Phylogenetic relationships and species differentiation of 39 *Legionella* species by sequence determination of the RNase P RNA gene *rnpB*

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The *rnpB* gene is ubiquitous in *Bacteria*, *Archaea* and *Eucarya* and encodes the RNA component of RNase P, an endoribonuclease P that consists of one RNA and one protein subunit (C5). In this study, partial *rnpB* genes were sequenced from 39 type strains and 16 additional strains of the genus *Legionella*. Models of the putative secondary structures of the RNase P RNA in the genus *Legionella* are proposed and possible interactions between RNase P RNA and C5 are discussed. The phylogenetic relationships within the genus *Legionella* were examined and *rnpB* sequences indicated six main clades that together comprised 27 of the 39 species examined. The phylogenetic relationships were further inferred by analysing combined datasets of sequences from the *rnpB*, *mip*, 16S rRNA and *rpoB* genes. It is concluded that *rnpB* is suitable for use in phylogenetic studies of closely related species and that it exhibits the potential to discriminate between *Legionella* species.

INTRODUCTION

The genus *Legionella* comprises more than 50 species (Park *et al.*, 2004) and new species are frequently described (<http://www.dsmz.de/bactnom/bactname.htm>). Some *Legionella* species cause human disease, while others have been detected only in the environment (Fields *et al.*, 2002). The diseases caused by *Legionella* include the pneumonic form, Legionnaires' disease, and the extrapulmonary flu-like form, Pontiac fever. *Legionella pneumophila* serogroup (sg) 1 is the causative agent in up to 84 % of disease cases that are due to *Legionella* infection (Yu *et al.*, 2002). There may, however, be a bias towards detecting *L. pneumophila* sg 1,

since commonly used urinary antigen tests mainly detect this serogroup (Helbig *et al.*, 2003). Other *L. pneumophila* serogroups, as well as *Legionella longbeachae*, *Legionella micdadei*, *Legionella dumoffii* and *Legionella feeleii*, are also often associated with human disease (O'Connell *et al.*, 1996). In Australia, *L. longbeachae* is the leading cause of Legionnaires' disease (Alli *et al.*, 2003). Immuno-compromised individuals are especially susceptible to infection and may be colonized by species not normally associated with disease. Therefore, it is important for epidemiological investigations to identify species within the genus accurately.

In clinical diagnostics, *Legionella* bacteria are commonly identified by culture since they have very specific growth requirements. If bacteria grow on cysteine-enriched buffered charcoal-yeast extract (BCYE) agar, but not on BCYE agar without cysteine, the bacteria presumably belong to the genus *Legionella* (Murray *et al.*, 2002). To discriminate between species, phenotypic tests such as growth characteristics, auto-fluorescence and serological methods targeting membrane proteins are often used. These methods provide low resolution and antigen cross-reactivity limits the specificity of antibody tests (Verissimo *et al.*, 1996).

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Abbreviations: AFLP, amplified fragment length polymorphism; NAIM, nucleotide analogue modification interference; NJ, neighbour-joining; MLST, multilocus sequence typing; MP, maximum parsimony; sg, serogroup; SW, Shannon–Wiener.

The GenBank/EMBL/DDBJ accession numbers for the *rnpB* sequences of 55 *Legionella* strains are AJ781429–AJ781483.

Tables showing the alignment of *Legionella rnpB* gene sequences included in this study are available as supplementary material in IJSEM Online.

Several DNA-based classification systems have been described for *Legionella*, some of which target the sequence variation of specific genes such as *mip* (Ratcliff *et al.*, 1998), 16S rRNA (Fry *et al.*, 1991), *rpoB* (Ko *et al.*, 2002) and *gyrA* (Feddersen *et al.*, 2000). Analysis of transfer RNA intergenic spacer length polymorphism has been shown to be highly discriminatory in the identification of *Legionella* species (De Gheldre *et al.*, 2001). Amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) are very discriminatory techniques for subtyping within the species *L. pneumophila* (Gaia *et al.*, 2003), where they also distinguish within serogroups (Jonas *et al.*, 2000; Valsangiacomo *et al.*, 1995). Thus, DNA-based analyses have greatly improved *Legionella* phylogenetics and the

ability to discriminate between bacteria within the *Legionella* genus.

The *rnpB* gene encodes the catalytic RNA moiety of endoribonuclease P (RNase P), the enzyme that removes the 5'-leader of precursor tRNAs. In *Bacteria* and some *Archaea*, the RNA component alone exhibits catalytic activity *in vitro*; however, the protein subunit C5 is essential for activity *in vivo* and is encoded by the *rnpA* gene (Altman & Kirsebom, 1999; Pannucci *et al.*, 1999). Based on the secondary structure of RNase P RNA, two types of bacterial RNase P RNA have been identified, type A and type B. RNase P RNA derived from *Legionella* belongs to type A, which is suggested to be the ancestral form of RNase P RNA found in

Fig. 1. Minimum consensus secondary structure of bacterial type A RNase P RNA and deduced secondary structures of RNase P RNA from *Legionella pneumophila* ATCC 33152^T sg 1, *Legionella moravica* ATCC 42877^T and *Legionella israelensis* ATCC 43119^T. Lower case letters indicate nucleotides in primer sequences and nucleotides denoted N are tentative nucleotides flanking the primers. Boxed nucleotides show positions proposed to interact with the C5 protein.

bacteria. Type B RNase P RNA may have emerged later within the low G + C Gram-positive lineage (Haas & Brown, 1998).

In bacteria, *rnpB* comprises about 400 nt (Pace & Brown, 1995) and several conserved regions essential for the functioning of RNase P RNA have been identified. Certain regions with high variability have also been identified, for example, P3, P12 and P17 (see Fig. 1). The sequence variation of *rnpB* has been used previously to differentiate closely related species of *Chlamydiaceae* (Herrmann *et al.*, 2000) and *Streptococcus* (Tapp *et al.*, 2003).

In this report, we investigate the phylogeny within the genus *Legionella*, discuss the interaction between RNase P RNA and the C5 protein and evaluate *rnpB* as a tool for discriminating between *Legionella* species.

METHODS

Bacterial strains. The 55 *Legionella* strains (39 type strains and 16 additional reference strains) included in this study are listed in Table 1. Furthermore, 12 clinical and three environmental isolates (1777/97, 1780/97 and 1829/00) are detailed (see Fig. 5).

DNA preparation. DNA for PCR amplification was extracted either by using the Qiagen DNA mini kit or by using a phenol/chloroform-based method.

PCR amplification. The *rnpB* gene was amplified using the primer pair LP3 [5'-CA(INOSINE)AGTYGGTCAGGCAAT-3'] and BM1-2 (5'-TGTAACGACGCGCCAGTAAAGCCGGTTCTGT-3'). The reaction mixture consisted of 0.8 µM of each primer, 200 µM dNTPs, 2 mM MgCl₂, 2 U HotStar *Taq* DNA polymerase (Qiagen) and 20–100 ng template DNA, as measured from semiquantitative measurement on ethidium bromide-stained agarose gels. The reaction mixture was subjected to enzyme activation for 15 min at 95 °C followed by 37 cycles of amplification. Each amplification cycle consisted of denaturation for 30 s at 95 °C, primer annealing for 40 s at 50 °C and elongation for 40 s at 72 °C. A final step of 7 min at 72 °C was performed to ensure complete extension.

The 16S rRNA and *mip* genes of the environmental isolate 1829/00 were sequenced as described elsewhere (Johansson *et al.*, 1995; Ratcliff *et al.*, 1998).

Sequence analyses. Initially, partial *rnpB* gene sequences were PCR-amplified with primers binding to the conserved regions defined by P4. The products generated by these primers, however, did not include the hypervariable P3 loop.

To facilitate the design of the LP3 primer, the sequence between the promoter region and the P3 loop was determined for ten *Legionella* species by amplifying the 5'-flanking DNA of the P4 loop in a PCR with biotinylated primers and streptavidin-coated beads, as described elsewhere (Sorensen *et al.*, 1993). Sequencing of PCR products defined by LP3 and BM1-2 was performed on both DNA strands of all strains using polymer POP6 in an ABI 310 Genetic Analyser (Applied Biosystems). BM1-2 and LP3 were also used as primers in the sequencing PCR, in which the BigDye terminator-labelled cycle sequencing chemistry kit version 2.0 (Applied Biosystems) was used.

Phylogenetic analysis. The *rnpB* gene sequences were aligned using CLUSTAL W, but required some manual editing to align homologous sites according to the secondary structures of RNase P RNA. The 16S rRNA, *mip* and *rpoB* gene sequences were obtained from

GenBank and their accession numbers are presented in Table 1. The 16S rRNA and *mip* gene sequences varied in size, and sequences from some species were therefore trimmed at the ends to generate sequences with homologous sites in the CLUSTAL W alignments. The *rnpB* fragments included were 304–354 nt long. 16S rRNA genes were approximately 1350 nt in length. All *rpoB* fragments were 300 nt long and approximately 650 nt were included from *mip* sequences, including the hypervariable insert adjacent to the signal sequence. The combined dataset, consisting of *rnpB*, 16S rRNA, *mip* and *rpoB* gene sequences, was constructed by concatenating the alignments for the individual genes and included 39 *Legionella* species, of which 37 had sequences available for all four genes.

The alignments obtained (individual genes and concatenated alignments) were used for phylogenetic inference using a Bayesian approach as implemented in MrBayes 3.0B4 (Huelsenbeck & Ronquist, 2001). MrBayes uses Metropolis-coupled Markov chain Monte Carlo methods to calculate the posterior probabilities for the parameters of interest. Each analysis was run for 1×10^7 generations with four differently heated chains; generations before convergence (as monitored on tree likelihood and total tree length) were discarded as burn-in.

To select an adequate model for the Bayesian analysis, as well as pairwise distances, we used a hierarchical likelihood ratio test approach (Huelsenbeck & Crandall, 1997). To do this, we used PAUP* 4.0b8-10 Linux and Macintosh versions (Swofford, 2000) and the same test hierarchy (and thus model selection) as implemented in the modeltest program (Posada & Crandall, 1998) at $P < 0.01$. Neighbour-joining (NJ) trees under the Jukes-Cantor model were produced for each of the datasets/partitions separately as well as in combination and the parameters for each model were estimated using these trees. The same model was used for the pairwise distances (e.g. gamma shape, proportion invariant sites) and the parameters assigned were based on the maximum-likelihood estimate.

In addition to the Bayesian analysis, we performed bootstrap analyses as implemented in PAUP* 4.0b8-10, using maximum parsimony (MP) as optimality criterion and also using NJ. For the optimality criterion method, 1000 bootstrap replicates were performed and heuristic search algorithms were used, namely simple stepwise addition and tree bisection reconnection branch swapping.

To compare the variation in the different genes, we used the Shannon-Wiener information index, H , defined as

$$H = - \sum_{i=1}^4 p_i \log p_i$$

where p_i is the proportion of A, T, C and G (Shannon & Weaver, 1949; Wiener, 1949). The mean value for all sites in the same set of taxa was calculated for each dataset.

RESULTS AND DISCUSSION

Sequence comparison of *rnpB* in the genus *Legionella*

PCR amplicons between 304 and 354 bp (primers excluded) were obtained, constituting 89–91 % of the total *rnpB* gene sequences. Partial *rnpB* gene sequences of 39 type strains were compared and the pairwise similarities ranged between 75.1 and 100 %, with *Legionella micdadei* ATCC 33218^T and *Legionella maceachernii* ATCC 35300^T as the only species having identical *rnpB* gene sequences. These two species are difficult to distinguish from each other phenotypically and

Table 1. GenBank accession numbers for the *rnpB*, 16S rRNA, *mip* and *rpoB* gene sequences and sources of strains used

ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection University of Göteborg, Gothenburg, Sweden; SMI, Swedish Institute for Infectious Disease Control, Stockholm, Sweden.

Strain	Serogroup	GenBank accession no.				Source
		<i>rnpB</i>	16 rRNA	<i>mip</i>	<i>rpoB</i>	
<i>Coxiella burnetii</i>		AE016960	AE016960	U14170	U86688	GenBank
<i>L. adelaidensis</i> ATCC 49625 ^T =CCUG 31231 A ^T		AJ781468	Z49716	U91606	AF367721	SMI
<i>L. anisa</i> ATCC 35292 ^T =CCUG 29665 ^T		AJ781469	Z32635	U91607	AF367722	SMI
<i>L. birminghamensis</i> ATCC 43702 ^T =CCUG 31233 A ^T		AJ781467	Z49717	U91608	AF367723	SMI
<i>L. bozemaniae</i> ATCC 33217 ^T =CCUG 11880 ^T	1	AJ781464	Z49719	U91609	AF367724	SMI
<i>L. bozemaniae</i> ATCC 35545=CCUG 16416	2	AJ781465	Z49718	AF022308		SMI
<i>L. brunensis</i> ATCC 43878 ^T =CCUG 31114 ^T		AJ781463	Z32636	U92227	AF367725	SMI
<i>L. cherrii</i> ATCC 35252 ^T =CCUG 29666 ^T		AJ781461	Z49720	U91635	AF367726	SMI
<i>L. cincinnatiensis</i> ATCC 43753 ^T =CCUG 31230 A ^T		AJ781462	Z49721	U91636	AF367727	SMI
<i>L. dumoffii</i> ATCC 33279 ^T =CCUG 11881 ^T		AJ781460	Z32637	U91637	AF267728	SMI
<i>L. erythra</i> ATCC 35303 ^T =CCUG 29667 ^T		AJ781459	Z32638	U92203	AF367729	SMI
<i>L. fairfieldensis</i> ATCC 49588 ^T =CCUG 31235 A ^T		AJ781458	Z49722	U92204	AF367730	CCUG
<i>L. feeleei</i> ATCC 35072 ^T =CCUG 16417 ^T	1	AJ781457	X73395	U92205	AF367731	SMI
<i>L. geestiana</i> ATCC 49504 ^T =CCUG 44893 ^T		AJ781456	Z49723		AF367732	CCUG
<i>L. gormanii</i> ATCC 33297 ^T =CCUG 12267 ^T		AJ781455	Z32639	U91638	AF367733	SMI
<i>L. gratiana</i> ATCC 49413 ^T =CCUG 44894 ^T		AJ781454	Z49725	U92206	AF367734	CCUG
<i>L. hackeliae</i> ATCC 35250 ^T =CCUG 31232 A ^T		AJ781453	M36028	U92207	AF367735	SMI
<i>L. israelensis</i> ATCC 43119 ^T =CCUG 31115 ^T		AJ781452	Z32640	U92208	AF367736	SMI
<i>L. jamestowniensis</i> ATCC 35298 ^T =CCUG 29669 ^T		AJ781451	Z49726	U92228	AF367737	SMI
<i>L. jordanis</i> ATCC 33623 ^T =CCUG 16413 ^T		AJ781450	Z32667	U92209	AF367738	SMI
<i>L. lansingensis</i> ATCC 49751 ^T =CCUG 31227 B ^T		AJ781449	Z49727	U92210	AF367739	CCUG
<i>L. londiniensis</i> ATCC 49505 ^T =CCUG 44895 ^T		AJ781448	Z49728	U92229	AF367740	CCUG
<i>L. longbeachae</i> ATCC 33462 ^T =CCUG 46622 ^T	1	AJ781447	AY444740	X83036	AF367741	SMI
<i>L. longbeachae</i> ATCC 33484=CCUG 46623	2	AJ781466	AY444741	AF000958		SMI
<i>L. maceachernii</i> ATCC 35300 ^T =CCUG 31116 ^T		AJ781446	X60081	U92211	AF367742	SMI
<i>L. micdadei</i> ATCC 33218 ^T =CCUG 31229 A ^T		AJ781445	AF227162	S62141	AF367743	SMI
<i>L. moravica</i> ATCC 43877 ^T =CCUG 31117 ^T		AJ781444	Z49729	U92212	AF367744	SMI
<i>L. nautarum</i> ATCC 49506 ^T =CCUG 44900 ^T		AJ781443	Z49730	U92213	AF367745	CCUG
<i>L. oakridgensis</i> ATCC 33761 ^T =CCUG 16414 ^T		AJ781442	X73397	U92214	AF367746	SMI
<i>L. parisiensis</i> ATCC 35299 ^T =CCUG 29670 ^T		AJ781441	Z49731	U92215	AF367747	SMI
<i>L. pneumophila</i> ATCC 33152 ^T =CCUG 9568 ^T	1 subsp. <i>pneumophila</i>	AJ781429	M59157	AF022334	AF367748	SMI
<i>L. pneumophila</i> ATCC 33154=CCUG 13396	2 subsp. <i>pneumophila</i>	AJ781470		AF022316	AY036039	SMI
<i>L. pneumophila</i> ATCC 33155=CCUG 13397	3 subsp. <i>pneumophila</i>	AJ781471		AF095227	AY036040	SMI
<i>L. pneumophila</i> ATCC 33156 ^T =CCUG 13398 ^T	4 subsp. <i>fraseri</i>	AJ781481		AF022318	AY036041	SMI
<i>L. pneumophila</i> ATCC 33215	6 subsp. <i>pneumophila</i>	AJ781472		AF022320	AY036043	SMI
<i>L. pneumophila</i> ATCC 33216=CCUG 13399	5 subsp. <i>fraseri</i>	AJ781482		AF022319	AY036042	SMI
<i>L. pneumophila</i> ATCC 33823=CCUG 16411	7 subsp. <i>pneumophila</i>	AJ781473		AF022321	AY036044	SMI
<i>L. pneumophila</i> ATCC 35096=CCUG 16412	8 subsp. <i>pneumophila</i>	AJ781474		AF022322	AY036045	SMI
<i>L. pneumophila</i> ATCC 35251=CCUG 30657 A	15 subsp. <i>fraseri</i>	AJ781483				CCUG
<i>L. pneumophila</i> ATCC 35289=CCUG 30704	9 subsp. <i>pneumophila</i>	AJ781475		AF022323	AY036046	SMI
<i>L. pneumophila</i> ATCC 43130=CCUG 30660	11 subsp. <i>pneumophila</i>	AJ781477		AF022325	AY036048	SMI
<i>L. pneumophila</i> ATCC 43283=CCUG 30705	10 subsp. <i>pneumophila</i>	AJ781476		AF022324	AY036047	SMI
<i>L. pneumophila</i> ATCC 43290=CCUG 30661	12 subsp. <i>pneumophila</i>	AJ781478		AF022326	AY036049	SMI
<i>L. pneumophila</i> ATCC 43703=CCUG 44898	14 subsp. <i>pneumophila</i>	AJ781480		AF022328	AY036051	SMI
<i>L. pneumophila</i> ATCC 43736=CCUG 30662	13 subsp. <i>pneumophila</i>	AJ781479		AF022327	AY036050	SMI
<i>L. quateirensis</i> ATCC 49507 ^T =CCUG 44899 ^T		AJ781440	Z49732	U92216		CCUG
<i>L. quinlivanii</i> ATCC 43830 ^T =CCUG 31234 A ^T		AJ781439	Z49733	U92217	AF367749	SMI
<i>L. rubrilucens</i> ATCC 35304 ^T =CCUG 29671 ^T		AJ781438	Z32643	U92218	AF367750	SMI
<i>L. saintelensis</i> ATCC 35248 ^T =CCUG 29672 ^T	1	AJ781437	Z49734	U92219	AF367751	SMI
<i>L. santicrucis</i> ATCC 35301 ^T =CCUG 29673 ^T		AJ781436	Z49735	U92220	AF367752	SMI

Table 1. cont.

Strain	Serogroup	GenBank accession no.				Source
		<i>rnpB</i>	16 rRNA	<i>mip</i>	<i>rpoB</i>	
<i>L. shakespearei</i> ATCC 49655 ^T =CCUG 31228 A ^T	1	AJ781435	Z49736	U92221	AF367753	CCUG
<i>L. spiritensis</i> ATCC 35249 ^T =CCUG 31118 ^T		AJ781434	M36030	U92222	AF367754	SMI
<i>L. steigerwaltii</i> ATCC 35302 ^T =CCUG 29674 ^T		AJ781433	Z49737	U92223	AF367755	SMI
<i>L. tucsonensis</i> ATCC 49180 ^T =CCUG 31119 ^T		AJ781432	Z32644	U92224	AF367756	SMI
<i>L. wadsworthii</i> ATCC 33877 ^T =CCUG 16415 ^T		AJ781431	Z49738	U92225	AF367757	SMI
<i>L. worsleiensis</i> ATCC 49508 ^T =CCUG 44924 ^T		AJ781430	Z49739	U92226	AF367758	CCUG

were previously referred to as the *Tatlockia* species (Fox *et al.*, 1991); they are however distinguishable using *mip* gene sequencing.

Differentiation of *Legionella bozeman* and *Legionella anisa* has been proven difficult by transfer DNA intergenic spacer length polymorphisms (De Gheldre *et al.*, 2001). A close species relationship was found when comparing *rnpB* gene sequences derived from these two *Legionella* species. In sequence analysis, *L. anisa* ATCC 35292^T differs in four nucleotide positions compared with *L. bozeman* ATCC 35545 sg 2, while the corresponding differences in comparisons between *L. anisa* ATCC 35292^T and *L. bozeman* ATCC 33217^T sg 1, as well as between the two serogroups of *L. bozeman*, were seven nucleotide positions. The finding that *rnpB* gene sequences from *L. bozeman* sg 2 and *L. anisa* are more similar than those from the two serogroups of *L. bozeman* was surprising since sequence data from previous studies examining other genes, such as *mip* and *rpoB*, have indicated a more distant relationship between the two species (Ko *et al.*, 2002; Ratcliff *et al.*, 1998).

The *L. pneumophila* serogroups 4, 5 and 15, together constituting *L. pneumophila* subsp. *fraseri*, differed in 14–15 nt from the *rnpB* gene sequence of *L. pneumophila* ATCC 33152^T sg 1, while the sequence variation within subspecies *fraseri* was 1 nt. Similarly, the sequence variation within the subspecies of *L. pneumophila* subsp. *pneumophila* varied between 0 and 4 nt.

Secondary structures and intermolecular interactions of the RNase P RNA

The RNase P RNA gene has been sequenced and characterized from a large number of different bacteria (Brown, 1999). This information has been incorporated into models of the three-dimensional structures of both types of RNase P RNA, as well as in a complex with the protein subunit (Massire *et al.*, 1998; Tsai *et al.*, 2003). The majority of these previous studies on bacterial RNase P RNA have, however, focused on structural and functional differences between members of different bacterial genera (Haas & Brown, 1998). This study examined 39 species within one genus.

With the guidance of a type A RNase P RNA minimum consensus structure model (Brown, 1999), secondary structures of RNase P RNAs from three *Legionella* type strains were generated. As shown in Fig. 1, the secondary structures are very similar to those of some γ -Proteobacteria, e.g. *Pseudomonas aeruginosa* and *Escherichia coli*. Within the *Legionella* genus, RNase P RNA showed structural variations, particularly in P3 and P12 but also in the structural element between P16 and P17.

Two P3 variants were observed where the major variant was an 8 bp stem structure with a loop varying in size between three and six residues. In one species, *Legionella israelensis* ATCC 43119^T, the sequence suggested the existence of a P3 helix composed of 20 bp with a U-rich internal bulge and a loop with eight residues (Fig. 1). Moreover, we noted that the first 6 bp of P3 are highly conserved irrespective of *Legionella* species (boxed residues in Fig. 1; see also sequence alignment data in Supplementary Material in IJSEM Online). Likewise, comparison of RNase P RNA structures derived from closely related bacterial species, e.g. *Mycoplasma* species (Svard *et al.*, 1994), also reveals that the length of P3 varies and that residues in the boxed region (Fig. 1) in P3 are conserved. In contrast, a comparison of the length and sequence variation of the P3 domain derived from different bacterial branches indicates that the structure of the P3 is highly variable (Haas & Brown, 1998). The three-dimensional structural model of *E. coli* RNase P RNA (type A) in complex with the RNase P protein (C5) suggests that part of P3 interacts with the C5 protein (Tsai *et al.*, 2003). In this model, the nucleotides in P3 that are suggested to interact with C5 are positioned closer to the P3 loop (the size of P3 in *E. coli* RNase P RNA is similar to that of *L. israelensis* ATCC 43119^T). However, if P3 serves as a binding region for C5, then the available structural data (i.e. comparison of the P3 structure derived from closely related bacterial species, see above) suggest that the interaction between P3 and C5 should not depend on the length of P3. We therefore argue that it is possible that C5 binds to a specific sequence motif in P3. We suggest that it is likely that the 'boxed' residues in Fig. 1 serve as a binding motif for the C5 protein. The extra P3 residues in *L. israelensis* ATCC 43119^T (and in other RNase P RNAs with long P3 helices)

might be important for structural stabilization and/or be involved in interactions with other factors in the cell. However, this remains to be investigated.

In *Bacillus subtilis*, RNase P system chemical cleavage footprinting (Loria *et al.*, 1998) and nucleotide analogue modification interference studies (NAIM; Rox *et al.*, 2002) suggest that P12 and the C5 protein are in close proximity. Given that the RNase P holoenzyme consists of one RNA subunit and one protein subunit, neither of the two three-dimensional models are consistent with an interaction of C5 with both P12 and P3 (Chen *et al.*, 1998; Tsai *et al.*, 2003 and references therein). However, Fang *et al.* (2001) observed that the *B. subtilis* RNase P holoenzyme formed tetramers in solution that might rationalize the footprinting and NAIM data. For *E. coli* RNase P holoenzyme, no tetramers in solution have so far been observed (Fang *et al.*, 2001). Nevertheless, cross-linking between the RNA and the protein subunits in *E. coli* RNase P holoenzyme has been observed (Sharkady & Nolan, 2001). The residues at positions 144 and 145 in *L. pneumophila* sg 1 correspond to the

nucleotides in *E. coli* RNase P RNA that cross-linked to the C5 protein. In this study, we observed major structural variation in P12 in RNase P RNA derived from a large number of *Legionella* species with respect to size (i.e. number of base pairs), loop size and the number of bulges and their position in P12 (Fig. 1). Taken together, if P12 constitutes a binding site for the RNase P protein, it might be reoriented as proposed elsewhere (Sharkady & Nolan, 2001) or perhaps these data might reflect flexibility in the structure of RNase P RNA. Moreover, there is a possibility of identifying C5 amino acid residues that bind to the RNA in P12 by characterizing the *rnpA* gene from the corresponding *Legionella* species and looking for co-variation.

Another interesting structural feature is the suggested stem-loop structure between P16 and P17. This structural element has previously been described in RNase P RNA derived from *Planctomycetes* and α -*Proteobacteria* (Brown, 1999), but it has not been observed in RNase P RNA from γ -*Proteobacteria*. Although the function of this stem-loop is not known, it is located very close to the domain of RNase P

Fig. 2. Majority rule consensus trees from Bayesian analyses of *mpB* and 16S rRNA gene sequences (posterior probabilities >0.9). Numbers below branches indicate bootstrap values >50% obtained by NJ and by parsimony analyses (in parentheses).

Site-to-site rate variation was modelled separately for each of the genes *rnpB*, 16S rRNA, *rpoB* and *mip*. For the *rnpB* and *rpoB* genes, the most adequate model was TrNef + I + Γ , i.e. a Tamura Nei model (Tamura & Nei, 1993), with equal base frequencies and invariant sites plus a gamma rate distribution. For the *mip* gene, the GTR + I + Γ model, i.e. General

The MP, NJ and Bayesian analyses of each gene separately, and for the four genes combined, resulted in similar topologies, although differences were observed. Tree topologies showed that the number of conflicts, above a Bayesian posterior probability of 95, was highest for the comparison between *rpoB* and *mip* (eight conflicts), followed by the 16S rRNA gene and *rpoB* (five conflicts), *rnpB* and *mip* (four conflicts) and *rpoB* and *rnpB* (three conflicts). There were two conflicts between the 16S rRNA gene sequence and *mip* and between 16S rRNA and *rnpB*.

Node support (posterior probabilities for Bayesian analyses and bootstrap proportions for MP and NJ) was used to evaluate the phylogenetic utility of the information embedded in the gene sequences. The majority rule consensus trees from the Bayesian analyses of *rnpB* and 16S rRNA gene sequences are shown in Fig. 2. Branches with Bayesian posterior probabilities of <0.90 are collapsed and the numbers below the branches indicate the score obtained by bootstrap analysis using NJ and parsimony algorithms.

Bayesian analysis of 16S rRNA gene sequences from 150 different species representing approximately 55 genera showed that the *Legionella* species examined form a well-supported clade and thus constitute a monophyletic group (data not presented).

The phylogenetic analysis of combined datasets (one comprising *rnpB* together with *mip* and the other with all four genes) included 39 *Legionella* species, of which 37 had sequences available for all four genes. The phylogenetic analysis of the four genes together (Fig. 3) and *rnpB* together with *mip* (not presented) revealed very similar branching with 18 and 17 well-supported nodes, respectively, i.e. with posterior probabilities of ≥ 0.95 . The numbers of

well-supported nodes in the analyses of each gene sequence alone were 13 for *mip*, 12 for 16S rRNA, 11 for *rnpB* and five for *rpoB*. The well-supported nodes together comprised 29 species in the analysis of all four genes, 28 species for *rnpB* together with *mip*, 25 species for *rnpB*, 22 species for *mip*, 19 species for 16S rRNA and 11 species for *rpoB*. Interestingly, the well-supported nodes derived from *rnpB* and *mip* gene sequences comprised more species than the well-supported nodes from 16S rRNA and *rpoB* gene sequences and therefore contributed most of the phylogenetic signal in the dataset including all four genes.

In our analysis of *rpoB*, we found five fewer nodes of substantial support compared with a previous study (Ko *et al.*, 2002; data not shown). In their presented NJ trees, there were several nodes that were in conflict compared with an analysis of *mip* gene sequences. In contrast, such conflicts were almost absent in our comparison between *rpoB* and the other gene sequences. Thus *rpoB* did not improve the resolution of the phylogenetic tree regarding species, but showed six nodes with significant support within the *L. pneumophila* species. This is more than was found for the *rnpB* and *mip* phylogeny, where only four nodes were observed. However, the *rpoB* tree is

Fig. 4. (a) Results from the Shannon-Wiener (SW) analysis of the *rpoB*, *rnpB*, *mip* and 16S rRNA gene sequences. The SW index shows the sequence variation along the individual genes. (b) Frequencies of sites with specific SW indices are shown. The mean SW index is 0.13 for the 16S rRNA gene sequence, 0.38 for *rnpB*, 0.48 for *rpoB* and 0.57 for *mip*.

single copy gene which has been shown to be useful in the differentiation of closely related bacteria (Herrmann *et al.*, 2000; Svard *et al.*, 1994; Tapp *et al.*, 2003).

To conclude, the sequence variation of *rnpB* separated 37 of 39 *Legionella* species included in this study. The variation in putative secondary structures provides the possibility of understanding the interaction between RNase P RNA and the C5 protein. Phylogenetic analysis showed that *rnpB* clustered more species in nodes with strong branch support than did any of the other three genes, and when combined with *mip* the phylogenetic utility was further enhanced. Thus, the combination of strictly conserved and hypervariable elements suggests *rnpB* as useful for species identification and for the phylogenetic analysis of closely related species.

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