Comparison of the 23S ribosomal RNA genes and the spacer region between the 16S and 23S rRNA genes of the closely related *Mycobacterium avium* and *Mycobacterium paratuberculosis* and the fast-growing *Mycobacterium phlei*

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The 23S rDNA sequences of *Mycobacterium paratuberculosis*, *M. avium* and *M. phlei* and the sequences of the spacer regions between the 16S and 23S rRNA genes were determined. The overall 23S rDNA sequence identity between *M. paratuberculosis* and *M. avium* was 99.7% (nine mismatches), showing the very close relatedness of these mycobacteria. Evolutionary distances between the five known mycobacterial 23S rRNA/rDNA sequences and those of other Gram-positive G + C-rich bacteria were determined. The 23S rDNA sequences of mycobacteria showed two inserted regions compared to the other bacteria. A mycobacterial unique region contained one mismatch between *M. paratuberculosis* and *M. avium*. An *Actinomycetales*-specific insertion, consisting of 111 nucleotides, was completely identical for *M. paratuberculosis* and *M. avium*. The sequence of the intergenic spacer region between 16S and 23S rDNA had a length of 278 bp for *M. paratuberculosis* and *M. avium* with only two mismatches. The spacer region of the fast-growing *M. phlei* was 85 bp longer. No tRNA-encoding region was found in the spacer region.

Keywords: 23S rRNA gene, intergenic spacer region, mycobacteria

INTRODUCTION

Mycobacterium avium and *M. intracellulare*, members of the *M. avium* complex (MAC), are important opportunistic pathogens in humans. Infections with these bacteria have become a major problem in immunodeficient people, especially in AIDS patients (Armstrong, 1985). *M. paratuberculosis* is the causative agent of paratuberculosis, an economically important intestinal disease of ruminants and it is also associated with Crohn's disease in humans (Chiodini, 1989). *M. paratuberculosis* has been included in the MAC, because of the close relatedness with the mycobactin-dependent *M. avium* strains (Mathews & McDiarmid, 1979; Hurley *et al.*, 1988; McFadden *et al.*, 1992; Thorel *et al.*, 1990).

Mycobacteria may be divided in four groups, based on growth rate and pigmentation (Runyon *et al.*, 1974). Further taxonomic classification of mycobacteria is complicated, because of the variety of specialized tests needed.

The comparison of ribosomal RNA (rRNA) sequences or the genes encoding rRNA (rDNA) has proved to be a powerful tool for the phylogenetic classification of bacteria (Woese, 1977). Phylogenetic studies based on the analysis of 16S rRNA of mycobacteria confirmed the classical division in fast- and slow-growers (Stahl & Urbance, 1990). However, in those cases where the 16S rRNA sequences of closely related species are almost identical, these sequences are not sufficient for phylogenetic analysis (Fox *et al.*, 1992). For instance, the 16S rRNA sequences of *M. paratuberculosis* and *M. avium* are 99.9% identical (Stahl & Urbance, 1990; Rogall *et al.*, 1990). Species-specific probes for *M. paratuberculosis* based on the 16S rRNA sequence could not be derived, because of the high level of 16S rRNA sequence identity with *M. avium* (Van der Giessen *et al.*, 1992).

Recently, the sequence of the gene coding for the large 23S rRNA subunit of *M. leprae* was determined (Liesack *et al.*, 1991). Comparison with selected regions of the 23S

rRNA of other mycobacterial species showed that a *M. leprae*-specific probe could be developed. Further, sequence analysis of the intergenic spacer region between the 16S and 23S rRNA subunit genes showed a high degree of variability even between closely related species (Suzuki *et al.*, 1988; Liesack *et al.*, 1991).

We present sequences of the genes encoding the 23S rRNA of *M. avium*, *M. paratuberculosis* and *M. phlei* including the sequences of the intergenic spacer regions. These sequences were compared with previously determined 23S rRNA sequences of bacteria stored in the EMBL database, in particular *M. leprae*, other Gram-positive, G+C rich actinomycetes, *B. stearothermophilus* and two Gram-negative bacteria (Gutell *et al.*, 1992). Our aim was to compare the 23S rDNA sequences of the closely related *M. avium* and *M. paratuberculosis* and to examine the possibility of deriving species-specific oligonucleotide probes for *M. paratuberculosis*.

METHODS

Bacterial strains and DNA extraction. DNA was extracted from *M. avium* strain 23435 serotype 8 (Institute Pasteur, Paris), *M. paratuberculosis* strain J2A (CVL, Weybridge), *M. phlei* strain R82 (CVL, Weybridge), *M. fortuitum* (CVI, Lelystad) and *M. chelonae* (CVI, Lelystad) as described by Van der Giessen *et al.* (1992).

Polymerase chain reaction and cloning of the 23S rDNA gene and the intergenic spacer region. The 23S rDNA genes of the mycobacteria were amplified in three parts. Part 1 was amplified with primers P1 [5'-GAT(G/T)TCGGAATGGG-(C/G)AACCC-3'] position 109–129 and P2 (5'-GACCAGTG-AGCTATTACGC-3') position 1193–1211 of the *M. avium/paratuberculosis* 23S rDNA sequence. Part 2 was amplified with primers P3 (5'-GGCTTAGAAGCAGCCATCC-3') position 1164–1182 and P4 (5'-GGTCGGAACCTACCCGAC-3') position 2153–2170. Part 3 was amplified with primers P5 (5'-GTCGGGTAAGTTCGACC-3') position 2153–2170 and P7 (5'-GCTTTCGGCCAGTTAGTGCCAG-3') position 3079–3100. The intergenic region was amplified with primers I1 (5'-TGCGGTGGATCACCTCCTT-3') position 1522–1541 of the 16S rRNA gene and I2 (5'-GGTTCCCCATTCGGAATC-3') position 109–128 of the 23S rRNA gene. Conditions of the PCR were: 35 cycles (1 min, 95 °C; 2 min, 58 °C; 3 min, 72 °C); total volume 100 µl containing 100 ng template DNA, 200 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, gelatin 0.1 g l⁻¹, 200 µM dNTP, 1 µM of both primers and 2.5 Units of Taq polymerase (Promega), covered with 100 µl paraffin oil. PCR products of part 1 were inserted in PstI-digested poly-G-tailed vector pUC19 (Pharmacia-LKB). PCR products of parts 2, 3 and the intergenic region were ethanol-precipitated, made blunt by incubation with Klenow fragment of DNA polymerase I and 1.67 mM dNTP and ligated into EcoRV-digested vector Bluescript KS M13– (pBS, Stratagene). Plasmid vectors were electroporated and propagated in *E. coli* K12 strain PC2495, a *recA*⁻ derivative of JM101 (Yanisch-Perron *et al.*, 1985).

Sequencing of the intergenic spacer region and the 23S rDNA genes. Plasmid DNAs were isolated using the Qiagen plasmid kit according to the manufacturer's manual. The sequence of the cloned 23S rDNA was determined by using the dideoxy chain termination method (Sanger *et al.*, 1977), an automated laser fluorescent DNA sequencer (Pharmacia) and the autoread sequencing kit using T7 DNA polymerase (Pharmacia). Besides

the fluorescein isothiocyanate (FITC)-labelled universal sequence primers (5'-CCCTCGAGGTCGACGGTATCG-3' and 5'-GCCGCTCTAGAACTAGTGGATC-3') the following FITC-labelled internal primers were used to determine the 23S rDNA sequences. S1 (5'-AGTAGCGGCGAGCGAA-3') position 245–260; S2 (5'-TAGCTGGTTCTCCCCGAAA-3') position 948–956; S5 (5'-GGAGTGAGGAATGCAGGCATG-3') position 1408–1427; S6 (5'-GGGGCTGCGTGGGACC-3') position 1657–1672; S7 (5'-CCCCAAACCAACACAGGTGG-3') position 1886–1905; S8 (5'-AACTGGGGCGGTTGCTCC-3') position 2543–2561 of the 23S rDNA sequence compared to *E. coli*.

Data analysis. Sequences were aligned using PILEUP and CLUSTALV computer programs (Devereux *et al.*, 1984; Higgins & Sharp, 1988). Phylogenetic analyses were performed with distance matrix and maximum parsimony methods, as described in the Felsenstein-Phylip package version 3.3 (Felsenstein, 1985). Phylogenetic trees were made with data distance matrix FITCH (Fitch & Margoliash, 1967), NJTREE (Saitou & Nei, 1987), UPGMA (Sneath & Sokal, 1973), and KITCH (Fitch & Margoliash, 1967) phylogenetic computer programs.

The 23S rRNA/rDNA sequences of *M. leprae*, *M. kansasii*, *Micrococcus luteus*, *Streptomyces griseus*, *Streptomyces ambofaciens*, *Bacillus stearothermophilus*, *Frankia* sp., *Pseudomonas aeruginosa* and *E. coli* were derived from the EMBL and GenBank databases.

RESULTS AND DISCUSSION

The determined sequences of 23S rDNA of *M. paratuberculosis* and *M. avium* have a length of 3100 bp and are thus only slightly shorter than the 23S rDNA gene of *M. leprae* (3122 bp) (Liesack *et al.*, 1991) and *M. phlei* (3121 bp). The sequences were submitted to the EMBL Data Library and will be available under the accession numbers X74495, X74494 and X74493, respectively.

The 23S rDNA sequences and their intergenic spacer regions between the 16S and 23S rDNA of *M. avium* serotype 8, *M. paratuberculosis* J2A and *M. phlei* were determined in order to examine the possibility of deriving species-specific DNA probes for *M. paratuberculosis*. *M. avium* serotype 8 strain was sequenced because this is one of the serotypes which is indistinguishable from *M. paratuberculosis* based on 16S rRNA analysis (Böddinghaus *et al.*, 1990). The alignment of the 23S rDNA mycobacterial sequences and the 23S rRNA/rDNA sequences of other Gram-positive bacteria with a high G+C content revealed a high resemblance. Evolutionary distances of the 23S rDNA sequences were generated by the distance matrix analysis and the FITCH program using the total length of 3200 nucleotides of the multiple sequence alignment (Table 1). Gaps were not considered in the calculations. Results obtained with the other programs were not significantly different from those of the FITCH program. The 23S rDNA sequences of *M. avium* and *M. paratuberculosis* were 99.7% identical. A similar high level of identity was found in the 16S rRNA subunits (99.9%) (Stahl & Urbance, 1990; Rogall *et al.*, 1990). However, when 23S rDNA sequences of *M. avium* and *M. paratuberculosis* were compared to those of other mycobacteria, they appeared to be less similar than the 16S rRNA/DNA sequences (Table 2). A tree of the clustering relations of

Table 1. Evolutionary distances ($\times 100$) based on the 23S rRNA genes of the Gram-positive high-G + C-containing bacteria, and two Gram-negative bacteria, *P. aeruginosa* and *E. coli*

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
1. <i>M. paratuberculosis</i>	0											
2. <i>M. avium</i>	0.3	0										
3. <i>M. kansasii</i>	3.8	3.7	0									
4. <i>M. leprae</i>	4.8	4.8	4.7	0								
5. <i>M. phlei</i>	8.7	8.7	8.9	10.1	0							
6. <i>Frankia</i> sp.	19.9	19.9	19.9	20.5	19.4	0						
7. <i>S. ambofaciens</i>	20.7	20.7	20.9	21.8	20.3	14.3	0					
8. <i>S. griseus</i>	20.4	20.3	20.4	21.2	20.3	14.1	4.5	0				
9. <i>Mic. luteus</i>	20.1	20.1	20.0	20.2	20.8	19.2	18.3	18.2	0			
10. <i>B. stearothermophilus</i>	30.8	30.9	30.8	30.8	30.9	28.5	30.9	31.3	32.3	0		
11. <i>P. aeruginosa</i>	37.2	37.2	37.0	36.7	36.8	34.3	35.8	36.5	37.3	30.7	0	
12. <i>E. coli</i>	38.9	38.9	39.1	39.6	39.6	38.2	38.6	38.7	36.7	34.5	17.5	0

Table 2. Percentage identity of mycobacteria based on the 16S rRNA sequences (top right) (Stahl & Urbance, 1990) and the 23S rDNA sequences (lower left)

	1.	2.	3.	4.	5.
1. <i>M. paratuberculosis</i>	0	99.9	98.1	97.2	95.3
2. <i>M. avium</i>	99.7	0	98.3	97.1	95.4
3. <i>M. kansasii</i>	96.2	96.3	0	96.7	95.3
4. <i>M. leprae</i> *	95.2	95.2	95.3	0	94.2
5. <i>M. phlei</i>	91.3	91.3	91.1	89.9	0

* 16S rRNA sequence (accession no. M61681) identity determined by using the FITCH computer program (Fitch & Margoliash, 1967).

the 23S rRNA/rDNA sequences constructed using the PILEUP computer program showed the close relatedness of *M. paratuberculosis* and *M. avium* (Fig. 1).

A unique insertion sequence in the 23S rDNA gene observed in some mycobacteria (Liesack *et al.*, 1991) was also detected in *M. paratuberculosis*, *M. avium* and *M. phlei* at position 766–787 of the multiple sequence alignment (Fig. 2). This sequence differed between the mycobacterial species; there was one mismatch between *M. paratuberculosis* and *M. avium* (Fig. 2). In the secondary structure model (Höpfel *et al.*, 1989), the insertion is located between helices 27 and 31 (Liesack *et al.*, 1991). The predicted secondary structure for the insertion sequence in *M. paratuberculosis*, *M. avium* and *M. phlei* is depicted in Fig. 3. The sequence of this region might be used to derive specific oligonucleotide probes, which can distinguish closely related species.

Helix 54 of the 23S rRNA/rDNA sequences of the *Actinomycetales* is much longer than that of the 23S rRNA/rDNA sequences of other bacteria (Roller *et al.*, 1992), and has been proposed to be an ideal target for diagnostic probes and selective PCR primers (Stackebrandt *et al.*, 1991). A specific *M. leprae* oligonucleotide

Fig. 1. Tree showing the clustering relations of the Gram-positive G+C-rich bacteria, together with *B. stearothermophilus*, *E. coli* and *P. aeruginosa* using the PILEUP computer program.

probe was derived from this region (Liesack *et al.*, 1991). Our data show that this extended helix is also present in the newly determined mycobacterial sequences. However, not a single mismatch was found between the inserted 111 nucleotides of *M. paratuberculosis* and *M. avium* (Fig. 4). This again stresses the close relatedness between *M. paratuberculosis* and *M. avium*. Only nine mismatches were observed between the complete 23S rRNA genes.

	751	800
1. <i>M. paratuberculosis</i>	AGTCTGAATA GGGCGCAT-- --CCCTTTTG ---GGGTGTA GTGGCGTGT	
2. <i>M. avium</i>	AGTCTGAATA GGGCGCAT-- --CCCTTTTG ---GGGTGTA GTGGCGTGT	
3. <i>M. kansasii</i>	AGTCTGAATA GGGCGTAT-- --CGCGCGCG AGCGTGTGTA GTGGCGTGT	
4. <i>M. leprae</i>	AGTCTGAATA GGGCGTAT-- --CACGTGTG AGCGTGTGTA GTGGCGTGT	
5. <i>M. phlei</i>	AGTCTGAATA GGGCGTATCC AACCTGTGG GTTGTGTGTA GTGGTGTST	
6. <i>Frankia</i> sp.	AGTCCGAAGA GGGCG----- --TTGA GTCCGATGTC	
7. <i>S. ambofaciens</i>	AGTCCGAATA GGGCG----- --TTCA GTAGCAGCCT	
8. <i>S. griseus</i>	AGTCCGAATA GGGCG----- --GTTGA GTTGACGCT	
9. <i>M. luteus</i>	AGTCTGAATA GGGCG----- --ATTGA GTCCGCTGT	
10. <i>B. steartothermophilus</i>	AGTCTGAATA GGGCGAA-- --AA GTACGTCGTC	
11. <i>P. aeruginosa</i>	AGTCTTAATA GGGCG----- --TTTA GTCCGTGGT	
12. <i>E. coli</i>	AGTCTTAAT GGGCG----- --TTAA GTTGACGGT	

Fig. 2

	U	U		U	U		U	U		U	U
	C	-	G		C	-	G		C	-	G
	C	-	G		C	-	G		C	-	G
	C	-	G		C	-	G		C	-	G
	U	.	G		U	.	G		U	.	G
	A	-	U		A	-	U		A	-	U
	C	-	G		C	-	G		C	-	G
	G	.	UAGU		G	.	UAGU		G	.	UAGU
<i>M. paratuberculosis</i>				<i>M. avium</i>				<i>M. phlei</i>			

Fig. 3

Fig. 2. Unique mycobacterial insertion sequence of the 23S rDNA gene corresponding to position 762–784 of the multiple sequence alignment of the 23S rDNA of G + C-rich bacteria, together with *E. coli* and *P. aeruginosa*. Dashes indicate gaps.

Fig. 3. Predicted secondary structure of the 23S rRNA sequences located between helices 27 and 31 of *M. paratuberculosis*, *M. avium* and *M. phlei*.

	1551	1600
1. <i>M. paratuberculosis</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
2. <i>M. avium</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
3. <i>M. kansasii</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
4. <i>M. leprae</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
5. <i>M. phlei</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
6. <i>Frankia</i> sp.	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
7. <i>S. ambofaciens</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
8. <i>S. griseus</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
9. <i>M. luteus</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
10. <i>B. steartothermophilus</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
11. <i>P. aeruginosa</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	
12. <i>E. coli</i>	GGGTTGATAT TCCCGTACCC GTGTATGGG GTCCCTGATG AATC-A-GCG	

	1601	1650
1. <i>M. paratuberculosis</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
2. <i>M. avium</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
3. <i>M. kansasii</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
4. <i>M. leprae</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
5. <i>M. phlei</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
6. <i>Frankia</i> sp.	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
7. <i>S. ambofaciens</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
8. <i>S. griseus</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
9. <i>M. luteus</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
10. <i>B. steartothermophilus</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
11. <i>P. aeruginosa</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	
12. <i>E. coli</i>	GTACTAACCA CCCAAACCG GATCGACCAT -TCCCTTCG GGGG-GTGG	

	1651	1700
1. <i>M. paratuberculosis</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
2. <i>M. avium</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
3. <i>M. kansasii</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
4. <i>M. leprae</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
5. <i>M. phlei</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
6. <i>Frankia</i> sp.	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
7. <i>S. ambofaciens</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
8. <i>S. griseus</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
9. <i>M. luteus</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
10. <i>B. steartothermophilus</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
11. <i>P. aeruginosa</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	
12. <i>E. coli</i>	CGATTCTGGG CTGCGTGGG CTTTCGCTGG TAGTAGTCAA GCAATGGGGT	

Fig. 4

	16S rDNA
<i>M. paratuberculosis</i>	TGCGGTTGGATCACCCTCTTTCTAAGGAGCACCACGAAAA-----GCACCCCAACT
<i>M. avium</i>	TGCGGTTGGATCACCCTCTTTCTAAGGAGCACCACGAAAA-----GCACCCCAACT
<i>M. leprae</i>	TGCGGTTGGATCACCCTCTTTCTAAGGAGCACCACGAAAA-----GCACCCCAACT
<i>M. phlei</i>	TGCGGTTGGATCACCCTCTTTCTAAGGAGCACCACGAAAA-----GCACCCCAACT

<i>M. paratuberculosis</i>	GGT--GGGG--TGCG-AGCCGTGAGGGGTTC--CGTCTGTAGTGGACGGGGCCGGGT
<i>M. avium</i>	GGT--GGGG--TGCG-AGCCGTGAGGGGTTC--CGTCTGTAGTGGACGGGGCCGGGT
<i>M. leprae</i>	AGTTTAGGG--TGTA-AGCCGTGAGGGGTTC--CATCTGTAGTGGATGAGACCGGGT
<i>M. phlei</i>	GAGGTAGGGAGTTGCGCGGTGGGAGAGTGCCTGGCGCTGTAGTGGTGTTCGGGTGGGT

<i>M. paratuberculosis</i>	GCACAACA-----GC-----AAAT-----GATTGCCAGACACT
<i>M. avium</i>	GCACAACA-----GC-----AAAT-----GATTGCCAGACACT
<i>M. leprae</i>	GCACAACA-----GC-----AAAT-----GATTGCCAGACACT
<i>M. phlei</i>	GCACAACA-----GC-----AAAT-----GATTGCCAGACACT

<i>M. paratuberculosis</i>	ATTGGGCGCTGAG--ACAAC--ACTCG-----GTCGCTCC-----
<i>M. avium</i>	ATTGGGCGCTGAG--ACAAC--ACTCG-----GTCGCTCC-----
<i>M. leprae</i>	GTTGGGCTGAG--GCAAC--ACTCG-----GCTTGATT-----
<i>M. phlei</i>	ATTGGGCTTTAAACAACAGCCGCTTCGCCCCGTGGTGGGGGTGTCTCCGGTTCGGG

<i>M. paratuberculosis</i>	-----GTGTGGAGTCCCTCCATCTTGGTGGGGGTGGTGTGTTGAGTATGGATAGT
<i>M. avium</i>	-----GTGTGGAGTCCCTCCATCTTGGTGGGGGTGGTGTGTTGAGTATGGATAGT
<i>M. leprae</i>	-----GGGTGTTGCTCCCTCATCTTGGTGGGGGTGGTGTGTTGAGTATGGATAGT
<i>M. phlei</i>	GTGCGGGTGTGTTGTGCTGCTTGTGGTGGGGGTGGTGTGTTGAGTATGGATAGT

<i>M. paratuberculosis</i>	GGTTCGAGCATCTAGATGAGCGCATGGTC--TCCGTGGC--CGGCGT--TCATCGAAA
<i>M. avium</i>	GGTTCGAGCATCTAGATGAGCGCATGGTC--TCCGTGGC--CGGCGT--TCATCGAAA
<i>M. leprae</i>	GGTTCGAGCATCTAAATGATGCGTGTGAGTATGATGAGTGGGCGATTCATTGAAA
<i>M. phlei</i>	GGTTCGAGCATCT-GATACTTGAGGCGCTCTTTTTCGGGGGT-CTTGGGTGTCGAA

Fig. 5

Fig. 4. Extended helix 54 of *Actinomycetales* corresponding to positions 1551–1700 of the multiple sequence alignment of the 23S rDNA sequence. Dashes indicate gaps.

Fig. 5. Sequence alignment of the intergenic spacer region between the 16S rDNA and the 23S rDNA gene of mycobacteria. Dashes indicate gaps.

The high sequence variability of the intergenic spacer regions, even between closely related organisms (Liesack *et al.*, 1991; Suzuki *et al.*, 1988), was not observed in *M.*

paratuberculosis and *M. avium*. This region comprises 278 bp, which is similar in length to the spacer regions of *M. bovis* BCG (Suzuki *et al.*, 1988), *M. tuberculosis*

Fig. 6. Agarose gel (1.5%) electrophoresis of PCR products from the intergenic spacer region of *M. paratuberculosis*, *M. avium*, *M. phlei*, *M. fortuitum*, *M. chelonae*, two negative PCR controls (no template) and lambda *Pst*I marker.

(Kempsell *et al.*, 1992) and *M. leprae* (Liesack *et al.*, 1991), and contained only two mismatches between *M. avium* and *M. paratuberculosis*. Thus, it was not possible to derive specific probes from this region to discriminate between these closely related species. The sequence of the spacer region of *M. avium* serotype 8 is identical to the internal transcribed spacer (ITS) sequences of the MAC strains belonging to the Mav-B sequevar as described by Frothingham & Wilson (1993). Interestingly, the *M. paratuberculosis* spacer sequence differs in two nucleotides from ITS sequences of the Mav-B sequevar but only in one nucleotide from ITS sequences of the Mav-A sequevar (Frothingham & Wilson, 1993), both in the same region, and therefore *M. paratuberculosis* seems more related to Mav-A strains based on the spacer region sequences. Considerable differences were found with the rapid growing *M. phlei*. The intergenic spacer region of *M. phlei* contained several insertions compared to the other mycobacteria increasing the length by 85 bp (Fig. 5). This difference was easily demonstrated by comparing the sizes of PCR fragments of this region. The sizes of the intergenic regions of two other fast-growing mycobacteria, *M. fortuitum* and *M. chelonae*, are comparable to that of *M. phlei* (Fig. 6). Although this insertion has approximately the size of a tRNA and these regions have been shown to encode a tRNA in some rRNA gene clusters (Loughney *et al.*, 1982; Bacot & Reeves, 1991), no tRNA-encoding region was found in the spacer region of *M. phlei*.

M. paratuberculosis and *M. avium* are phylogenetically very closely related, based on small 16S rRNA (Stahl & Urbance, 1990; Rogall *et al.*, 1990), the large 23S rRNA and their intergenic sequences. Although these data and results from other studies indicate that *M. paratuberculosis* and *M. avium* can be considered subspecies (Thorel *et al.*, 1990), there are nevertheless differences. Most *M. paratuberculosis* strains grow slower than *M. avium* and are

mycobactin dependent in conventional mycobacterial culture media. Only a few *M. avium* strains, including the wood pigeon isolates, now designated *M. avium* subsp. *silvaticum* (Thorel *et al.*, 1990) are mycobactin dependent. They can cause a disease in cattle which resembles paratuberculosis (Mathews & McDiarmid, 1979). These strains can be distinguished from *M. paratuberculosis* by a different insertion sequence (IS) (Kunze *et al.*, 1991). IS901 is related to but can be distinguished from IS900, which so far has been found in *M. paratuberculosis* only (McFadden *et al.*, 1987; Green *et al.*, 1989).

In conclusion, the sequences of the 23S rDNA of *M. paratuberculosis* and *M. avium* are very similar. These sequences contain only nine mismatches, which none-the-less could be used to differentiate these species. In contrast, the 23S rDNA sequences of other closely related mycobacteria were less similar than their 16S rRNA/DNA sequences, indicating that 23S rRNA is more suitable for finding differences between related organisms or for deriving specific oligonucleotide probes. The intergenic spacer region between the 16S and 23S rDNA gene, which has been found to be more diverse among closely related organisms than the rRNA genes themselves, was also almost identical between *M. paratuberculosis* and *M. avium*. The lengths of the intergenic spacers of the fast-growing mycobacteria are considerably greater than those of the slow-growing mycobacteria, but no tRNA-encoding region was found in any of the mycobacterial sequences determined.

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