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Extensive DNA Sequence Conservation throughout the *Mycobacterium tuberculosis* Complex

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The *Mycobacterium tuberculosis* complex includes the four species *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. We sequenced 13 *M. tuberculosis* complex strains in the 16S-to-23S rDNA internal transcribed spacer (ITS). The ITS has a high rate of nucleotide substitution. Previous reports found three nucleotide substitutions in the ITS between two *M. tuberculosis* complex strains. In contrast, we found the same ITS sequence in all 13 *M. tuberculosis* complex strains (including all four species and *M. bovis* BCG). This finding confirms the conservation of 16S rDNA sequence and the high DNA-DNA relatedness found in previous studies. By the usual criteria, the four species of the *M. tuberculosis* complex would be considered a single species. In a phylogenetic analysis based on the ITS sequence, the four species of the *M. tuberculosis* complex were distinct from nontuberculous mycobacteria. The ITS contains at least seven potential sites for oligonucleotide probes with specificity for the *M. tuberculosis* complex.

The *Mycobacterium tuberculosis* complex includes the four species *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. These species are genetically very similar, with 85 to 100% DNA-DNA relatedness (10), but they differ in their epidemiology. *M. tuberculosis* causes tuberculosis in humans and animals which have contact with humans. *M. africanum* causes tuberculosis in humans in Africa (22). *M. bovis* is found in a wide range of wild and domestic animals, while *M. microti* causes tuberculosis in voles (22).

Slowly growing mycobacteria have a single rRNA operon (11, 13, 18). The operon is transcribed as a unit; a single transcript is then processed into mature 16S, 23S, and 5S rRNAs (11). The 16S rRNA gene (ribosomal DNA [rDNA]) is widely used for phylogenetic analysis at the genus and species levels. However, the internal transcribed spacer (ITS) between 16S rDNA and 23S rDNA is much more variable than 16S rDNA. In slowly growing mycobacteria, the ITS is about 280 bp long and does not contain the tRNA genes which are often found in other bacteria (18). The high nucleotide substitution rate of the ITS makes it a good site for phylogenetic analysis and a good target for diagnostic oligonucleotide probes directed at closely related species or strains (1, 8, 9, 13). The ITSs of 60 strains of eight species of slowly growing mycobacteria have been sequenced (8, 9, 11, 13, 18). Three ITS nucleotide substitutions were found between *M. tuberculosis* H37Rv^T (the T indicates type strain) and *M. bovis* BCG Calmette (11, 18).

We sequenced the ITSs of 13 strains of the *M. tuberculosis* complex. The strains chosen for analysis included the type strain of each species as well as a strain of *M. bovis* BCG. Ten of the 13 strains were previously analyzed by restriction endonuclease digestion of chromosomal DNA and then Southern hybridization with the insertion element IS*6110* (4). Two of the strains chosen had identical IS*6110* fingerprints (TMC 410 and TMC 1024), two of the strains had fingerprints which differed slightly (TMC 102 and TMC 201), and other strains had highly variable fingerprints (e.g., T2, T3, and T5). The strains chosen were thus expected to represent strains within the *M. tuberculosis* complex with various degrees of genetic conservation and variability.

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MATERIALS AND METHODS

ITS amplification by PCR. Heat-killed lysates of the type strains of *M. africanum* and *M. microti* were obtained from the Clinical Microbiology Laboratory of the Veterans Affairs Medical Center, Durham, N.C. DNAs from *M. tuberculosis* and *M. bovis* were obtained from the University of Arkansas for Medical Sciences, Little Rock (4). The ITS was amplified by using 30 cycles of PCR as described previously (8). The primers (T3-Ec16S.1390p and T7-Mb23S.44n) were complementary at their 3′ ends to conserved regions of 16S and 23S rDNAs, which flank the ITS (8). Each primer also incorporated a T3 or T7 phage RNA polymerase promoter at the 5′ end (not used in the present work). Either purified DNA (1 to 50 ng) or a broth culture lysate (10 μl) was used in each 100-μl reaction mixture. Samples were mixed with PCR reagents in a room not used for PCR product analysis. Multiple interspersed negative controls (reagents only, no DNA) were included. Successful amplification was confirmed by electrophoresis on ethidium bromide-stained 2% agarose gels. No amplification products were observed in the negative controls. Positive amplification controls were 100 ng of DNA from *M. avium* ATCC 25291^T.

Direct sequencing of PCR products. PCR products were purified by using the GENECLEAN II Kit (Bio-101, La Jolla, Calif.) and were sequenced by using the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.). The sequencing primers included the complementary portions of the PCR primers (Ec16S.1390p and Mb23S.44n) and the internal primer Mt16S.1501p (5′-CCGTA CCGGAAGGTGCGGCTG-3′). Each sequencing reaction included 100 ng of PCR product and 10 pmol of sequencing

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primer in a total volume of 20 μl. Each reaction underwent 25 cycles (94°C for 30 s, 45°C for 2 s, 60°C for 4 min) in the GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Norwalk, Conn.). The sequencing products were purified through Centri-Sep columns (Princeton Separations, Adelphia, N.J.) and were loaded onto 6% denaturing polyacrylamide gels in an automated sequencer (373A DNA Sequencer; Applied Biosystems). Sequences were combined by using the ASSEMGEL program of PCGENE release 6.5 (IntelliGenetics, Mountain View, Calif.). Discrepancies were resolved by examination of chromatographs from the automated sequencer.

Phylogenetic analysis. Sequences were aligned by using the CLUSTAL program of PCGENE. Previously published mycobacterial ITS sequences were included in the alignment (8, 9, 11, 13, 18). The alignment was adjusted manually. Only the ITS sequence (excluding the flanking portions of 16S and 23S rDNA sequences) was used for phylogenetic analysis. The phylogenetic tree reported here was constructed by using parsimony analysis with a branch-and-bound search by using PAUP version 3.1.1 (19). The data were resampled by using 100 bootstrap replications (19). Other phylogenetic trees were generated by using PHYLIP version 3.5c (5) and the following methods: maximum likelihood, Fitch-Margoliash, neighbor-joining, and parsimony.

Nucleotide sequence accession numbers. The sequences of the type strains of *M. tuberculosis* (9), *M. bovis*, *M. africanum*, and *M. microti* and of *M. bovis* BCG Glaxo were submitted to GenBank. The accession numbers are listed in Table 1. GenBank accession numbers for other sequences are as follows: Mav-A, L07855; Mav-B, L07856; Mav-C, L07857; Mav-D, L07858; Min-A, L07859; MAC-A, L07847; MAC-B, L07848; MAC-C; L07849; MAC-D, L07851, MAC-E, L07852; MAC-F, L07853; Msc-B, L07854; MAC-H, L15620; Mce-A, L15621; Msc-A, L15622; Mxe-A, L15624; Mle-A, X56657.

RESULTS

Sequence results. Complete ITS sequences together with flanking partial 16S and 23S rDNA sequences were obtained for the 13 strains listed in Table 1. The sequences of all 13 *M. tuberculosis* complex strains were identical. The sequences of the four type strains and *M. bovis* BCG Glaxo are aligned in Fig. 1 with the sequences of six nontuberculous mycobacteria (8, 9, 13).

Comparison with previously published *M. tuberculosis* complex sequences. Other investigators have reported the sequences of the ITSs and flanking rDNAs for *M. tuberculosis* H37Rv^T and *M. bovis* BCG Calmette (11, 18). These two sequences are shown in Fig. 1. They differ from each other at 16S rDNA position 1444 and at ITS positions 12, 13, and 104, and both have an extra G after ITS position 173 in comparison with the sequences of our *M. tuberculosis* complex strains. The discrepancies probably represent sequencing errors or cloning artifacts in the previous reports. We obtained the same sequence in all 13 *M. tuberculosis* complex strains, including one of the previously published strains (*M. tuberculosis* H37Rv^T). The nucleotides that we identified at the discrepant ITS positions were present in most of the other mycobacterial species whose sequences are known (Fig. 1). Compressions in manual sequencing gels may have caused some of the discrepancies (e.g., GC versus CG, 5 G residues versus 4 G residues). We avoided the problem of cloning artifacts by direct sequencing (6).

Comparison of *M. tuberculosis* complex sequences with those of other mycobacteria. Sequences from six nontuberculous mycobacterial species are included in the alignment in Fig. 1. The 3′ ends of the 16S rDNAs were relatively well conserved among these species (*M. tuberculosis* 16S rDNA positions 1401 to 1536). In contrast, the ITSs had many nucleotide substitutions, insertions, and deletions. The highly variable sequence of the ITS makes it a good target for diagnostic oligonucleotide probes (1, 8, 13). Figure 1 shows seven potential probe sites in the ITS for the *M. tuberculosis* complex. The sequences of more mycobacterial species are needed to confirm the specificities of these sites. Table 2 lists the numbers of nucleotide differences between pairs of ITS sequences from various species of slowly growing mycobacteria. The four species of the *M. tuberculosis* complex were distinct from the other species, differing by at least 32 nucleotides.

TABLE 1. *M. tuberculosis* complex strains sequenced in the 16S-to-23S rDNA internal transcribed spacer

Species	Strain name	Accession no.[a]		
		TMC	ATCC	GenBank[b]
M. tuberculosis	H37Rv (type)	102	27294	L15623
	H37Ra	201	25177	
	T1[c]			
	T2[c]			
	T3[c]			
	T4[c]			
	T5[c]			
M. bovis	Type	410	19210	L26327
	Ravenel	401	35720	
	AN-5	412	35726	
	BCG Glaxo	1024	35741	L26328
M. africanum	Type		25420	L26330
M. microti	Type	1619	19422	L26329

[a] Abbreviations: TMC, Trudeau Mycobacterial Culture Collection; ATCC, American Type Culture Collection.
[b] GenBank accession numbers for the five sequences which were submitted are listed.
[c] Recent clinical isolate (4).

The phylogenetic tree in Fig. 2 displays the relationships among the 18 different ITS sequences obtained from 71 strains of slowly growing mycobacteria (8, 9, 13). This tree was selected on the basis of maximum parsimony (19). Four other ITS trees were generated by other methods and showed minor variations, especially in the placement of the *M. scrofulaceum* sequences. A recent phylogenetic analysis based on 16S rDNA included sequence data for all seven species listed in Table 2 (3). The branching order for those seven species was consistent in the 16S rDNA and ITS trees. This suggests that recombination does not occur frequently in the rRNA operon in slowly growing mycobacteria (8). The ITS tree has longer branch lengths than the 16S rDNA tree, reflecting the greater variability of the ITS sequence. ITS sequence analysis thus provides a potentially finer means of discriminating among closely related mycobacterial species or strains (8).

DISCUSSION

All 13 strains of the four species of the *M. tuberculosis* complex that we tested had identical ITS sequences, suggesting a very close evolutionary relationship. These four species also have identical 16S rDNA sequences (12) and identical partial *dnaJ* gene sequences (20). Together, these sequence results support the idea that the *M. tuberculosis* complex represents a

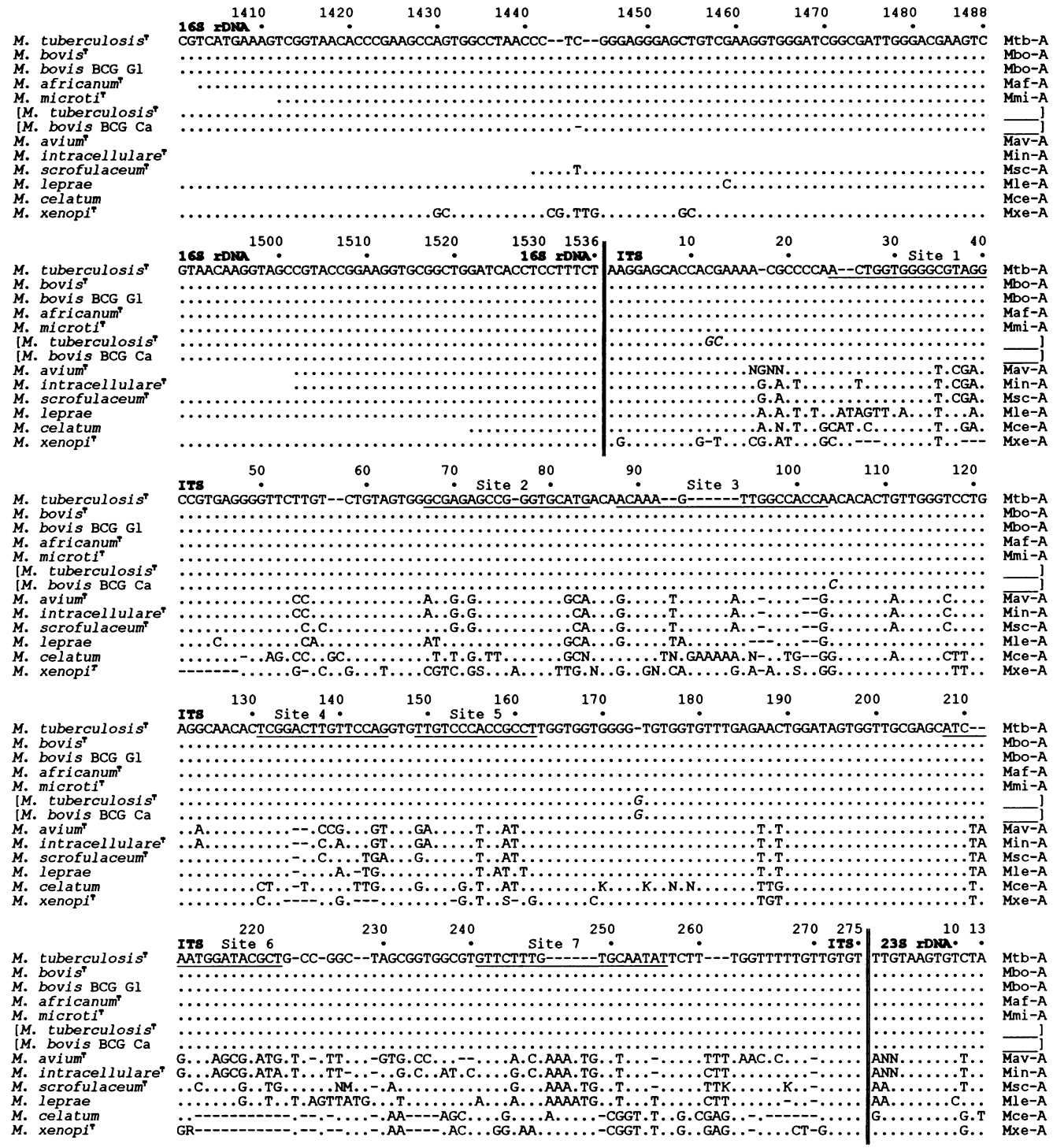

FIG. 1. Sequence alignment, including alignment of the sequences of the complete 16S-to-23S rDNA ITS and flanking partial 16S and 23S rDNAs. The sequences of only 5 of the 13 *M. tuberculosis* complex strains sequenced are displayed. The sequence of the *M. tuberculosis* type strain is given in full. The dots represent identity with that sequence. The dashes represent deletions. Potential oligonucleotide probe sites with specificity for the *M. tuberculosis* complex are underlined. Previously published sequences which differ from the sequences obtained in the present study are bracketed. Abbreviations corresponding to the phylogenetic tree in Fig. 2 follow each sequence line (see Fig. 2 legend for definitions of abbreviations). ^{T}, type strain; BCG, bacillus Calmette-Guérin; Gl, Glaxo; Ca, Calmette; K, G or T; M, A or C; N, any nucleotide; R, A or G; S, C or G.

TABLE 2. Nucleotide differences between pairs of 16S-to-23S rDNA ITS sequences

Species	No. of nucleotide differences from species:					
	M. tuberculosis	*M. avium*	*M. intracellulare*	*M. scrofulaceum*	*M. leprae*	*M. celatum*
M. tuberculosis[a,b]						
M. avium[b]	51					
M. intracellulare[b]	48	17				
M. scrofulaceum[b]	32	28	26			
M. leprae	41	50	44	38		
M. celatum	53	53	51	44	59	
M. xenopi[b]	58	66	67	60	64	46

[a] There were no nucleotide differences between *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*.
[b] Type strain.

single species. Generally, a bacterial species includes strains which have 70% or greater DNA-DNA relatedness (21). The four species of the *M. tuberculosis* complex have DNA-DNA relatedness of 85 to 100% (10), and so they would be considered a single species by this criterion. The division of the *M. tuberculosis* complex into four species is probably an artifact of the great historical interest in this pathogen.

Even when the members of the *M. tuberculosis* complex are considered a single species, their sequences are more conserved than those of some other mycobacterial species. Figure 2 contrasts the sequence conservation of the *M. tuberculosis* complex with the sequence variability of the *M. avium* complex (the 12 ITS sequences from Mav-A to Mav-E). The *M. avium* complex includes the species *M. avium*, *M. intracellulare*, and

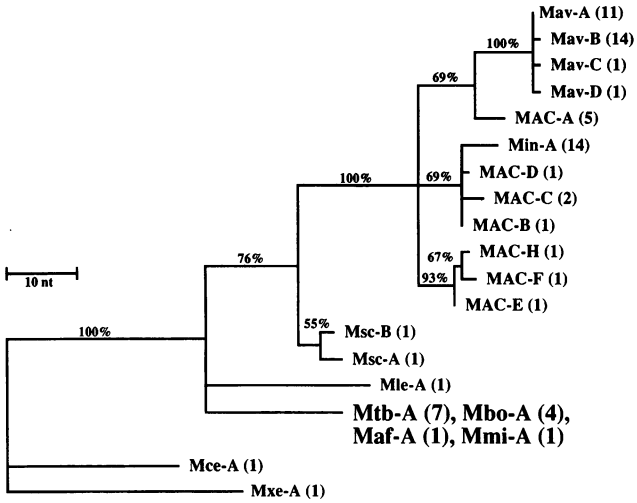

FIG. 2. Phylogenetic tree of 18 complete 16S-to-23S rDNA ITS sequences obtained from 71 mycobacterial strains. The numbers in parentheses indicate the number of strains with each sequence. Multiple isolates with the same sequence associated with the same patient are counted as one strain (9). The following abbreviations are used: Mav, *M. avium*; MAC, *M. avium* complex; Min, *M. intracellulare*; Msc, *M. scrofulaceum*; Mle, *M. leprae*; Mtb, *M. tuberculosis*; Mbo, *M. bovis*; Maf, *M. africanum*; Mmi, *M. microti*; Mce, *M. celatum*; Mxe, *M. xenopi*. Horizontal lengths represent genetic distances; bar, 10 nucleotide (nt) differences. Vertical lengths are not meaningful. Sequences grouped on the same branch are hypothesized to share a common ancestry. The percentage of bootstrap replications which yielded each grouping is indicated. This percentage is a measure of how well the sequence data support the grouping.

strains not assigned to either species. Strains of the *M. avium* complex differ in their ITS sequences by up to 21 nucleotides. Also, two strains of *M. scrofulaceum* differ in their ITS sequences by five nucleotides (Msc-A and Msc-B in Fig. 2 [8, 9]). Strains of *M. intracellulare*, *M. kansasii*, *M. gordonae*, and *M. celatum* have substantial sequence variability even in the more conserved 16S rDNA (3, 12, 15).

Several alternate explanations may account for the high degree of sequence conservation among *M. tuberculosis* complex strains. First, the *M. tuberculosis* complex may have evolved relatively recently. Second, the prolonged clinical latency of tuberculosis may lead to a slow evolutionary clock. Third, members of the *M. tuberculosis* complex are obligate parasites. In contrast, other mycobacterial species are widely distributed in the environment. This may result in a smaller total number of *M. tuberculosis* complex organisms and fewer opportunities for mutations. Fourth, bacteria in the *M. tuberculosis* complex normally live inside host macrophages. This limits the opportunity for recombination with other species or strains and may result in genetic homogeneity. Fifth, the *M. tuberculosis* complex is presumably well adapted to the relatively constant environment of the host. Most mutations probably provide no selective advantage, and thus are not preserved. In contrast, other mycobacteria propagate in various environments, allowing selection of various mutations.

Members of the *M. tuberculosis* complex are commonly identified by using a commercial chemiluminescent oligonucleotide probe (AccuProbe; Gen-Probe, Inc., San Diego, Calif.). The target of this probe is the hypervariable region of 16S rRNA (2). Several recent reports have identified strains of *M. celatum* and *M. terrae* which give false-positive results with the *M. tuberculosis* complex AccuProbe and which have sequences similar to that of the *M. tuberculosis* complex in that region (2, 16, 17, 23). The seven ITS probe sites identified in Fig. 1 may be more specific for the *M. tuberculosis* complex. The ITS of one *M. celatum* strain which gives a positive *M. tuberculosis* complex AccuProbe result has been sequenced. It differs substantially from the *M. tuberculosis* complex at all of the potential ITS probe sites (Fig. 1). The ITS may be a useful target for the simultaneous identification of multiple mycobacterial species. PCR could be performed by using primers complementary to flanking regions of 16S and 23S rDNAs with specificity for the genus *Mycobacterium*. The PCR product could then be identified at the species level by hybridization with a panel of oligonucleotide probes, direct sequencing (12), or possibly, restriction endonuclease digestion (14).

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