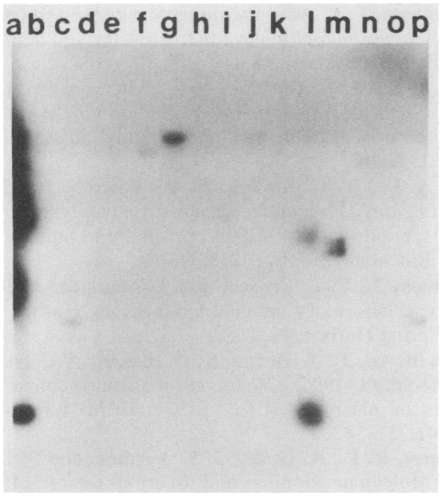

FIG. 4. Southern hybridization of DNA preparations from *Legionella* species with an *L. micdadei mip* probe. Genomic DNA was purified from each strain, digested with *Eco*RI, and electrophoresed in 0.8% agarose. A Southern blot was made, hybridized with ^{32}P-labeled pBA6004 under high-stringency conditions, 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 (≥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.

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