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Distribution of *mip*-related sequences in 39 species (48 serogroups) of *Legionellaceae*

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SUMMARY

The macrophage infectivity potentiator gene (*mip*) from *Legionella pneumophila* is a major virulence factor of the species. Thus, *mip*-detection by amplification has been proposed to assess the presence of *L. pneumophila* in clinical and environmental samples. The distribution of *mip*-related sequences within the *Legionellaceae* was studied by DNA amplification using *mip*-specific primers followed by Southern blot hybridization with an internal probe. Thirty-nine species (48 serogroups) of *Legionellaceae* were screened in this attempt. Using this approach, sequences related to *mip* were observed in 89% of the tested species including the most recently described *L. fairfieldensis*, *L. lansingensis* and *L. shakespearei*. In several cases, cloning and sequencing of the amplified products confirmed the high levels of similarity between the sequence found in non-*pneumophila* species with that of the *L. pneumophila mip* gene. This confirms previous reports that *mip* related genes are widespread among *Legionellaceae* and therefore specific detection of the species *L. pneumophila* cannot be based on *mip*-targeted amplification.

INTRODUCTION

Among the 39 species of the family *Legionellaceae*, *L. pneumophila* accounts for most of the cases of Legionnaire's disease, whilst the other species of *Legionella* are detected almost exclusively in the environment. The superior potential of *L. pneumophila* to cause disease has been attributed in part to the presence of a macrophage infectivity potentiator (MIP) in this species [1, 2]. The MIP protein is a 24 kDa surface protein, which is required for optimal intracellular infection of both protozoa (the reservoir for legionella in the environment [3–6]) and human macrophages (the primary host cells for multiplication of legionella in humans [2, 7, 8]). The *mip* gene which encodes the MIP protein is present in the 14 serogroups of *L. pneumophila* and related sequences to *mip* have also been detected by Southern blot and immunoblot analysis in several other *Legionella* species [9]. The *mip*-analogue of *L. micdadei*, which has been cloned and sequenced has 71% DNA homology with that of *L. pneumophila* [10]. Despite the fact that the *mip* gene is widespread among *Legionella* species, DNA amplification using *mip*-specific primers was proposed for the specific detection of *L. pneumophila* [11] or alternatively for that of *L. pneumophila*, *L. micdadei* and *L. bozemanii* [12, 13]. The aim of the present study was to examine fully the distribution of *mip*-related sequences within the *Legionellaceae*, in order to (i) evaluate the specificity of this approach in the recognition of *L. pneumophila*

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and (ii) confirm whether the *mip* gene can be considered as a general trait of both pathogenic and non-(yet)-pathogenic *Legionella* species.

MATERIALS AND METHODS

Bacterial strains

Strains representing the 39 species and 48 serogroups of *Legionella* are indicated in Table 1. The bacteria were cultivated on BCYE-α agar plates or on GVPC agar plates when required [14]. Non-*Legionella* species served as negative controls for the *mip* gene. They were *Pseudomonas aeruginosa* ATCC 27853, *Haemophilus influenzae* ATCC 33291, *Escherichia coli* ATCC 25922, *Aeromonas hydrophila* ATCC 7966, *Bacteroides fragilis* ATCC 23745, *Proteus mirabilis* ATCC 29906, *Proteus vulgaris* ATCC 13315, *Staphylococcus aureus* CCM885, *Acinetobacter baumanii* ATCC 17959, *Stenotrophomonas maltophilia* ATCC 13637 and *Flavobacterium aquatile* ATCC 11947.

DNA extraction

Legionella cells were incubated in 500 μl of lysis buffer (50 mM KCl, 10 mM Tris-HCl, 2·5 mM $MgCl_2$, 0·45% (vol/vol) Nonidet NP40, 0·45% (vol/vol) Tween 20 (pH 8)) containing 100 μg/ml of proteinase K (Sigma), for 2 h at 55 °C followed by 10 min at 95 °C. Nucleic acids were purified by the addition of phenol-chloroform iso-amyl alcohol and precipitated by the addition of ethanol. The pellet was then resuspended in 200 μl sterile distilled water and stored at −20 °C until used. Non-*Legionella* species were processed following a thermal lysis procedure to release the bacterial DNA [12]. The bacteria were diluted in 500 μl of sterile distilled water in a 1·5 ml microcentrifuge tube, which was heated at 100 °C for 5 min and then chilled on ice for 5 min. This procedure was repeated twice. After 5 min of centrifugation at 5000 g and 4 °C, the supernatant was stored at −20 °C until needed.

Synthetic oligonucleotides and DNA amplification

The *mip*-specific primers were Lpm-1 and Lpm-2 which were designed in a previous study [15] to allow the amplification of an internal 630 bp DNA fragment in the *mip* gene. These primers were shown to give an amplification product from *L. pneumophila* serogroups 1–14, *L. micdadei* and *L. bozemanii* serogroup 1 [12]. A third oligonucleotide, termed Lpm-3, internal to the amplified product initiated by Lpm-1 and Lpm-2, was designed previously [12] and used as a detection probe in a Southern blot experiment. For DNA amplification, 10 μl of each sample were subjected to 45 cycles of amplification in a 100 μl volume containing 2·5 U of *Taq* polymerase (Perkin Elmer), 1 mM of each primer, 0·2 mM of each of the four dNTP, 10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$ and 0·1 mg/ml of gelatin. Reaction mixtures were overlaid with 50 μl of mineral oil and after an initial denaturation step at 92 °C for 20 min, each amplification cycle was as follows: 2 min (denaturation) at 92 °C, 2 min (annealing) at 62 °C, and 2 min (extension) at 74 °C. Controls were included in each set of amplifications, namely, a reaction mixture with no DNA added and a sample containing DNA from *L. pneumophila* serogroup 1 ATCC 33152 as a positive control.

Detection of the amplification products

Following amplification, 10 μl of each sample was electrophoresed through 1·5% agarose gel in 0·5 × TBE buffer as described [12]. The gels were then stained with ethidium bromide and photographed under UV light. The DNA was vacuum transferred to nylon membranes (Boehringer Mannheim) and cross-linked to the membrane by heating for 60 min at 80 °C. The oligonucleotide Lpm-3 was digoxigenin end-labelled following the manufacturer's recommendations (Boehringer Mannheim). Hybridization and washing were performed under stringent conditions using the reagents and conditions provided by the manufacturer (Boehringer Mannheim). The chemoluminescence used for detection was generated by hydrolysis of CSPD® substrate (Tropix Inc.) followed by exposure of the membranes to Hyperfilm ECL (Amersham).

Cloning and sequencing of *mip* analogues

After agarose gel electrophoresis of the amplified products, specific DNA bands were excised from the gel, purified with the Geneclean Kit II (BIO 101 Inc.) and cloned in *E. coli* using the TA Cloning® vector, following the instructions of the manufacturer (Invitrogen). DNA sequencing of the recombinant plasmid with Sequenase version 2.0 (Amersham) was

Table 1. *Detection of* mip*-related DNA sequences in* Legionellaceae *by PCR and Southern blot hybridization*

Legionella species	Serogroup	Strain	Source (ATCC)	DNA amplification	Hybridization
L. pneumophila	1	Philadelphia 1	33152	+	+
L. pneumophilia	1	Wadsworth (130b)	CNRL*	+	+
L. pneumophila	'15'	Lansing 3	35251	+	+
L. adelaidensis		1762-AUS-E	49625	−	−
L. anisa		WA-316-C3	35292	+	+
L. birminghamensis		1407-AL-H	43702	+	+
L. bozemanii	1	WIGA	33217	+	+
L. bozemanii	2	Toronto 3	35545	+	+
L. brunensis		441-1	43878	+	+
L. cherrii		ORW	35252	+	+
L. cincinnatiensis		72-OH-H	43753	+	+
L. dumoffii		NY-23	33279	+	+
L. erythra	1	SE-32A-C8	35303	+	+
L. erythra	2	LC217	NCTC11977	+/−	+
L. fairfieldensis		1725-AUS-E	49588	+	+
L. feeleii	1	WO-44C	35072	+	+
L. feeleii	2	691-WI-H	35849	+	+
L. geestiana		1308	49504	+/−	+w
L. gormanii		LS-13	33297	+	+
L. gratiana		LYON 8420412	49413	+w	+
L. hackeliae	1	Lansing 2	35250	+w	+
L. hackeliae	2	798-PA-H	35999	−	+/−
L. israelensis		Bercovier-4	43119	−	−
L. jamestowniensis		JA-26-G1-E2	35298	+	+
L. jordanis		BL-540	33623	+	+
L. lansingensis		1677-MI-H	49751	+/−	+
L. londiniensis	1	1477	49505	+/−	+w
L. londiniensis	2	Mulhouse B26	CNRL*	+w	+w
L. longbeachae	1	Long Beach 4	33462	+	+
L. longbeachae	2	Tucker 1	33484	+/−	+
L. maceachernii		PX-1-G2-E2	35300	+	+w
L. micdadei		Tatlock	33218	+	−
L. moravica		316-36	43877	+	+
L. nautarum		1224	49506	+	+
L. oakridgensis		OR-10	33761	+	+
L. parisiensis		PF-209C-C2	35299	+/−	−
L. quateirensis		1335	49507	−	+
L. quinlivanii	1	43830	43830	+	+w
L. quinlivanii	2	LC870	NCTC12434	+	+w
L. rubrilucens		WA-270A-C2	35304	+/−	−
L. sainthelensi	1	Mt St Helens-4	35248	+	+w
L. sainthelensi	2	1489-CA-H	49322	+/−	−
L. santicrucis		SC-63-C7	35301	+	+
L. shakespearei		214	49655	+w	+w
L. spiritensis		Mt St Helens-9	35249	−	−
L. steigerwaltii		SC-18-C9	35302	+	+w
L. tucsonensis		1087-AZ-H	49180	+	+w
L. wadsworthii		81-716A	33877	+	+
L. worsleiensis		1347	49508	+	+

+ for positive signal; +w for weak signal; − for absence of signal; +/− for a very weak signal.
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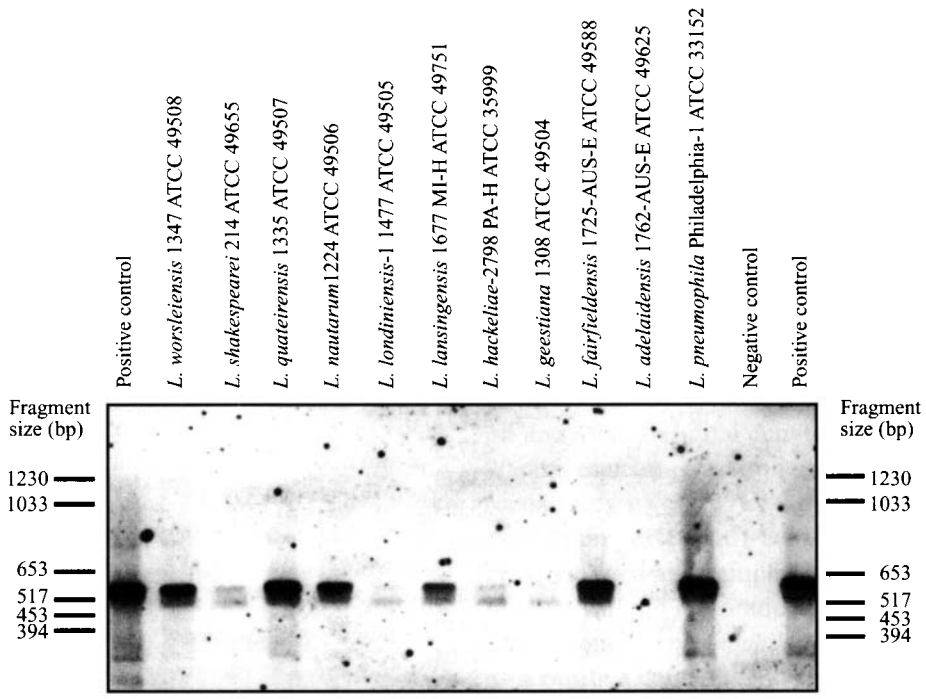

Fig. 1. Southern blot analysis using a *mip*-specific probe. PCR products using Lpm-1 and Lpm-2 primers were run on agarose gel, transferred to nylon membrane and hybridized to a probe (Lpm-3) internal to the *mip* gene. Positive control, purified DNA from *L. pneumophila* Philadelphia-1 ATCC 33152; negative control, water; the fragment size (DNA weight molecular marker 6, Boehringer Mannheim) is indicated in bp.

by the dideoxynucleotide method of Sanger and colleagues [16] on double stranded DNA templates using SP6 and T7 promoter oligonucleotides as primers and subsequently using various internal oligonucleotide primers. The DNA sequences corresponding to the *mip* amplified fragments of *L. fairfieldensis* and *L. worsleiensis* have been submitted to GenBank, under accession number U60163 and U60164, respectively.

RESULTS

PCR using *mip*-specific primers

The primers designed by Engleberg and colleagues [15] shown to amplify DNA from *L. pneumophila*, *L. micdadei* and *L. bozemanii* [12], were re-evaluated to study the distribution of *mip*-analogues within the whole family of *Legionellaceae*. The amplification protocol was slightly modified in comparison with that used by Jaulhac and colleagues [12], i.e. 45 *vs.* 40 cycles, in order to increase the sensitivity of the method. Using these parameters, the specificity did not appear to be altered since none of the non-*Legionella* species produced an amplification signal (data not shown). Results concerning the 48 strains that represented the 39 species and 48 serogroups of *Legionellaceae* are presented in Table 1. An amplification product was detected on ethidium bromide-stained agarose gel electrophoresis for most of the *Legionella* strains studied (42 out of 48 strains). Only six species, *L. adelaidensis*, *L. hackeliae* serogroup 2, *L. israelensis*, *L. quateirensis*, *L. parisiensis* and *L. spiritensis*, did not present an amplification product. In almost every case, the positive and negative results of ethidium bromide-stained agarose gel were confirmed by Southern blot hybridization using the internal probe of the *mip* gene (Fig. 1). However, three species, *L. micdadei*, *L. rubrilucens* and *L. sainthelensi* serogroup 2 produced a detectable amplification product that did not hybridize with the probe. Among the six species which did not present a DNA band on agarose gel (see above), only *L. quateirensis* gave a hybridization signal. The recently described species which, to our knowledge, had never been tested previously for the presence of *mip* analogues such as *L. fairfieldensis*, *L. lansingensis* and *L. shakespearei* showed an amplification signal.

Sequencing of amplicons

In order to gain some knowledge on the degree of sequence divergence between *mip* analogues among

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the *Legionella* species, the amplification product from several of the tested species was cloned and sequenced. Based on the strong PCR signals with *L. fairfieldensis* and *L. worsleiensis* those PCR products were chosen to be cloned and sequenced. Hence, the 630 bp-sequence of the PCR product from *L. pneumophila* 1 (strain Wadsworth), *L. fairfieldensis* (reference strain) and *L. worsleiensis* (reference strain) were compared with each other and with the published sequence of the *mip* gene from *L. pneumophila* 1 [15] (strain Philadelphia 1 ATCC 33152 and strain Wadsworth) and *L. micdadei*. The results given by sequencing the amplicon from *L. pneumophila* (strain Wadsworth) [17] were identical to that reported for the *mip* gene of this strain, confirming the validity of the method. The sequences of the amplicons from *L. fairfieldensis* and *L. worsleiensis* showed 99·3% and 98·6% homology respectively with that of the *L. pneumophila* serogroup 1 (strain Philadelphia 1 ATCC 33152) *mip* gene.

DISCUSSION

Our results confirm the previously expected prevalence of *mip*-sequences through the genus *Legionella* [18]. With a single pair of oligonucleotides directed to the *L. pneumophila mip* sequence, we were able to amplify DNA from 87% (34) of the tested species (39), reflecting the high degree of relatedness between the different *mip* analogues, at least in the region targeted by the oligonucleotide primers. In three cases, an amplification product was detectable on ethidium bromide-stained agarose gel but did not hybridize to the probe. This may reflect sequence divergence in the region corresponding to the probe, a possibility that is confirmed in the case of *L. micdadei* in which the published sequence of its *mip* analogue [10] showed six mismatches within the 25-nucleotide sequence of our detection probe. The possibility that our findings were the result of non-specific amplification can be ruled out with several arguments: (i) absence of amplification of DNA from non-*Legionella* species, (ii) positive Southern blot hybridization of the amplicons with a *mip*-internal probe in 89% of cases, and (iii), sequencing three amplicons (including that of *L. pneumophila* reference strain) that showed a high degree of homology (above 98%) of the sequences between the different species. Using their protocol, Jaulhac and colleagues were able to amplify DNA only from *L. pneumophila*, *L. bozemanii* serogroup 1 and *L. micdadei* [12]. The difference between our results and those published by Jaulhac and colleagues [12] using the same primers may be explained by several modifications of the protocol: an extended number of cycles (45 *vs.* 40), a different brand of *Taq* polymerase (Perkin-Elmer *vs.* Beckman), and a different thermalcycler (PHC-3 Techne *vs.* PHC-2 Techne). Finally, under the conditions used in the present study, the *mip*-targeted amplification appears to be a genus-specific method for the detection of legionella rather than a species-specific assay for a limited number of species [13]. However, it remains to be determined whether the *mip* analogues detected in the various species serve a similar function in the intracellular life cycle to the *L. pneumophila* MIP protein.

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REFERENCES

1. Cianciotto NP, Eisenstein BI, Mody CH, Toews GB, Engleberg NC. A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. Infect Immun 1989; **57**: 1255–62.
2. Cianciotto NP, Eisenstein BI, Mody CH, Engleberg NC. A mutation in the *mip* gene results in an attenuation of *Legionella pneumophila* virulence. J Infect Dis 1990; **162**: 121–6.
3. Rowbotham TJ. Preliminary report on the pathogenicity of *Legionella pneumophila* for fresh water and soil amoebae. J Clin Pathol 1980; **33**: 1179–83.
4. Vandenesch F, Surgot M, Bornstein N, et al. Relationship between free *amoeba* and *Legionella*: studies *in vitro* and *in vivo*. Zbl Bakt 1990; **272**: 265–75.
5. Fields BS, Barbaree JM, Shotts EB, et al. Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. Infect Immun 1986; **53**: 553–9.
6. Fields BS. *Legionella* and Protozoa: interaction of a pathogen and its natural host. In: Barbaree JM, Breiman RF, Dufour AP, eds. *Legionella*: Current status and emerging perspectives. Washington D.C.: American Society for Microbiology, 1993: 129–36.
7. Fields BS, Barbaree JM, Sanden GN, Morrill WE. Virulence of a *Legionella anisa* strain associated with Pontiac fever: an evaluation using protozoan, cell culture, and guinea pig models. Infect Immun 1990; **58**: 3139–42.
8. Cianciotto NP, Stamos JK, Kamp DW. Infectivity of *Legionella pneumophila mip* mutant for alveolar epithelial cells. Curr Microbiol 1995; **30**: 247–50.

9. Cianciotto NP, Bangsborg JM, Eisenstein BI, Engleberg NC. Identification of *mip*-like genes in the genus *Legionella*. Infect Immun 1990; **58**: 2912–8.
10. Bangsborg JM, Cianciotto NP, Hindersson P. Nucleotide sequence analysis of the *Legionella micdadei mip* gene encoding a 30-kilodalton analog of the *Legionella pneumophila* Mip protein. Infect Immun 1991; **59**: 3836–40.
11. Jaulhac B, Harf C, Nowicki M, Monteil H. Detection of *Legionella* spp. in environmental water samples and free-living amoeba by using DNA amplification. In: Barbaree JM, Breiman RF, Dufour AP, eds. *Legionella*: Current status and emerging perspectives. Washington D.C.: American Society for Microbiology, 1993: 151–3.
12. Jaulhac B, Nowicki M, Bornstein N, et al. Detection of *Legionella* spp in bronchoalveolar lavage fluids by DNA amplification. J Clin Microbiol 1992; **30**: 920–4.
13. Nowicki M, Bornstein N, Jaulhac B, Piemont Y, Monteil H, Fleurette J. Rapid detection of *Legionellae* in clinical and environmental samples by polymerase chain reaction. In: Barbaree JM, Breiman RF, Dufour AP, eds. *Legionella*: Current status and emerging perspectives. Washington D.C.: American Society for Microbiology, 1993: 178–81.
14. Edelstein PH, Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. J Clin Microbiol 1981; **14**: 294–303.
15. Engleberg NC, Carter C, Weber DR, Cianciotto NP, Eisenstein BI. DNA sequence of *mip*, a *Legionella pneumophila* gene associated with macrophage infectivity. Infect Immun 1989; **57**: 1263–70.
16. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; **74**: 5463–7.
17. Ludwig B, Rahfeld J, Schmidt B, et al. Characterization of Mip proteins of *Legionella pneumophila*. FEMS Microbiol Lett 1994; **118**: 23–30.
18. Cianciotto NP, O'Connell W, Dasch GA, Mallavia LP. Detection of *mip*-like sequences and Mip-related proteins within the family *Rickettsiaceae*. Curr Microbiol 1995; **30**: 149–53.