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Identification of *mip*-Like Genes in the Genus *Legionella*

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The *mip* gene of *Legionella pneumophila* serogroup 1 strain AA100 encodes a 24-kilodalton surface protein (Mip) and enhances the abilities of *L. pneumophila* to parasitize human macrophages and to cause pneumonia in experimental animals. To determine whether this virulence factor is conserved in the genus *Legionella*, a large panel of *Legionella* strains was examined by Southern hybridization and immunoblot analyses for the presence and expression of *mip*-related sequences. Strains representing all 14 serogroups of *L. pneumophila* contained a *mip* gene and expressed a 24-kilodalton Mip protein. Although the isolates of the 29 other *Legionella* species did not hybridize with *mip* DNA probes under high-stringency conditions, they did so at reduced stringency. In support of the notion that these strains possess *mip*-like genes, these species each expressed a protein (24 to 31 kilodaltons in size) that reacted with specific Mip antisera. Moreover, the cloned *mip* analog from *Legionella micdadei* encoded the cross-reactive protein. Thus, *mip* is conserved and specific to *L. pneumophila*, but *mip*-like genes are present throughout the genus, perhaps potentiating the intracellular infectivity of all *Legionella* species.

Members of the genus *Legionella* normally inhabit natural aquatic environments in which, it is believed, they survive as intracellular parasites of freshwater protozoa (18, 19, 30, 35). However, legionellae are also responsible for a significant percentage of both community-acquired and nosocomial pneumonias and can account for 1 to 30% of all pneumonias (29, 35). Although 18 of the 30 species of *Legionella* have been implicated in human disease, *Legionella pneumophila*, first recognized as the causative agent of Legionnaires disease, is the species most frequently associated with pneumonia (8, 17). It is assumed that *L. pneumophila* enters the distal airways in aerosol form and is then phagocytized by the resident alveolar macrophages (22, 35). Within the macrophage, *L. pneumophila* inhibits both phagosome acidification and phagosome-lysosome fusion and effectively replicates within a membrane-bound, ribosome-studded endosome (22). Severe lung damage and pneumonia eventually result from both the increase in bacterial burden and the release of tissue-destructive substances from the bacteria, the damaged or infected host cells, or both (9, 35). Despite our understanding of the cellular events of pathogenesis, the critical bacterial genes and gene products remain poorly understood (9).

Recently, using the method of site-specific mutagenesis, we determined that the *mip* gene is required for the ability of a *L. pneumophila* serogroup 1 (SG1) strain to infect alveolar macrophages and to cause pneumonia in guinea pigs (9a, 10). The *mip* gene (for macrophage infectivity potentiator) encodes a 24-kilodalton (kDa) surface protein (Mip) and plays a crucial role in the resistance of *L. pneumophila* to intracellular killing (10, 14). Preliminary experiments had suggested that this form of Mip was specific to strains of *L. pneumophila* (13, 16).

Although several of the other *Legionella* species have been shown to infect mononuclear cells in vitro (23, 33; D. Havlicheck, J. White, and L. Ade, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, B-137, p. 52; J. A. Elliott, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, B-131, p. 51), the pathogenic mechanisms mediating infection by these species are unknown. There is some evidence to suggest that different pathogenic mechanisms may be used by different species and even among different strains of *L. pneumophila* (2, 7, 9, 16, 25–28). Limited evidence suggests that *L. micdadei*—after *L. pneumophila*, the *Legionella* species most frequently associated with disease—does not employ complement-mediated opsonophagocytosis to enter macrophages, replicate within a ribosome-studded phagosome, or inhibit phagosome-lysosome fusion, all in contrast to *L. pneumophila* (28, 33). To more fully understand *Legionella* intracellular parasitism and pathogenesis, it is therefore important to determine whether the bacterial factors critical for *L. pneumophila* are also critical in other legionellae. Consequently, we sought to determine whether *mip*, the first defined virulence factor of *L. pneumophila*, is present and expressed in other legionellae. Here, we demonstrate that the *mip* gene is conserved and specific to *L. pneumophila* but that *mip* analogs exist in all legionellae. These data confirm the significance of *mip* in the natural history of legionellosis and suggest that some components of intracellular parasitism and virulence may be conserved among the various legionellae.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains representing the 14 serogroups of *L. pneumophila* are indicated in Table 1. The *mip* gene had been cloned and characterized from *L. pneumophila* SG1 strain AA100 (10, 14, 15). The recombinant plasmid pSMJ31.42, which contains the *mip*

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TABLE 1. *L. pneumophila* strains

Serogroup[a]	Strain	Source[b]
1	AA100[c]	Edelstein
1	Philadelphia 1, ATCC 33152	CDC
2	Togus 1, ATCC 33154	CDC
3	Bloomington 2, ATCC 33155	CDC
4	Los Angeles 1, ATCC 33156	CDC
5	Dallas 1E, ATCC 33216	CDC
6	Chicago 2, ATCC 33215	CDC
7	Chicago 8, ATCC 33823	CDC
8	Concord 3, ATCC 35096	CDC
9		MDPH
10		MDPH
11		MDPH
12		MDPH
13	B2A3105	CDC
14	1169-MN-H	CDC

[a] The 14 serogroups of *L. pneumophila* are distinguished by the lipopolysaccharide antigen (11).
[b] CDC, Centers for Disease Control; MDPH, Michigan Department of Public Health.
[c] Also referred to as strain 130b, AA100 is a clinical isolate from a Los Angeles epidemic.

gene, was previously described (14). Strains representing the 29 other *Legionella* species are indicated in Table 2. Legionellae were grown on buffered charcoal-yeast extract agar for 48 to 72 h at 37°C (15). *Escherichia coli* SC181 (20) and JM109 (36) were used in cloning experiments. These strains were grown on L agar, and when appropriate, ampicillin or kanamycin was added to a concentration of 50 μg/ml.

***mip* DNA probes.** To test for the presence of *mip*-related sequences in strains of *Legionella*, two *mip*-specific DNA probes were derived from pSMJ31.42. The 5′ probe was bounded at one end by the *Eco*RI site located near the middle of *mip* (14) and included the adjacent 0.54 kilobase pair (kb) of upstream DNA. Consequently, this DNA probe included the first half of the *mip* coding region (approximately 0.3 kb), the *mip* promoter region, and the 0.14 kb of DNA upstream of the −35 region of the *mip* promoter. Although the 5′ probe is, technically speaking, not specific to the *mip* coding region, DNA sequence analysis of this region of DNA indicates that there are no overlapping open reading frames (genes) contained in this probe (14). In contrast, the 3′ probe was completely internal to the *mip* coding region. This DNA probe begins at the same *Eco*RI site noted above but includes the adjacent 0.33 kb of downstream DNA. The final approximately 70 base pairs of the *mip* coding region are not represented in these DNA probes (14). DNAs were radiolabeled with ^{32}P by using a random primer labeling kit (Amersham Corp.).

DNA isolation and Southern hybridization analyses. Genomic DNA was isolated from strains of *Legionella* as previously described (15). Plasmid DNA was isolated by the alkaline lysis method (1). Restriction enzyme digestion of genomic DNA and agarose gel electrophoresis were performed as described elsewhere (10). Southern hybridizations and autoradiography were performed as described previously, with the following modifications employed to achieve different stringency conditions (1). To achieve high-stringency conditions (T_m − 14°C), filters were hybridized with the probe at 37°C in the presence of 2.6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 60% deionized formamide and were subsequently washed at 50°C in 0.08× SSC–0.1% sodium dodecyl sulfate. To achieve reduced-stringency conditions (T_m − 47°C), filters were hybridized with the probe at 37°C in the presence of 5× SSC and 18% deionized formamide and were washed at 50°C in 5.3× SSC–0.1% sodium dodecyl sulfate. The high- and low-stringency conditions used permit hybridization with approximately 10 and 30% base-pair mismatching, respectively.

TABLE 2. *Legionella* species

Species	Strain[a]	Implicated in disease (reference)	Hybridization[b] and size (kDa) of Mip protein
L. anisa	SG1, WA 316-C3	Yes (17)	+, 26[c]
L. birminghamensis	SG1, 1407-AL-H	Yes (17)	+, 25
L. bozemanii	ATCC 33217	Yes (17)	+, 27[c]
L. brunensis	441-1	No (34)	+,
L. cherrii	SG1, 0 RW	Yes (17)	+, 27[c]
L. cincinnatiensis	SG1, 72-OH-H	Yes (17)	+, 27[c]
L. dumoffii	ATCC 33279	Yes (17)	+, 26
L. erytha	SE-32A	No (8)	+, 28
L. feeleii	SG1, SG2	Yes (17)	+, 26
L. gormanii	ATCC 33297	Yes (17)	+, 27[c]
L. gratiana	Lyon 8420412	No (6)	ND, 26[c]
L. hackeliae		Yes (17)	+, 31
L. israelensis		No (8)	+, 27
L. jamestowniensis	JA-26	No (8)	+, 30
L. jordanis	SG1, BL-540	Yes (17)	+, 30
L. longbeachae	ATCC 33462	Yes (17)	+, 27[c]
L. maceachernii		Yes (17)	+, 29
L. micdadei	ATCC 33218	Yes (17)	+, 30
L. moravica	316-36	No (34)	+, 24[c]
L. oakridgensis		Yes (17)	+, 25
L. parisiensis	PF-209	No (8)	+, 24
L. pneumophila	SG1 through SG14	Yes (17)	+++, 24[c]
L. quinlivanii	1442-AUS-E	No (5)	+, 27
L. rubrilucens	WA-270	No (8)	+, 40 and 29
L. sainthelensi	SG1, Mt. St. Helens 4	Yes (17)	+, 27[c]
L. santicrusis	SC-63	No (8)	+, 27[c]
L. spiritensis	MSH-9	No (8)	+, 25
L. steigerwaltii	SG1, SC-18-C9	No (8)	+, 27[c]
L. tucsonensis	SG1, 1087-AZ-H	Yes (32)	ND, 26
L. wadsworthii	81-716	Yes (17)	+, 26

[a] Most strains were obtained from the Centers for Disease Control. The exceptions were strains of *L. oakridgensis*, *L. feeleii*, *L. maceachernii*, and *L. hackeliae*, which were obtained from the Michigan Department of Public Health.
[b] +++, Hybridization to the *mip* probe (derived from *L. pneumophila* AA100) under conditions of high stringency; +, hybridization only under reduced stringency conditions; ND, not determined.
[c] Strains in which the Mip or Mip-related protein reacted with the anti-Mip monoclonal antibody 12F4.

Antibodies and immunoassays. The anti-Mip monoclonal antibody 12F4 was previously described (10). To isolate polyclonal, monospecific anti-Mip serum, a rabbit was immunized by subcutaneous injection of approximately 130 μg of Mip in Freund complete adjuvant. Then, every 2 weeks for the next 6 weeks, a booster of 100 to 120 μg of Mip in Freund incomplete adjuvant was administered. The Mip protein used for these immunizations was purified from a recombinant strain of *E. coli* containing the cloned *mip* gene (M. Hurley, et al., unpublished data). Anti-*L. micdadei* serum was previously described (3). Sera were filter sterilized and heat treated (56°C for 30 min) before use.

To assay for the presence of Mip proteins in strains of *Legionella*, whole-cell lysates were prepared and reacted

with anti-Mip antibodies. Briefly, bacteria from each strain were harvested from fresh agar plates, suspended in water to the same concentration (optical density at 600 nm), and boiled for 5 min in the presence of sodium dodecyl sulfate and 2-mercaptoethanol (24). Then, an equal volume of each lysate was electrophoresed in a 15% sodium dodecyl sulfate-acrylamide gel. After the separated proteins were transferred to filter paper, immunoblots were made as before (10). Horseradish peroxidase-conjugated goat anti mouse or anti-rabbit antibody (Cappel Laboratories) was used as the secondary antibody, and diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) was used as the color reaction substrate.

Construction and screening of *L. micdadei* genomic library. Genomic DNA from *L. micdadei* was subjected to partial digestion with *Sau*3A, and the resulting mixture of fragments was ligated into *Bam*HI-digested, dephosphorylated cosmid vector pHC79 (21). The ligation mixture was introduced into competent *E. coli* SC181 cells by using a lambda bacteriophage DNA packaging kit (Boehringer Gmbh, Mannheim, Federal Republic of Germany). To isolate clones which express *L. micdadei* antigens, ampicillin-resistant colonies were screened by immunoblotting with *E. coli* SC181-absorbed, rabbit anti-*L. micdadei* serum. Although several antigen-expressing clones were identified, clone 1254D4 was chosen for further study since it expressed a protein of the same molecular weight (30 kDa) as the Mip-related protein of *L. micdadei* (see below). The 1254D4 strain contained a 17-kb recombinant plasmid (pBA20) with a 10.5-kb insert of *Legionella* DNA.

To isolate the 30-kDa protein gene, *Hin*dIII fragments of pBA20 were subcloned randomly into *Hin*dIII-digested, dephosphorylated pBGS18+ (31), and the ligation mixture was transformed into competent *E. coli* JM109. From this set of subclones, a 14-kb plasmid, pBA6001, that still expressed the 30-kDa antigen was isolated. Further subcloning of pBA6001 finally localized expression of the *L. micdadei* 30-kDa protein to a 1.5-kb fragment of DNA cloned in vector pBGS18+ (pBA6004). *E. coli* JM109(pBA6004) was assayed for both the expression of Mip-related proteins and the presence of *mip*-related DNA sequences.

RESULTS

Conservation of *mip* gene in *L. pneumophila*. The *mip* gene was first cloned and characterized from an SG1 strain (AA100) of *L. pneumophila* (10, 14). Although clinical isolates most often belong to SG1, strains of SG2 through SG14 have all been associated with cases of Legionnaires disease (8). To determine whether *mip* is conserved in these other pathogenic strains, genomic DNAs from strains of the other 13 serogroups of *L. pneumophila* as well as the SG1 Philadelphia 1 strain were hybridized with *mip* DNA probes. DNAs from all strains hybridized at high stringency to both the 5′ (Fig. 1) and 3′ (Fig. 2) *mip* probes, indicating that the *mip* gene is conserved in *L. pneumophila*. Each strain contained a single *Eco*RI fragment that hybridized to the 5′ *mip* probe and a single *Eco*RI fragment that hybridized to the 3′ *mip* probe. In some cases, the same *Eco*RI fragment hybridized to both *mip* probes, e.g., the SG3, SG4, SG8, and SG13 strains (lanes d, e, i, and n, respectively, in Fig. 1 and 2). These data suggest that these fragments contain the entire *mip* gene and that their DNAs have a polymorphism at the *Eco*RI site within the AA100 coding sequence. For strain Philadelphia 1 and strains representing SG5, SG6, SG7, SG10, SG11, and SG12, the fragment that hybridized with the 5′ probe comigrated with the 1.2-kb hybridizing fragment of strain AA100 (Fig. 1). In contrast, strains representing SG2, SG3, SG4, SG8, SG9, and SG13 each had a 2.7-kb hybridizing fragment, whereas the SG14 strain had a unique-size hybridizing fragment. Similarly, there was variability in the size of the *Eco*RI fragment that hybridized with the 3′ *mip* probe (Fig. 2). These data indicate that, though not necessarily serogroup specific, restriction site polymorphism does exist in or around the *mip* gene in these independent isolates. The DNAs from strain Philadelphia 1 and SG3 appeared to hybridize less intensely with the 5′ *mip* probe than did the others (Fig. 1, lanes b and d). Similarly, the

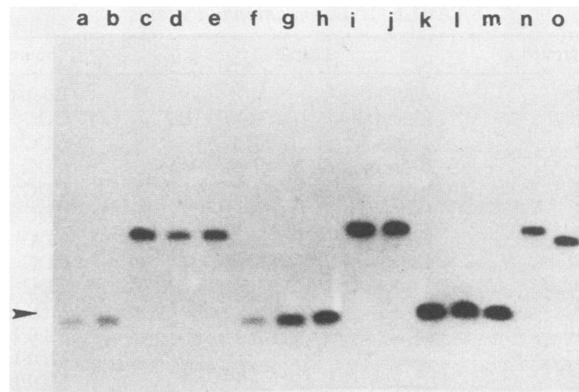

FIG. 1. Hybridization of DNAs from all serogroups of *L. pneumophila* with the 5′ *mip* DNA probe. DNA was purified from each isolate, digested to completion with *Eco*RI, and electrophoresed in 0.8% agarose. In ethidium bromide-stained gels, each strain exhibited a unique *Eco*RI restriction digestion pattern. A Southern blot was made, hybridized with the ^{32}P-labeled *mip* probe under high-stringency conditions, and autoradiographed. Lanes: a, SG1 strain AA100; b, SG1 strain Philadelphia 1; c, SG2; d, SG3; e, SG4; f, SG5; g, SG6; h, SG7; i, SG8; j, SG9; k, SG10; l, SG11; m, SG12; n, SG13; o, SG14. Note that the probe hybridized to a 1.2-kb *Eco*RI fragment of AA100 (arrow, lane a) and that the hybridization signal was weak because of a smaller amount of DNA in the lane.

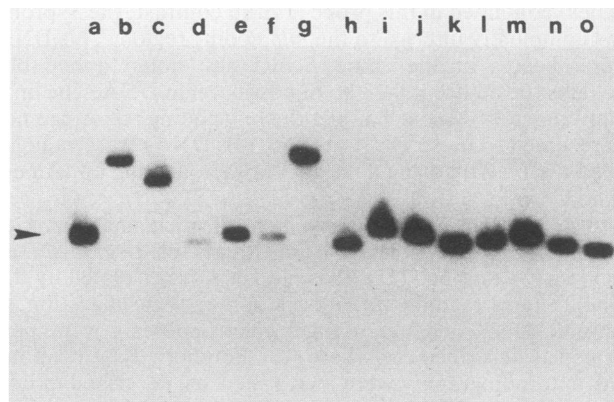

FIG. 2. Hybridization of DNAs from all serogroups of *L. pneumophila* with the 3′ *mip* DNA probe. A Southern blot and an autoradiograph were prepared as described for Fig. 1. Lanes: a, SG1 strain AA100; b, SG1 strain Philadelphia 1; c, SG2; d, SG3; e, SG4; f, SG5; g, SG6; h, SG7; i, SG8; j, SG9; k, SG10; l, SG11; m, SG12; n, SG13; o, SG14. The 3′ probe hybridized to a 3.0-kb *Eco*RI fragment of AA100 (arrow, lane a). The hybridization signal for SG3 (lane d) was weak because of a smaller amount of DNA in the lane.

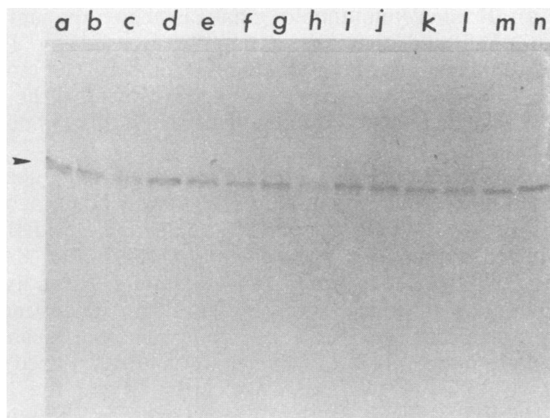

FIG. 3. Immunoblot analysis of *L. pneumophila* strains with anti-Mip monoclonal antibody 12F4. Lanes: a, SG1 strain AA100; b, SG2; c, SG3; d, SG4; e, SG5; f, SG6; g, SG7; h, SG8; i, SG9; j, SG10; k, SG11; l, SG12; m, SG13; n, SG14. Though not included in this immunoblot, SG1 strain Philadelphia 1 also produced a 24-kDa Mip protein. The arrow indicates the 24-kDa Mip protein of *L. pneumophila* strains.

DNA from the SG5 strain hybridized relatively weakly to both the 5′ and 3′ *mip* probes (Fig. 1 and 2, lane f). Nevertheless, the data indicate that all serogroups of *L. pneumophila* contain a *mip* gene that is very closely related to the *mip* gene of SG1 strain AA100.

To determine whether or not these strains expressed a Mip protein, immunoblots were performed with anti-Mip antibodies. All serogroups expressed a 24-kDa protein that reacted with the anti-Mip monoclonal antibody 12F4 (Fig. 3) and polyclonal, monospecific anti-Mip serum (data not shown). Incidently, a derivative of strain AA100 that had been rendered avirulent by prolonged passage on artificial media still expressed a 24-kDa Mip protein (data not shown). Taken together, the Southern and immunoblot analyses confirm that the *mip* gene is conserved and expressed in all 14 serogroups of *L. pneumophila*.

Detection of *mip*-like genes in other species of *Legionella*. To determine whether or not the *mip* gene was present in other species of *Legionella*, genomic DNA was isolated from 27 of the 29 other species of *Legionella* and hybridized with the *mip* probes (Table 2). There was no hybridization to the *mip* probes under high-stringency conditions, which permitted only 10 to 20% base-pair mismatching (data not shown). These data confirm that *mip* is, formally speaking, specific to the *L. pneumophila* species. However, under reduced-stringency conditions, DNAs from all species hybridized to the 5′ *mip* probe (Fig. 4 and 5). Strains examined included both those that have been associated with human disease (Fig. 4) and those that have not (Fig. 5). Generally, a single *Eco*RI fragment hybridized with the probe; however, the size and intensity of that fragment was variable. In contrast to the results obtained with the 5′ probe, the 3′ *mip* probe hybridized very weakly to these same DNA samples (data not shown). These data suggest that all *Legionella* strains have DNA sequences that are homologous to *mip*; these sequences will be referred to as *mip*-like genes.

Expression of Mip-related proteins in *Legionella* species. To determine whether or not the presence of *mip*-like genes in strains of *Legionella* is associated with the expression of Mip-related proteins, immunoblot analysis was performed. Of the 29 species examined, 28 expressed a protein that reacted with the polyclonal anti-Mip serum (Table 2 and Fig. 6). These Mip-related proteins varied in size from approximately 24 to 31 kDa, and 11 of them also reacted with the anti-Mip monoclonal antibody 12F4 (Table 2). *L. rubrilucens* was unique in that it appeared to express two Mip-related proteins (Fig. 6, lane n). Although it contained *mip*-related DNA sequences, *L. brunensis* did not appear to express a Mip-related protein (Fig. 6, lane s). These data indicate that

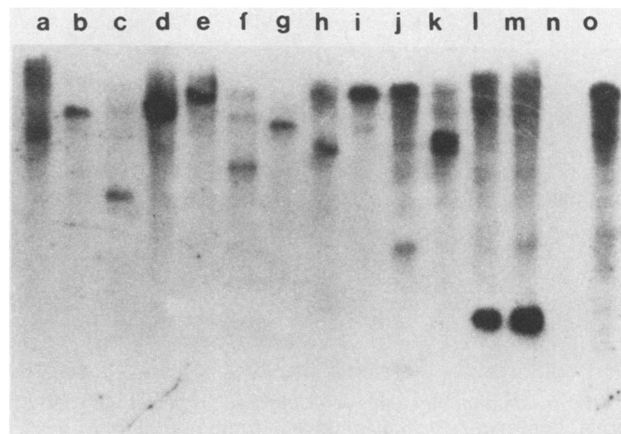

FIG. 4. Hybridization of DNAs from other *Legionella* species associated with human disease with the 5′ *mip* DNA probe. Purified bacterial DNA digested with *Eco*RI was processed as described in the legend to Fig. 1. In ethidium bromide-stained gels, each species had a unique *Eco*RI digestion pattern. Hybridization was performed under reduced-stringency conditions. 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* SG1; m, *L. feeleii* SG2; n, alkaline lysis plasmid preparation from *L. feeleii*; o, *L. oakridgensis*. Since not all DNA samples were digested completely by the restriction enzyme, faint bands or smearing of the signal was observed in some of the lanes.

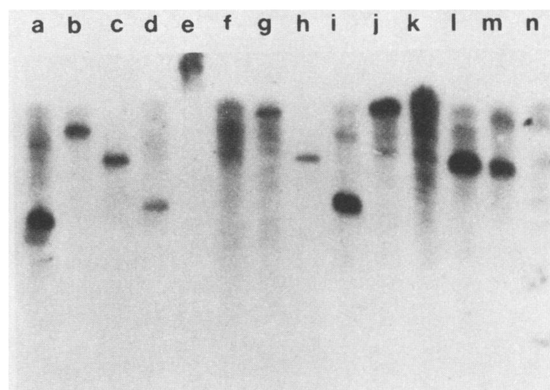

FIG. 5. Hybridization of DNAs from *Legionella* species not yet associated with human disease with the 5′ *mip* probe. Purified bacterial DNA digested with *Eco*RI was processed as described in the legend to Fig. 1. In ethidium bromide-stained gels, each species had a unique *Eco*RI digestion pattern. Hybridization was performed under reduced-stringency conditions. Lanes: a, *L. cherrii*; b, *L. sainthelensi*; c, *L. anisa*; d, *L. jamestowniensis*; e, *L. rubrilucens*; f, *L. erytha*; g, *L. spiritensis*; h, *L. parisiensis*; i, *L. steigerwaltii*; j, *L. santicrusis*; k, *L. israelensis*; l, *L. moravica*; m, *L. brunensis*; n, *L. quinlivanii*. Since not all DNA samples were digested completely by the restriction enzyme, faint bands or smearing of the signal was observed in some of the lanes. *Eco*RI did not appear to digest DNA isolated from *L. rubrilucens* (lane e).

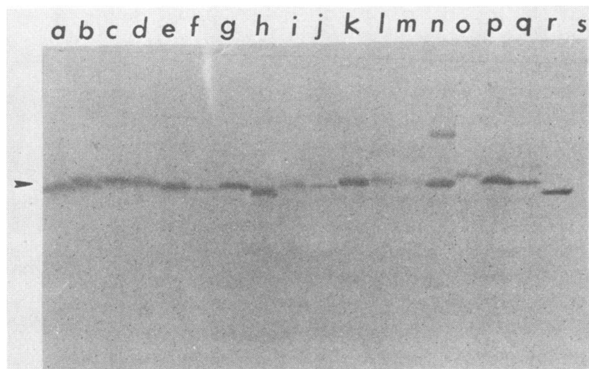

FIG. 6. Immunoblot analysis of *Legionella* species with anti-Mip serum. Lanes: a, *L. pneumophila* SG1 strain AA100; b, *L. spiritensis*; c, *L. dumoffii*; d, *L. feeleii* SG1; e, *L. birminghamensis*; f, *L. oakridgensis*; g, *L. wadsworthii*; h, *L. parisiensis*; i, *L. israelensis*; j, *L. quinlivanii*; k, *L. erythra*; l, *L. micdadei*; m, *L. maceachernii*; n, *L. rubrilucens*; o, *L. hackeliae*; p, *L. jordanis*; q, *L. jamestowniensis*; r, *L. tucsonensis*; s, *L. brunensis*. The arrow indicates the 24-kDa Mip protein of *L. pneumophila* (lane a). Additional lysates of *L. brunensis* were prepared and assayed for Miplike proteins on several different occasions.

Mip-related proteins are present throughout the genus *Legionella*.

Cloning of *mip*-like gene of *L. micdadei*. To confirm that the Mip-related proteins are encoded by the *mip*-like genes, we sought to clone a *mip*-like gene from another *Legionella* species. *L. micdadei*, which produces a 30-kDa Mip-related protein, was chosen for this analysis (Fig. 6, lane 1, and Fig. 7B, lane c). A recombinant plasmid, pBA6004, containing 1.5 kb of *L. micdadei* DNA, that expressed a 30-kDa protein antigen in *E. coli* JM109 had been identified (Fig. 7A, lane a). Further immunoblot analysis using the anti-Mip serum confirmed that the antigen expressed by JM109 (pBA6004) was related to Mip (Fig. 7B, lane a). The mobility of the *L. micdadei* protein was, like that of Mip, the same under reducing and nonreducing conditions (data not shown).

To determine whether or not the cloned sequence that encoded the 30-kDa Mip-related protein was homologous to *mip*, pBA6004 was digested with *Sst*I and *Hin*dIII and hybridized with *mip*, i.e., probe pSMJ31.42 (Fig. 8A). A 1.0-kb *Sst*I-*Hin*dIII fragment of *L. micdadei* DNA hybridized with *mip* at reduced stringency (lane c), suggesting that it contained the *mip*-like gene (compare lanes a and c). However, since only the 5′ *mip* probe had hybridized to *L. micdadei* DNA (Fig. 4), it is also possible that the 1.0-kb *Sst*I-*Hin*dIII fragment contains the 5′ portion of the *mip*-like gene and that the 3′ portion is on the adjacent 0.5-kb *Hin*dIII-*Hin*dIII fragment.

To confirm that pBA6004 contains *mip*-related sequences, the cloned *L. pneumophila mip* gene was digested with *Hae*III and hybridized with pBA6004 (Fig. 8B). The 0.84-kb *Hae*III fragments of pSMJ31.42 containing *mip* hybridized with the *L. micdadei* probe under low (lane b) but not high (lane a) stringency. When pSMJ31.42 was doubly digested with *Hae*III and *Eco*RI to cleave *mip* into its 5′ and 3′ halves, only the 0.45-kb *Hae*III-*Eco*RI fragment containing the 5′ portion of *mip* hybridized to the *L. micdadei* probe (data not shown). Taken together, the immunoblot and hybridization analyses of pBA6004 confirm that, at least in one *Legionella* species, the Mip-related proteins are encoded by the *mip*-like genes.

DISCUSSION

The conservation of the *mip* gene and Mip protein within a number of pathogenic *L. pneumophila* strains supports our

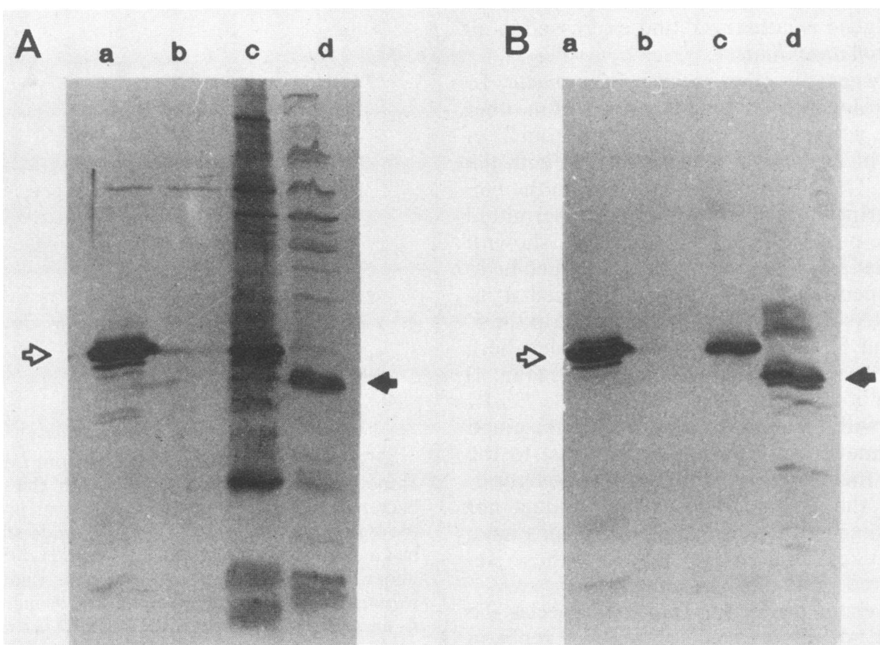

FIG. 7. Immunoblot analysis of the cloned *L. micdadei mip*-like gene. (A) Immunoblot with anti-*L. micdadei* serum. (B) Immunoblot with polyclonal anti-Mip serum. Lanes: a, *E. coli* JM109(pBA6004); b, *E. coli* JM109(pBGS18+); c, *L. micdadei*; d, *L. pneumophila* SG1. The closed arrow indicates the 24-kDa Mip protein of *L. pneumophila* (lane d). The open arrow indicates the 30-kDa antigen of *L. micdadei* (lanes a and c). Note that the 24-kDa Mip protein of *L. pneumophila* is recognized by anti-*L. micdadei* serum (A, lane d).

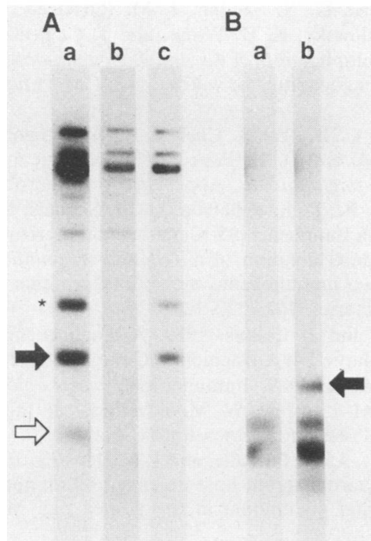

FIG. 8. Hybridization analysis of the cloned *L. micdadei mip*-like gene. (A) Identical samples of pBA6004 DNA digested with *Sst*I and *Hin*dIII. The probes and hybridization conditions used were pBA6004 at high stringency (lane a), pSMJ31.42 at high stringency (lane b), and pSMJ31.42 at reduced stringency (lane c). When digested with *Sst*I and *Hin*dIII, the 1.5 kb of *L. micdadei* DNA cloned into pBA6004 was cut into a 1.0-kb *Sst*I-*Hin*dIII fragment (closed arrow) and its adjacent 0.5-kb *Hin*dIII-*Hin*dIII fragment (open arrow) (see lane a). In some instances, the internal *Hin*dIII site was not cleaved, and then a 1.5-kb *Sst*I-*Hin*dIII partial-digest fragment containing the entire segment of cloned DNA (*) was produced. The other, higher-molecular-weight bands in lane a are fragments containing vector sequences. Since there is homology between the vectors used to construct pSMJ31.42 and pBA6004, the vector fragments also hybridized to the pSMJ31.42 probe in lanes b and c. (B) Identical samples of pSMJ31.42 digested with *Hae*III and hybridized with pBA6004 at high stringency (lane a) or reduced stringency (lane b). The entire *mip* gene except for its final 80 base pairs was contained on a 0.84-kb *Hae*III fragment (arrow) (14). The remaining, smaller hybridizing bands represent fragments of vector DNA.

earlier conclusion that *mip* is an important component of *L. pneumophila* pathogenesis. The absence of high-stringency hybridization of the *mip* probes to DNAs from the other *Legionella* species confirms that *mip* is specific to *L. pneumophila*. However, by performing reduced-stringency hybridizations, we have identified an analog of *mip* in all species of *Legionella*. Until we better understand the function of these DNA sequences and their structural relationship to *mip*, we have chosen to refer to them as *mip*-like genes.

In the vast majority of *Legionella* species, there was a correlation between hybridization to *mip* probes and cross-reaction with anti-Mip antibodies, suggesting that the Mip-related proteins are encoded by the *mip*-like genes. We confirmed this by cloning the *mip*-like gene of *L. micdadei*. Two strains representing the species *L. gratiana* and *L. tucsonensis* were not tested for homology to *mip* but undoubtedly have *mip*-like genes since they contained Mip-like proteins. Generally, the presence of a *mip*-like gene was associated with the presence of a single Mip-like protein that was 24 to 31 kDa in size. *L. rubrilucens* was unique in that it expressed two Mip-like proteins, suggesting that it may contain two *mip*-like genes. We could not confirm this, since the DNA isolated from that strain was not digestible with the restriction enzyme used in the analysis. *L. brunensis* was also unique in that it did not express a Mip-related protein and may contain a mutation in its *mip*-like gene.

Hybridization between *mip* and its analogs was most readily detected when probes derived from the 5′ portion of the genes were used, suggesting that the DNA sequences in the 5′ portion of *mip* have been conserved to a greater extent than those in the 3′ portion. The variability in the size and antibody reactivity of the Mip-related proteins also indicates that significant differences exist in the structure of the *mip* gene family.

The *mip* gene family appears to be limited to the genus *Legionella*; i.e., probes now known to contain *mip* do not hybridize under any conditions to DNAs from strains of *E. coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Streptococcus pneumoniae* (13). Moreover, we did not detect any Mip-related proteins in strains of *Acinetobacter*, *Escherichia*, *Flavobacterium*, *Haemophilus*, *Mycoplasma*, *Pasteurella*, or *Pseudomonas* (16). Therefore, *mip* (and Mip) could serve as a probe for detecting legionellae in clinical and environmental samples. Moreover, depending on the hybridization conditions employed or the segment of *mip* included, a *mip* probe could be used as either a *L. pneumophila* species-specific probe or a *Legionella* genus-specific probe. Recently, the *mip* gene has been exploited by another group to develop a polymerase chain reaction kit specific for *L. pneumophila* (R. Miller, personal communication). Finally, *mip* could also be employed as a tool for studying the taxonomy of the legionellae.

Our data indicate that Mip has a role in the resistance of *L. pneumophila* SG1 strain AA100 to intracellular killing in the macrophage (9a, 10). We strongly suspect that Mip serves a similar function in the intracellular life cycle of other strains of *L. pneumophila*. It is tempting to speculate that the Mip-like proteins also have a role in macrophage infection by the other *Legionella* species and that some aspects of the intracellular life cycle are shared among species. However, confirmation of these hypotheses will require the construction of more *Legionella* mutants.

It is not clear why different strains of *Legionella* (serogroups of *L. pneumophila* and other species) cause different incidences of human disease. It has been postulated that only *L. pneumophila* and to a lesser extent the other pathogenic legionellae possess the virulence factors necessary to cause disease. Alternatively, it is has been proposed that all species are potentially pathogenic but that environmental or ecological parameters such as species distribution in natural waters or ability of the species to survive in water systems and aerosols dictate their attack rates; e.g., *Legionella* strains can differ in terms of both their tolerance to salt (4) and desiccation (12) and their protozoal host ranges (30). The data reported here suggest that the differences in pathogenicity among strains of legionellae cannot be explained by differences in expression of a Mip-like protein per se, though the structural differences between the Mip-related proteins could reflect differences in the function of these proteins.

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