Comparison of *Mycobacterium* 23S rRNA Sequences by High-Temperature Reverse Transcription and PCR

BENJAMIN B. STONE,* RAYMOND M. NIETUPSKI,† GARY L. BRETON,
AND WILLIAM G. WEISBURG

GENE-TRAK, Inc., Framingham, Massachusetts 01701

We describe a modified rRNA sequence analysis method which we used to determine the phylogenetic relationships among 58 species belonging to the genus *Mycobacterium*. We combined the sensitivity of the reverse transcriptase PCR for amplifying nanogram amounts of template rRNA material with the elevated extension temperatures used for the thermostable DNA polymerase from *Thermus thermophilus*. A 70°C reverse transcription extension step permitted improved read-through of highly structured rRNA templates from members of the genus *Mycobacterium*, which have G+C contents of 66 to 71 mol%. The nucleic acid sequences of the amplified material were then determined by performing thermal cycle sequencing with α -³²P-labeled primers, again with extension at 70°C. Nonspecifically terminated bands were chased by using terminal deoxynucleotidyl transferase. Our method had a template requirement of nanogram amounts or less of purified RNA or 2,000 CFU of intact cells and had sufficient sensitivity so that lyophils obtained from the American Type Culture Collection could be used as source material. Sequences from a 250-nucleotide stretch of the 23S rRNA were aligned, and phylogenetic trees were evaluated by using the De Soete distance treeing algorithm and *Rhodococcus bronchialis* as the outgroup. Our 23S rRNA trees were compared with previously published 16S rRNA trees, including the comprehensive trees developed by the University of Illinois Ribosomal Database Project, and included 15 species not evaluated previously. Most of the groups were in general agreement and were consistent with relationships determined on the basis of biochemical characteristics, but some new relationships were also observed.

The genus *Mycobacterium* contained 56 validated species (18) as of 1991, and recently Shinnick and Good (34) listed 71 species. In addition, there are several subspecies and many trivially named strains. Members of this genus are frequently categorized as being slow growers (more than 7 days is required to see colonies on a plate) or fast growers (less than 7 days is required) (36). Several important human pathogens, including *Mycobacterium tuberculosis* and *Mycobacterium leprae*, are members of the slowly growing group, as are a number of opportunistic pathogens (Table 1) (15). In recent reports workers have described methods for using nucleic acid sequences to identify various specific members of the genus *Mycobacterium* (4, 5, 20, 21, 29, 31, 37, 38). However, not all known members of the genus have been examined; the organisms that have not been examined include some strains which have recently been shown to be problematic in probe-based assays (7, 12, 14).

Our interest in nucleic acid-based probe diagnoses for infectious diseases led us to examine members of the genus *Mycobacterium* for unique rRNA sequences that can be used to identify potential pathogens. To do this, we needed to evaluate the phylogenetic relationships of pathogenic and environmental strains to develop probes with appropriate specificities. However, several technical difficulties hindered this effort.

One of the technical difficulties that we encountered was the fact that the very low growth rates and low cell yields of many members of the genus *Mycobacterium* made it difficult to obtain sufficient quantities of purified rRNA for reverse transcriptase (RT) sequencing by the method of Lane et al. (23), as did the BL-3 level containment requirements of several patho-

gens. In addition, cloning the ribosomal DNAs (rDNAs) from all 58 species which we studied would have been extremely laborious. Another technical problem was the fact that the high G+C contents of mycobacterial genomes frequently inhibited efficient and reliable primer extension by RT through stable secondary structures at 37°C.

In recent years there have been a number of reports describing various methods for improving the method that Lane et al. (23) used for rRNA sequence analysis. The improvements that have been described include separating the labeling step from the termination step (11), altering the concentrations of specific deoxynucleoside triphosphates (40), utilizing low numbers of cells (27, 39), sequencing DNA PCR amplicons (6) with an additional 70°C extension step (4), and coupling DNA amplification with thermal cycle sequencing (30, 33). None of the methods that have been described combined all of the characteristics for developing sequence information desired by us. We sought a method in which small amounts of RNA template material could be used in combination with high extension temperatures. In this paper we describe such a method and the phylogenetic relationships of 58 members of the genus *Mycobacterium* based on a 250-nucleotide, variable region of the 23S rRNA subunit. The organisms which we used included 17 species which have not been included yet on the complete 16S rRNA tree developed by the Ribosomal Database Project (RDP) at the University of Illinois; 15 of these species have not had their 16S rRNA sequences evaluated in a phylogenetic context yet.

MATERIALS AND METHODS

Growth of bacterial strains. Organisms (Table 1) were grown on Middlebrook 7H10 agar. Cell pellets or lyophils were processed by shaking them with zirconium beads and phenol as described previously (3).

Nucleic acid purification. RNA was purified from cell lysates (3) through a cesium trifluoroacetic acid gradient as described previously (3). When DNA was used as the template material, it was cloned from PCR-amplified ribosomal

* Corresponding author. Mailing address: GENE-TRAK, Inc., 31 New York Avenue, Framingham, MA 01701. Phone: (508) 935-1205. Fax: (508) 879-6462.

† Present address: 6 Cherry St., Millbury, MA 01527.

TABLE 1. Strains used in this study

Species	Strain ^a	Growth rate ^b	Template ^c	GenBank accession no.
Strict pathogens^d				
<i>Mycobacterium africanum</i> ^e	ATCC 25420 ^T	S	R/D	U24502
<i>Mycobacterium asiaticum</i>	ATCC 25276 ^T	S	R	U24505
<i>Mycobacterium bovis</i>	ATCC 35745	S	D	U24509
<i>Mycobacterium haemophilum</i> ^e	GT3804	S	R	U24526
<i>Mycobacterium leprae</i>		S ^f		X56657
<i>Mycobacterium malmoeense</i>	ATCC 29571 ^T	S	R	U24531
<i>Mycobacterium shimoides</i> ^e	ATCC 27962 ^T	S	R	U24547
<i>Mycobacterium simiae</i>	ATCC 25275 ^T	S	R	U24548
<i>Mycobacterium tuberculosis</i>	H37Ra (= ATCC 25177)	S	R/D	U24555
<i>Mycobacterium ulcerans</i> ^e	GT4380	S	R	U24556
Opportunistic pathogens				
<i>Mycobacterium avium</i>	GT3246	S	D	U24508
<i>Mycobacterium celatum</i> ^e	ATCC 51131 ^T	S	R/D	U24510
<i>Mycobacterium chelonae</i>	GT3248	F	R	U24511
<i>Mycobacterium farcinogenes</i>	ATCC 35753 ^T	S	R	U24517
<i>Mycobacterium fortuitum</i>	ATCC 6841 ^T	F	R/D	U24519
<i>Mycobacterium genavense</i>	GT5011	S	R/D	U24523
<i>Mycobacterium intracellulare</i>	ATCC 13950 ^T	S	D	U24527
<i>Mycobacterium kansasii</i>	ATCC 12478 ^T	S	D	U24528
<i>Mycobacterium marinum</i>	ATCC 927 ^T	S	R	U24532
<i>Mycobacterium microti</i>	ATCC 11152	S	R	U24533
<i>Mycobacterium paratuberculosis</i> ^g	ATCC 19689	S	R	U24539
<i>Mycobacterium porcinum</i> ^e	ATCC 33776 ^T	F	R	U24541
<i>Mycobacterium scrofulaceum</i>	ATCC 19981 ^T	S	D	U24545
<i>Mycobacterium senegalense</i>	ATCC 35796 ^T	F	R	U24546
<i>Mycobacterium szulgai</i>	ATCC 35799 ^T	S	R	U24551
<i>Mycobacterium xenopi</i>	ATCC 19250 ^T	S	R	U24558
Rare, potential pathogens				
<i>Mycobacterium flavescens</i>	ATCC 14474 ^T	F	R	U24518
<i>Mycobacterium gordonae</i>	ATCC 35756	S	R/D	U24525
" <i>Mycobacterium lactis</i> " ^{e,h}	ATCC 27356		R	U24530
<i>Mycobacterium lepraemurium</i> ^{e,g}	ATCC 35779	S		ND ⁱ
<i>Mycobacterium neoaurum</i>	ATCC 27595 ^T	F	R	U24535
<i>Mycobacterium smegmatis</i>	ATCC 14468	F	R	U24549
<i>Mycobacterium thermoresistibile</i>	ATCC 19527 ^T	F	R	U24553
Nonpathogenic environmental mycobacteria				
<i>Mycobacterium agri</i> ^e	ATCC 27406 ^T	F	R	U24503
<i>Mycobacterium aichiense</i>	ATCC 27280 ^T	F	R	U24504
<i>Mycobacterium aurum</i>	ATCC 23366 ^T	F	R	U24506
<i>Mycobacterium austroafricanum</i> ^e	ATCC 33464 ^T	F	R	U24507
<i>Mycobacterium chitae</i>	ATCC 19627 ^T	F	D	U24512
<i>Mycobacterium chubuense</i>	ATCC 27278 ^T	F	R	U24513
<i>Mycobacterium diernhoferi</i>	ATCC 19343	F	R	U24514
<i>Mycobacterium duvalii</i> ^e	ATCC 43910 ^T	F	R	U24515
<i>Mycobacterium fallax</i>	ATCC 35219 ^T	F	R	U24516
<i>Mycobacterium gadium</i>	ATCC 27726 ^T	F	R	U24520
" <i>Mycobacterium gallinarum</i> " ^{e,h}	ATCC 19710		R	U24521
<i>Mycobacterium gastri</i>	ATCC 15754 ^T	S	R/D	U24522
<i>Mycobacterium gilvum</i>	ATCC 43909 ^T	F	R	U24524

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genes by using the method of Weisburg et al. (41) and 23S rDNA eubacterial primers derived from the study of Lane (22). To determine the sensitivity of the amplification procedure, quantified cells were extracted by the procedure of Barns et al. (3). The supernatant from the phenol extractions was ethanol precipitated by using yeast tRNA as the carrier. The pellets were redissolved in 100 µl of 10 mM Tris (pH 8)–1 mM EDTA buffer, and 1 µl was used as template material for rTth RT amplification.

Nucleic acid amplification. For reverse transcription and subsequent PCR we followed the recommendations of Perkin-Elmer-Cetus, Norwalk, Conn., and used the rTth DNA polymerase and reagents in a GeneAmp thermostable rTth RT RNA PCR kit (Perkin-Elmer-Cetus). Reverse primer 3284 (Table 2) was annealed at 70°C for 10 min in the presence of 1 mM MnCl₂. Following chelation with EGTA [ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], the DNA was amplified for 40 cycles with denaturation at 94°C for 20 s and annealing with forward primer 3415 and extension at 70°C for 20 s. The following three species required slightly less stringent annealing conditions (65°C): *Mycobacterium gilvum*, *Mycobacterium smegmatis*, and *Mycobacterium rhodesiae*. Am-

plicons were purified from buffer and primers by using Ultra Free-MC 30,000 mw spin filters (Millipore, Bedford, Mass.).

Nucleic acid sequencing. The primers used for amplification and sequencing (Table 2) were designed to hybridize primarily to high-G+C-content gram-positive organisms and to be stable for annealing at 70°C. Primer 3415 and reverse primer 3284 generated a 369-bp amplicon from *M. tuberculosis*. For thermal cycle sequencing of linear, double-stranded amplicons we used α-³²P-labeled primers, as well as the reagents and protocols described in an AmpliTaq cycle sequencing kit (Perkin-Elmer-Cetus). Most ambiguities were resolved by sequencing the opposite strand. Nondideoxy-terminated pause sites were alleviated with a terminal deoxynucleotidyl transferase chase at 37°C for 30 min as described previously (22). The sequences of DNA clones were determined by using a Sequenase kit (U.S. Biochemicals, Cleveland, Ohio).

Nucleic acid sequence analysis. Sequences (*Escherichia coli* positions 519 to 729) were manually aligned with a VAX minicomputer by using the Olsen sequence editor (28) and were printed by using a MegAlign (version 3.02) package obtained from DNASTAR, Madison, Wis. Two hypervariable stem-loop

TABLE 1—Continued

Species	Strain ^a	Growth rate ^b	Template ^c	GenBank accession no.
<i>Mycobacterium komossense</i>	ATCC 33013 ^T	F	R	U24529
<i>Mycobacterium moriokaense</i> ^e	ATCC 43059 ^T	F	R	U24534
<i>Mycobacterium nonchromogenicum</i>	ATCC 19530 ^T	S	R	U24536
<i>Mycobacterium obuense</i>	ATCC 27023 ^T	F	R	U24537
<i>Mycobacterium parafortuitum</i> ^c	ATCC 19686 ^T	F	R	U24538
<i>Mycobacterium phlei</i>	ATCC 11758 ^T	F	R	U24540
<i>Mycobacterium poriferum</i> ^e	ATCC 35087 ^T	F	R	U24542
<i>Mycobacterium pulveris</i> ^e	ATCC 35154 ^T	F	R	U24543
<i>Mycobacterium rhodesiae</i> ^e	ATCC 27024 ^T	F	R	U24544
<i>Mycobacterium sphagni</i>	ATCC 33027 ^T	F	R	U24550
<i>Mycobacterium terrae</i>	GT3247	S	R	U24552
<i>Mycobacterium tokaiense</i> ^e	ATCC 27282 ^T	F	R	U24554
<i>Mycobacterium triviale</i>	ATCC 23292 ^T	S	R	ND
<i>Mycobacterium vaccae</i>	ATCC 15483 ^T	F	R	U24557
Nonmycobacteria				
<i>Corynebacterium glutamicum</i>	ATCC 13032 ^T		R	
<i>Micrococcus luteus</i>	ATCC 381		R	
<i>Rhodococcus bronchialis</i>	ATCC 25592 ^T		R/D	U24559

^a ATCC, American Type Culture Collection; GT, GENE-TRAK Collection.

^b S, slow; F, fast.

^c R/D, RNA or DNA; R, RNA; D, DNA.

^d Pathogens classified as described by Good (15).

^e Not included on the RDP 16S rRNA tree.

^f *M. leprae* is a slowly growing obligately intracellular parasite.

^g *Lyophil* obtained from the American Type Culture Collection.

^h Not a valid species.

ⁱ ND, not determined.

structures occur in this region, and these sequences were aligned by determining homologous structures as base-complementary stems. The secondary structure of *M. leprae* (16) was used as the basis for these alignments. A sequence mask was used to remove ambiguously aligned positions from the analysis. Phylogenetic trees were developed in the Genetic Data Environment with a SUN SPARCstation by using the De Soete distance treeing algorithm (10). A 16S rRNA tree for the genus *Mycobacterium* was provided by the RDP (23a, 24).

Nucleotide sequence accession numbers. The GenBank accession numbers of the 23S rRNA sequences shown in Fig. 1 are given in Table 1.

RESULTS

Sensitivity of RT amplification. Natural total RNAs were prepared from various strains, including *M. tuberculosis* H37Ra (avirulent). We assumed that one-half of the mass of each total RNA was 23S rRNA. This material was serially diluted, and 1- μ l aliquots were used as templates for RT PCR. We routinely detected at least 1 ng in each experiment when we used various sources, including *Corynebacterium glutamicum*, *Micrococcus luteus*, and *Rhodococcus bronchialis* (data not shown). A maximum level of sensitivity of 10 pg was observed in the RT PCR with *M. tuberculosis* (data not shown); this represented between 10⁶ and 10⁷ initial rRNA template molecules. DNA PCR in which we used AmpliTaq DNA polymerase (Perkin-Elmer-Cetus) and primers 3415 and 3284 (Table 2) were performed with these same samples to demonstrate that the RNA, not contamination by trace amounts of DNA, was the template material. We detected no products by ethidium

bromide staining following agarose gel electrophoresis (data not shown).

We were concerned about the relatively poor sensitivity in our experiments compared with the data of Meyers and Gelfand (26), who observed levels of sensitivity between 100 and 1,000 copies with transcribed RNA. Synthetic transcripts of a clone of the 5' one-half of the 23S rRNA gene from *M. tuberculosis* H37Ra (avirulent) were prepared by using T7 RNA polymerase (Promega, Madison, Wis.), and the amounts of these transcripts were determined by measuring the A_{260} . Next, serial dilutions were prepared, and 1- μ l aliquots were used as template material. We found that the level of sensitivity obtained with the synthetic RNA was at least 3 orders of magnitude higher than the level of sensitivity obtained with the natural material (data not shown). Since the transcript included only about 1,600 nucleotides, 1 fg represented 10³ molecules. These results more closely approached the results of Meyers and Gelfand (26).

There are several possible reasons for the difference in sensitivity between the natural and synthetic rRNAs. Posttranscriptional modifications of the natural rRNA, such as methylation, could have inhibited efficient copying by the polymerase. It is also possible that the secondary and/or tertiary structure of the full-length natural rRNA was more stable, which could have further impeded copying. The synthetic transcript included only the first one-half of the 23S rRNA molecule.

TABLE 2. Amplification and sequencing primers targeted to high-G+C-content gram-positive eubacteria

Primer	No. of nucleotides	G+C content (mol%)	<i>E. coli</i> positions	Sequence (5' to 3')
3415	32	53	457–488	AGTACCGTGA GGGAAAGGTG AAAAGTACCC CG
3359	27	48	553–579	GTGATGGCGT GCCTTTTGWG GAATGAG
3429	27	56	751–725	TTTCAACCTA AGTGGGTTCG GGCCTCC
3284	22	59	791–770	GATTGGCCTT TCACCCCTAC CC

	10	20	30	40	50	60	70	80	90	100	
1	..A.G..	..G.A..	GG...ACG..	..TA..		..CTT..	..CT..	..A...T.T..	..G.T.A..	..C.T.T.A	E.coli
1	..A.G..	..G.A..	GG...ACG..	..T.T..		..GT..	..CT..	..A...T.T..	..G.T.A..	..C.T.T.A	R.bronchialis
1				..CT..	..T..		..G..				M.africanum
1							..G..				M.asiaticum
1				..CTT..		..G..					M.bovis
1				..TT..		..G..	T..				M.haemophilum
1				..CTTTT..		..G..					M.leprae
1	G..	A..	CT.CTTTTTTT..	G..							M.malmloense
1				..CT.CTTTTTTT..	G..						M.shimoidae
1				..CTT..		..G..					M.sintiae
1	TTGCTTCAATCC-GTCAGAGCCCTCTTTCTC-	TCG..			GAGGAGGGTGTGATGC-	GTGCTTTTGAAGAATGAGCCTGCGATGAGG					M.tuberculosis
1				..CT..		..G..					M.tulciensis
1				..CT..		..G..					M.avium
1		A..		..CTT..		..G..					M.celatum
1	..C.T..	T.GA..	CTTTG..	TC.T.G..							M.farcinogenes
1	..C.T..	T.GA..	CTTG..	TC.T.G..							M.forcurnum
1				..CTT..		..G..					M.gonaveense
1				..CT..		..G..					M.intracellulare
1				..CT..		..T..					M.kansasii
1				..CT..		..T..					M.marinum
1						..G..					M.microti
1						..G..					M.paratuberculosis
1		T.GA..	CTTTG..	TC.T.G..							M.porcinum
1				..CTT..		..G..					M.scrofulaceum
1	..C.T..	CT.C..	T.T..	G.T.G..							M.senegalense
1				..CTT..		..G..					M.szulgai
1		A..C..	CGT..	G..							CT M.xenopi
1		C..	CT.GA..	TTTT..	TT.T.G..						M.flauiensis
1				..CT..		..T..					M.gordonae
1				..CT.C..	CTT..	G.T.G..					CC M.lactis
1				..CT..	C.TGC..	T.G..					M.neoaerum
1	..C.T..	G..	CT.GA..	CGT..	TC.T.G..						M.smeagmatis
1	..C.TG..	G..	T.GGC..	TGTG..	G.T.T.G..						CT M.thermoresistibilis
1		C..	CT.C..	TT..	T.G..						M.agri
1				..CT..	CT.C..	T.G..					M.aichiense
1				..CT.C..	T.T..	G.T.G..					M.aerum
1				..CT.C..	T.T..	G.T.G..					M.austroafricanum
1				..CT.G..	TTTA..	C.T.G..					M.chitae
1				..T.GA..	CTT..	TC.T.G..					M.chubuense
1				..CT.GA..	TC.T.G..						M.dierckhoferi
1				..CT.GA..	CTTTG..	TT.T.G..					M.dnavalli
1		G..A..	TGT..			C..				T.T.	M.fallax
1				..CT.GC..	TTAT..	T.T.G..					M.gadium
1				..CT.C..	T.T..	G.T.G..					M.gallinarum
1						..T..					M.gastri
1				..C.T..	T.T..	G.T.G..					M.gilvum
1				..CT.GA..	CTT..	TC.T.G..					M.komossense
1		A..	CT.GAC..	GTTT..	TT.T.G..						M.moriokaense
1				..CT.CC..	TGT..	G.T.G..					CC M.nonchromogenicum
1				..CT.GA..	CTTTG..	TT.T.G..					M.obuense
1				..CT.C..	T.T..	G.T.G..					M.parafortuitum
1				..CT.C..	T.T..	G.T.G..					M.philii
1		G..A..	CTT.TT..	G..		C..				T.T.	M.poriferae
1		A..C..	TTTGTG..	T.G..							CC M.pulveris
1				..CT..	CTTTG..	T.G..					M.rhodesiae
1				..CT.C..	T.T..	G.T.G..					M.sphagni
1				..CT.CC..	T.T..	G.T.G..					CC M.serpenti
1				..CT.C..	T.T..	G.T.G..					M.tokaiense
1				..CT.C..	T.T..	G.T.G..					M.vacciae

	110	120	130	140	150	160	170	180	190	200	
75	TT	. . . A	. . . GAATA	. . . A . G . C .	. . . T . CT			T			E.coli
76	TTT	. . . A	. . . GAATA	. . . T . G . C .	. . . T . CT			A			T.bronchialis
79		. . . G						CA		GC	M.afrikanum
80		. . . G						CAT . C . C	. . . G . A	. . . G . G . TG	M.asiaticum
81		. . . G						TAT . GT	. . . C . C		M.bovis
82		. . . G						TAT . GT	. . . TG . G		M.bacillum
83		. . . C						TAT . C . C . A	. . . T . TT	. . . T . G . G . C	M.leprae
84		. . . G	. . . G					TAT . C . C . A	. . . T . TT	. . . T . G . G . C	M.malmoense
85		. . . G	. . . G					TAT . C . C . A	. . . T . TT	. . . T . G . G . C	M.shimoidae
86		. . . G	. . . G					TAT . C . C . A	. . . T . TT	. . . T . G . G . C	M.siniae
74	GACATGTCCGACGGTTAACGGTGTGGGGTGGCCGACGGAAGC				GAGTCTGTAATAGGGCC		CC . CACACGC	ATACGC	GGTGTGTG	AATATGT	M.tuberculosis
75	. . . G . A				. . . C . C . C . C		TAT . C . C . C	. . . G . G . C			M.kansas
76	. . . C . G . C	. . . A . C			. . . CAT . C . G . C		TAT . G . G . C	. . . T . G	. . . G . G . TG		M.avium
77	. . . G . C	. . . C . C			. . . TAT . G . G . C		GGCA	. . . C .	. . . TG		M.celatum
78	. . . C . G . C	. . . T			. . . COCA						M.chelonae
81	. . . G	. . . G			TAT . CACACA	. . . G . G	. . . TG	TG	TG		M.farcinogenes
82	. . . G				TAT . CGCACA	. . . G . G	. . . TG	TG	TG		M.foruitum
83	. . . G				TAT . C . C . C						M.gordona
74	. . . C . G . C	. . . C . C			. . . CAT . C . C . TT			. . . G . G . TG			M.intracellulare
75	. . . G	. . . A . C			. . . TAT . G . G . C		GGCA	. . . C .	. . . TG		M.kansasii
76	. . . G				TAT . C . C . G . A			GG			M.marinum
88		. . . C	. . . C				GC				M.microti
89	. . . G	. . . C			TAT . CACACA	. . . G . G	. . . TG	TG	TG		M.paratuberculosis
90	. . . C . G . C	. . . G . C			. . . CAT . C . C . TT			. . . G . TG			M.scrofulaceum
79	. . . G				TAT . CACACA	. . . G . G	. . . TG	TG	TG		M.senegalense
74	. . . G	. . . G			TAT . C . C . G . A		. . . G . G . TG				M.szulgai
75	. . . G	. . . G			TAT . GT	. . . G . G	. . . TG		. . . C .	. . . TG	M.xenopi
76	. . . G	. . . G			TAT . GTCA	. . . C . G	. . . TG	. . . C .	. . . TG		M.flavescens
77	. . . G	. . . G			TAT . C . C . C						M.gordona
79	. . . G . C . C	. . . G			TAT . TC	. . . G . TG					M.lactis
78	. . . G				TAT . CGCACA	. . . G . G	. . . TG	TG	TG		M.necaurum
80	. . . G	. . . G			TAT . CACACA	. . . G . G	. . . TG	TG	TG		M.smegmatis
85	. . . T . G	. . . GT . G	. . . T		TAT . CA . TC	. . . G . G	. . . TG	TG	TG		M.thermoresistibilis
89	. . . G	. . . G	. . . G	. . . TG	TAT . C . C . G	. . . TG	. . . TG	TG	TG		M.agri
90	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . C . G	. . . TG	TG	TG		M.chimere
79	. . . G	. . . G	. . . G	. . . TG	TAT . CA . GCA	. . . C . G	. . . TG	TG	TG		M.aurum
79	. . . G	. . . G	. . . G	. . . TG	TAT . CA . TC	. . . G . G	. . . TG	TG	TG		M.austroafricanum
80	. . . G	. . . G	. . . G	. . . TG	TAT . C . C . G	. . . GA	. . . G	. . . TG	TG		M.chitae
80	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . C . G	. . . TG	TG	TG		M.chubense
80	. . . G	. . . G	. . . G	. . . TG	TAT . CA . TC	. . . G . G	. . . TG	TG	TG		M.dienhoferi
81	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . G	. . . TG	TG	TG		M.durvalii
75	. . . C . G . G	. . . G	. . . A . T		. . . C			T			M.fallax
81	. . . G	. . . G	. . . G	. . . TG	TAT . CA . TT	. . . G	. . . AA . TG	TG	TG		M.gadium
74	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . G	. . . TG	TG	TG		M.gallinarum
74	. . . G	. . . A . C			TAT . G	. . . G . A	. . . C .	. . . TG			M.gastri
79	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . C . G	. . . TG	TG	TG		M.gordona
80	. . . G	. . . G	. . . G	. . . TG	TAT . C . C . G . TA	. . . G . G . TG					M.komossense
82	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . C . G	. . . TG	TG	TG		M.moriokaense
79	. . . G . C . C	. . . G			TAT . CA . TC	. . . G . TG	. . . G	. . . TG	TG		M.nonchromogenicum
81	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . C . G	. . . TG	TG	TG		M.obuense
79	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . C . G	. . . TG	TG	TG		M.paraforuitum
80	. . . G	. . . G	. . . G	. . . TG	TAT . CA . GT	. . . G . G	. . . TG	TG	TG		M.pulveris
75	. . . C . G . G	. . . G	. . . A . T		. . . C			T			M.pulveris
79	. . . G	. . . G	. . . G	. . . TG	TAT . CA . ACA	. . . C . G	. . . TG	TG	TG		M.pulveris
79	. . . G	. . . G	. . . G	. . . TG	TAT . CA . GT	. . . G . G	. . . GC . TG	TG	TG		M.rhodesiae
80	. . . G . C . C	. . . G	. . . A . T		. . . T			. . . G . TG	TG		M.sphagni
79	. . . G . C . C	. . . G	. . . G	. . . TG	TAT . TC	. . . G	. . . G	. . . TG	TG		M.terrae
79	. . . G	. . . G	. . . G	. . . TG	TAT . C . C	. . . G . GA	. . . G	. . . TG	TG		M.tokiosense
79	. . . G	. . . G	. . . G	. . . TG	TAT . CACACA	. . . G	. . . TG	TG	TG		M.vacciae

FIG. 1. Alignment of 23S rRNA sequences. The region shown includes homologous *E. coli* positions 519 through 729. All of the sequences are shown as the DNA form (T in place of U). Dots represent sequence nucleotides which correspond to nucleotides in the *M. tuberculosis* sequence. Dashes are included for alignment purposes. The residues enclosed in boxes are residues that were determined with only one primer and one strand. Table 1 shows the sources of sequences and the nature of the template material (RNA or DNA). The core loop structure was derived from *M. leprae* (16). Stem regions are underlined with two lines; loop regions are underlined with one line.

We wanted to demonstrate the utility of our technique for amplifying rRNA fragments from very low yields of whole cells. We used *R. bronchialis* as a model instead of mycobacteria to eliminate the need for BL-3 containment during processing. Our results revealed a level of sensitivity of about 2,000 CFU (data not shown). Assuming that there are approximately 1,000 23S rRNA targets per CFU (1), this finding is consistent with the presence of 10^6 to 10^7 molecules of purified natural rRNA. These values do not account for possible losses during the extraction procedure and therefore are probably underestimates of the true level of sensitivity. Nevertheless, these values are useful for demonstrating the limits for potential screening programs. Single colonies should provide adequate material for amplification. We were able to amplify material from lyophilis of *Mycobacterium paratuberculosis*.

Sequence analysis. The mycobacterial 23S rRNA regions homologous to *E. coli* positions 519 to 729 are shown in Fig. 1. These regions were used to develop the phylogenetic trees shown in Fig. 2. In the detailed overview analysis (Fig. 2A) we included 17 species that have not been included yet on the 16S rRNA tree available from the RDP at the University of Illinois. Two of these species, *Mycobacterium celatum* (34) and *Mycobacterium africanum* (13), have been placed phylogenetically by other workers. Figure 2B shows the positions of a selection of species extracted from the whole group and reanalyzed to clarify the main groups, as well as improve the general reliability of the results. For the most part the groups of species on

these trees are similar to the groups on the rRNA 16S rRNA tree. Some specific differences are discussed below.

Mycobacterium chitae ATCC19627^T (T = type strain) clustered in the genus *Mycobacterium* near *Mycobacterium komossense*, as found on the 16S rRNA tree obtained from the RDP and as described in a reexamination of this strain by Pitulle et al. (29). This finding is not consistent with the data of Stahl and Urbance (36), who found that *M. chitae* is less closely related to the genus *Mycobacterium* than *Rhodococcus equi* is. We found that *Mycobacterium fallax* and *Mycobacterium poriferae* were separated from the main group of the genus *Mycobacterium*, whereas on the 16S rRNA trees *M. fallax* clusters with *M. chitae* and *Mycobacterium gadium*.

Figure 2C shows the results of an analysis of the fast-growing species, in which *M. tuberculosis* was used as the outgroup. The large cluster from *M. gilvum* to *Mycobacterium phlei* was quite similar to a large group on the RDP 16S rRNA tree. *Mycobacterium farcinogenes* was excluded from this analysis because of its slow growth rate, but both on the tree shown in Fig. 2A and on the 16S rRNA trees this organism was placed in this cluster. We found that *M. gadium* grouped with *Mycobacterium diernhoferi* and *Mycobacterium neoaurum* and is closely related to *M. chitae*. As noted above, on the RDP 16S rRNA tree *M. fallax* clusters close to these species, whereas on our tree it clustered separately, along with *M. poriferae*.

Figure 2D shows the results of an analysis of the slowly growing species, in which *M. smegmatis* was used as the out-

FIG. 2. Phylogenetic relationships based on partial 23S rRNA sequences. (A). Overview of 58 species of the genus *Mycobacterium*. Note that "*Mycobacterium lactis*" and "*Mycobacterium gallinarum*" are not officially recognized species. *R. bronchialis* was used as the outgroup. (B). Selected overview of the genus *Mycobacterium*. Reducing the number of species in the tree should have improved the general reliability of the relationships. (C). Selected subgroup of fast growers. *M. tuberculosis* was used as the outgroup. (D). Selected subgroup of slow growers. *M. smegmatis* was used as the outgroup.

group. *Mycobacterium marinum* clustered with *Mycobacterium gordonae* and close to *Mycobacterium asiaticum* and *Mycobacterium szulgai*. On the 16S rRNA trees *M. marinum* is closely related to *M. tuberculosis*. The recently described organism *Mycobacterium genavense* is very closely related to *Mycobacterium simiae*, as found on the RDP 16S rRNA tree and as determined by a mycolic acid profile analysis (9). *M. celatum* is identical to *Mycobacterium kansasii*, a finding which is inconsistent with the phenotypic placement of these organisms as a slowly growing nonphotochromogen and a slowly growing photochromogen, respectively (34). *Mycobacterium haemophilum*, whose phylogenetic position has not been described, exhibited a high level of 23S rRNA sequence similarity to *M. leprae* despite many dissimilar phenetic traits (34).

DISCUSSION

Method. The method described above was a synthesis of the following two established techniques for molecular analysis of RNA sequences: (i) reverse transcription coupled to PCR amplification in which the recombinant DNA polymerase from *Thermus thermophilus* is used, and (ii) thermal cycle sequencing of the resulting amplicons to avoid many of the problems associated with sequencing linear, double-stranded templates. In this method ³³P-end-labeled sequencing primers are used, and amplicons are rapidly separated from amplification primers by using microcentrifuge spin filters. Thus, there is no requirement for gel purification (2), exonuclease digestion (19, 32), biotin capture (25), cold temperature "snap" annealing (8), or separate asymmetric amplification of each strand (17). Our method has several distinct advantages which we used for phylogenetic analysis of the vast majority of species in the genus *Mycobacterium* (currently 69 type strains are listed by the American Type Culture Collection).

Very small amounts of template material are required for the method described in this paper. Routinely, less than 1 ng of total RNA (as little as 10 pg was used in this study) or fewer than 10⁴ cells are needed to generate amplicons, compared with the 4 to 10 µg of RNA needed for avian myeloblastosis virus-based RT sequencing (23). This is an improvement in sensitivity of at least 3 to 4 (up to 6) orders of magnitude. Compared with the sensitivity of PCR amplification of rDNA genes (5, 6), this is not a step forward. However, we initially targeted RNA (instead of the coding rDNA) because we assumed that the natural copy number (at least 1,000 copies per CFU for *M. tuberculosis* [1]) would improve the sensitivity of the amplification technique. This assumption was investigated experimentally as described above. Furthermore, we were slightly concerned about sequence variation in chromosomal rDNA genes in those species (presumably including the fast growers) that contain more than one copy. Using the rRNA molecules directly provided sequences from the functional final gene products.

A high temperature during the annealing and extension step permitted improved read-through of stable secondary structures, whereas avian myeloblastosis virus RT is used at 37°C. This is particularly important for high-G+C-content organisms. Our experience with avian myeloblastosis virus RT showed that our technique resulted in significant improvement in the ability to read sequence information. Despite this, some secondary structures were still a problem during sequencing extension with members of the genus *Mycobacterium*, even at 70°C. We determined that these structures were not gel artifacts since they could be chased away with terminal deoxynucleotidyl transferase.

Sequencing two strands results in improved resolution in

denaturing gels because of different specific secondary structures that form with different primers. Avian myeloblastosis virus RT sequencing offers fewer options for checking sequence information. However, it is important to note that the sequence information originates from a single strand, compared with material cloned from PCR amplification preparations.

While the latter approach is an option, it is more time consuming. In addition, because at least 1,000 template molecules are required, the method described in this paper shares the advantage of direct PCR amplification and subsequent thermal cycle sequencing of rDNA genes (6) in that single misincorporations should not detectably affect the sequence information. This can be a problem with material cloned from PCR amplification preparations.

Phylogenetic relationships. The data presented in this paper place for the first time 15 members of the genus *Mycobacterium* in a phylogenetic context based on rRNA sequence information. Several of these organisms, including *M. haemophilum*, *Mycobacterium shimoidei*, and *Mycobacterium ulcerans*, are considered pathogenic for humans. We compared our results with the comprehensive overview provided by the 16S rRNA phylogenetic tree of the RDP, as well as with the results of specific analyses described previously (13, 29, 31, 36). For the most part, our results corroborate the 16S rRNA tree data, although there are some exceptions.

It is important to note that a true phylogeny probably will not be derived from a single character, such as a fragment of the 23S rRNA sequence. A polyphasic approach (35) will help even out the deficiencies of using specific traits (e.g., molecular size, stability, "evolutionary clock"). Therefore, our 23S rRNA data are not intended to be used to rewrite the phylogeny of the genus *Mycobacterium*, but rather complement the data obtained previously. In the case of the 15 species which we studied whose 16S rRNA sequences have not been aligned yet, phenetic traits still should be given significant weight.

Stahl and Urbance (36) and Rogall et al. (31) found that the growth rates of species (slowly growing versus fast growing) appeared to be phylogenetically conserved on the basis of 16S rRNA sequence data. Figures 2A and B show that all of the organisms except two can be clustered on the basis of growth rates. Initially, *M. chelonae* (a fast grower) was grouped with the slowly growing species (Fig. 2A). However, when we examined a subset of the members of the genus (Fig. 2B), *M. chelonae* was placed in the fast-growing species cluster. This change in position probably resulted from the limited sequence examined (29).

M. farcinogenes, a slowly growing species, clustered with the fast growers, close to *Mycobacterium fortuitum* (Fig. 2A) and in a subcluster adjacent to *Mycobacterium senegalense*. This position is very similar to the position determined on the basis of 16S rRNA sequences (29). Although the taxonomic position of this species has not been determined by using other phenetic traits, the results of DNA hybridization studies place it near *M. senegalense* (34). Assuming that the extracted and reanalyzed positions of *M. chelonae* and its fellow fast-growing species in Fig. 2B are correct, this is the only example of a contradiction with the slow-fast phylogenetic split (31, 36). The results of several independent approaches (16S rRNA, 23S rRNA, DNA hybridization) have placed *M. farcinogenes* with the fast growers, and slow growth appears to be a recently acquired trait (29). It is possible that the loss of rapid growth potential in *M. farcinogenes* has been acquired independently.

The position of *M. chitae* ATCC19627^T which we determined in this study is similar to the position of this organism on the RDP 16S rRNA tree and the position determined by Pitulle et al. (29). Pitulle et al. reexamined the 16S rRNA

sequence of this strain and obtained dramatically different results than Stahl and Urbance obtained (36). Both groups of authors recommended a comprehensive reexamination of the authenticity of this strain. Since the phenetic traits and mycolic acid composition of *M. chitae* still place it in the genus *Mycobacterium* (18), there may have been a problem with the specific culture used by Stahl and Urbance (36).

The identical 23S rRNA sequences of *M. celatum* and *M. kansasii*, like the identical 16S rRNA sequences of *M. gastri* and *M. kansasii*, provide another example of the limitations of relying on a single trait to place species in groups. While both *M. celatum* and *M. kansasii* are opportunistic pathogens in immunocompromised hosts, they belong to different Runyon groups (groups III [nonphotochromogens] and I [photochromogens], respectively) and have different biochemical test profiles (34). Along the same lines, in this study *M. haemophilum* and *M. leprae* clustered closely, although they clearly are quite different bacteria (34). *M. leprae*, an obligately intracellular pathogen, clusters close to (RDP) or between (34) the *M. tuberculosis* complex and the *Mycobacterium avium* complex. Although *M. haemophilum*, a nonphotochromogen, is similar to the *M. avium* complex in several biochemical characteristics, it has a distinctive mycolic acid profile; furthermore, it requires complexed iron for growth and can be cultured in vitro. Thus, the 23S rRNA sequence similarities belie important phenetic differences, which should again promote caution in the development of a true phylogeny.

Another difference is the branching of *M. fallax* and *M. poriferae* away from the main line of the genus. While the 23S rRNA sequences exhibited the deepest split within the genus in this study, the 16S rRNA trees (29, 34) also exhibit a reasonably deep branch point for *M. fallax* (until now *M. poriferae* has not been placed taxonomically). The true evolutionary significance of these differences may be muted by some of the limitations inherent in using this data set.

First, the sequences are not totally nonbiased for selection. Note the differences in the *M. tuberculosis* complex sequences, the *M. avium* complex sequences, and the sequences of other species. This 23S rRNA region was chosen because of probe development considerations, as well as method development and phylogenetic evaluation considerations. Therefore, this region was not randomly chosen on the basis of conserved structures but rather was chosen on the basis of hypervariable regions.

Second, as noted above, only about 250 nucleotides were evaluated, and fewer positions in an alignment are never as good as as many as possible. Thus, the phylogenetic trees which we obtained are not completely robust. Future changes in groups and membership are likely when more sequence information becomes available.

Finally, short branch lengths can be questionable, as discussed by Pitulle et al. (29). Short internodal distances are less predictive than long internodal distances. This could explain the variable position of *M. chelonae* depending upon the subset of species with which it is analyzed. Of course, efforts to neatly categorize the species highlight the anthropocentric nature of the fast-versus-slow division, and it may be more appropriate to consider some species phylogenetically intermediate between the two groups.

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