The Division between Fast- and Slow-Growing Species Corresponds to Natural Relationships among the Mycobacteria

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Comparative 16S rRNA sequencing was used to infer the phylogenetic relationships among selected species of mycobacteria and related organisms. The phylogeny inferred reflects the traditional classification, with major branches of the phylogenetic tree in general correspondence to the four Runyon groups and with numerical classification analyses. All the mycobacterial species compared, with the exception of *M. chitae*, are closely related (average similarity values greater than 95%). The slow growers form a coherent line of descent, distinct from the rapid growers, within which the overt pathogens are clustered. The distant relationship between *M. chitae* and the remaining mycobacteria suggests that this organism is incorrectly classified with the mycobacteria. *M. paratuberculosis* 18 was indistinguishable from *M. avium-M. intracellulare-M. scrofulaceum* serovar 1 by this analysis.

Members of the genus *Mycobacterium* are widespread in nature, ranging from harmless inhabitants of water and soil to the agents of such devastating diseases as tuberculosis and leprosy. Although they were among the first bacteria described (13, 29), a working taxonomy was formulated only within the last 30 years (14, 49). The early classification was based on growth rate, pigmentation, and clinical significance (33). A fundamental taxonomic division was tied to growth rate; members of the mycobacteria were defined as either slow or rapid growers. The rapid growers show visible growth from dilute inocula within 7 days, and the slow growers require more than 7 days for visible growth (55). By these criteria, the genus was divided into the four Runyon groups: group I, slow-growing photochromogens; group II, slow-growing scotochromogens; group III, slow-growing nonphotochromogenic (nonpigmented) isolates; and group IV, the rapid growers. Numerical taxonomy offered additional criteria by which to define species but did not provide precise boundaries between some, such as between *M. avium* and *M. intracellulare* (27, 31, 35, 44–47).

More recently, other methods have been used to complement the numerical studies and to infer natural relationships among the mycobacteria. These include immunological techniques (3, 25, 48, 52), comparison of cell wall components (5, 16, 17, 28, 37, 43), comparison of homologous enzyme sequences (51–54), DNA-DNA homology (1–3, 12, 15, 22, 23, 36, 37), plasmid profiles (20, 26), and restriction endonuclease analyses (6, 7, 12, 32, 56). But these too have so far failed to provide a unified and unambiguous classification of the genus.

An approach not previously systematically applied to mycobacterial classification is the comparative sequencing of the 16S rRNAs. The use of comparative rRNA sequencing (in particular the 16S rRNA) to infer natural relationships among microorganisms is now generally accepted (57, 58). Among available methods for assessing phylogenetic relationships, this has proved to be the most generally applicable and the most incisive. We present here a detailed analysis of mycobacterial phylogeny based on comparative 16S rRNA sequencing.

MATERIALS AND METHODS

Cultures. Table 1 lists the cultures used in this study. Mycobacterial cultures were maintained on Lowenstein-Jensen medium and Middlebrook 7H10 agar with oleic acid-dextrose-catalase enrichment (OADC) (9). *Corynebacterium*, *Actinomyces*, and *Rhodococcus* cultures were maintained on Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar with 5% sheep blood. Mycobacterial cultures for sequence analysis were grown in Middlebrook 7H9 broth with OADC. *Corynebacterium*, *Actinomyces*, and *Rhodococcus* cultures for sequence analysis were grown in brain heart infusion broth. The cultures were incubated at 37°C on a rotary shaker to the mid-log phase and harvested by centrifugation (6,000 × g for 7 min). The cell pellets were washed three times in 0.85% saline with 0.05% Tween 80, frozen, and stored at −85°C before nucleic acid extraction.

Nucleic acid extraction. Cell pellets were suspended in breakage buffer (50 mM Tris hydrochloride, 20 mM $MgCl_2$, 50 mM KCl, 5 mM mercaptoethanol, pH 7.5) and broken by sonication in the presence of glass beads in an ultrasonic cleaner (Branson 1200). After centrifugation at 16,500× g for 30 min, the supernatant was layered on sucrose step gradients (5, 30, 50% in breakage buffer) and centrifuged for 3.5 h at 285,000× g (Beckman SW40.1). The gradients were fractionated, and the ribosome fraction was identified spectrophotometrically (peak A_{260}). Pooled fractions were extracted two times with phenol saturated with low-pH buffer (50 mM sodium acetate, 10 mM EDTA, 0.1% 8-hydroxyquinoline, pH 5.1) and two times with buffer-saturated phenol-chloroform (4:1). After two extractions with chloroform, RNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. After incubation at −20°C for 12 h, the nucleic acid was recovered by centrifugation, washed twice with 80% ethanol, and dissolved in distilled water (1 mg/ml) for use in sequencing reactions.

RNA sequencing. Dideoxynucleotide sequencing was done with reverse transcriptase and [γ-^{35}S]ATP by the method of Lane et al. (21). Sequences are available through GenBank (accession numbers M29552 through M29575) or the corresponding author. The *M. bovis* BCG sequence was previously published (41).

Phylogenetic analysis. Approximately 1,300 nucleotides of

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TABLE 1. Strains used for rRNA sequence analysis

Organism	Source
Mycobacterium asiaticum	ATCC 25276
Mycobacterium aurum	ATCC 23366
Mycobacterium chelonei subsp. abscessus	ATCC 19977
Mycobacterium chitae	ATCC 19627
Mycobacterium fallax	ATCC 35219
Mycobacterium flavescens	ATCC 14474
Mycobacterium gordonae	ATCC 14470
Mycobacterium kansasii	ATCC 12478
Mycobacterium neoaurum	ATCC 25795
Mycobacterium nonchromogenicum	ATCC 19530
Mycobacterium phlei	ATCC 11758
Mycobacterium senegalense	ATCC 35796
Mycobacterium terrae	ATCC 15755
Mycobacterium thermoresistible	ATCC 19527
Mycobacterium triviale	ATCC 23292
M. avium-M. intracellulare-M. scrofulaceum serovar 1 (43)	Human isolate[a]
M. avium-M. intracellulare-M. scrofulaceum serovar 4 (43)	Human isolate[a]
Mycobacterium sp. strain 3937	Primate enteritis[b]
Mycobacterium paratuberculosis 18	Source unknown[c,d]
Chromogen	Bovine feces[c,e]
Actinomyces pyogenes[f]	ATCC 19411
Corynebacterium renale	ATCC 19412
Rhodococcus equi	Equine lung[c]

[a] Courtesy of P. J. Brennan.
[b] Mycobactin dependent. Courtesy of R. J. Chiodini.
[c] Laboratories of Veterinary Diagnostic Medicine, University of Illinois, Urbana.
[d] Mycobactin independent. Original source is unknown.
[e] Saprophytic fecal isolate.
[f] Formerly Corynebacterium pyogenes (8, 30).

continuous sequence were used for all pairwise comparisons. Homologous nucleotide positions were brought into correspondence by aligning the sequences on conserved features of primary and secondary structure. The evolutionary distance (mutations per nucleotide position) separating each pair of organisms was estimated as $-3/4 \ln (1 - 4L/3)$, where L is the observed fractional sequence difference between aligned sequences (18). This treatment partially corrects the observed number of sequence differences for multiple and back mutations (18). Alignment gaps (insertions or deletions) were assigned one-half the value of a nucleotide difference. Deletions of five or more consecutive nucleotides were treated as five gaps.

A phylogenetic tree was inferred by a distance matrix method (10, 11). Briefly, the evolutionary distance estimates were fitted to an initial branching order, the optimal segment lengths were determined, and the goodness of fit of this topology to the evolutionary distance estimates was evaluated. The error of the tree was defined as the sum of the squares of the differences between the pairwise evolutionary distance estimates and the corresponding tree reconstructions of those distances (i.e., the sums of the tree branches which connect the pairs of organisms), with each difference being weighted by its corresponding statistical uncertainty (19).

Starting with this topology (branching order) and measure of its goodness of fit to the input distance data, an optimal topology was sought. A computer program was used to examine all rearrangements of a tree derived by moving any single group of organisms (subtree) to every alternative location in the tree or by interchanging any pair of groups (subtrees). This optimization program examines all the above rearrangements and then takes the best of the exam-

ined alternatives as the starting point for another cycle of searching for better branching orders (G. J. Olsen, Ph.D. thesis, University of Colorado Health Sciences Center, Denver, 1983). When a cycle of optimization fails to find a better tree (i.e., a branching order with a lower error than the starting tree), the best tree is assumed to have been found. The phylogeny shown in Fig. 1 was derived from the similarity values of Table 2.

In general, the tree was stable to change in the composition of sequences and different weightings of insertions and deletions in the calculation of similarity (instability was demonstrated for the branching order of M. senegalense-M. neoaurum). However, certain compositions of outgroup organisms included in the analysis did distort relationships inferred among the mycobacteria. Therefore, the final topology was arrived at in two stages. First, a global topology of the mycobacteria (excluding M. chitae) was constructed. The final tree and branch lengths among the mycobacteria reflect this analysis. The relationships of the mycobacteria to the remaining organisms (arthrobacter, actinomyces, corynebacteria, rhodococci) were established by constructing a tree including several representative mycobacteria (M. bovis, M. aurum, and M. phlei). The branch order and branch lengths outside the mycobacterial cluster reflect this analysis.

RESULTS AND DISCUSSION

Mycobacterial phylogeny and its relationship to mycobacterial taxonomy. The 16S rRNA similarity values (Table 2) show the mycobacteria to be a closely related, coherent group, distinct from the corynebacteria, actinomyces, and rhodococci examined. Average percent similarity values for the mycobacteria are greater than 95% (compared, for example, with a 93% similarity value separating Escherichia coli from Proteus vulgaris). Only M. chitae falls outside this cluster. The similarity values between the mycobacteria and members of the related genera are between 85 and 90%. Of those organisms compared, Rhodococcus equi was most closely related to the mycobacteria (90 to 93% similarity).

Relationships among the mycobacteria are most clearly displayed by the phylogenetic tree (Fig. 1). A striking feature is the natural coherence among slow-growing species; they define a distinct line of evolutionary descent. This supports the traditional separation of slow-growing from fast-growing species. Although the fast-growing species are polyphyletic as represented in the tree, the segment separating their two lines of descent is not long enough to establish this division with certainty. However, signature analysis (below) would unite the fast-growing species to the exclusion of the slow-growing line of descent. If the topology near the base of the mycobacterial radiation is correct, this suggests that fast growth is the more primitive state. The alternative (slow growth primitive) would necessitate two independent origins of fast growth.

Virulence also is an attribute reflected by the phylogeny; the overt pathogens (M. bovis, M. kansasii, M. avium-M. intracellulare-M. scrofulaceum complex, M. paratuberculosis) are closely related and clustered within the slow-growing line of descent. Thus, in good part, the specific relationships inferred are consistent with existing mycobacterial classification. M. chitae is not considered since these results suggest it is misclassified with the mycobacteria. Selected phenotypic characteristics corresponding to phylogenetic relationships among the mycobacteria are given in Table 3.

One branch of related rapid growers consists of M. phlei, M. flavescens, M. thermoresistible, M. fallax, and an isolate
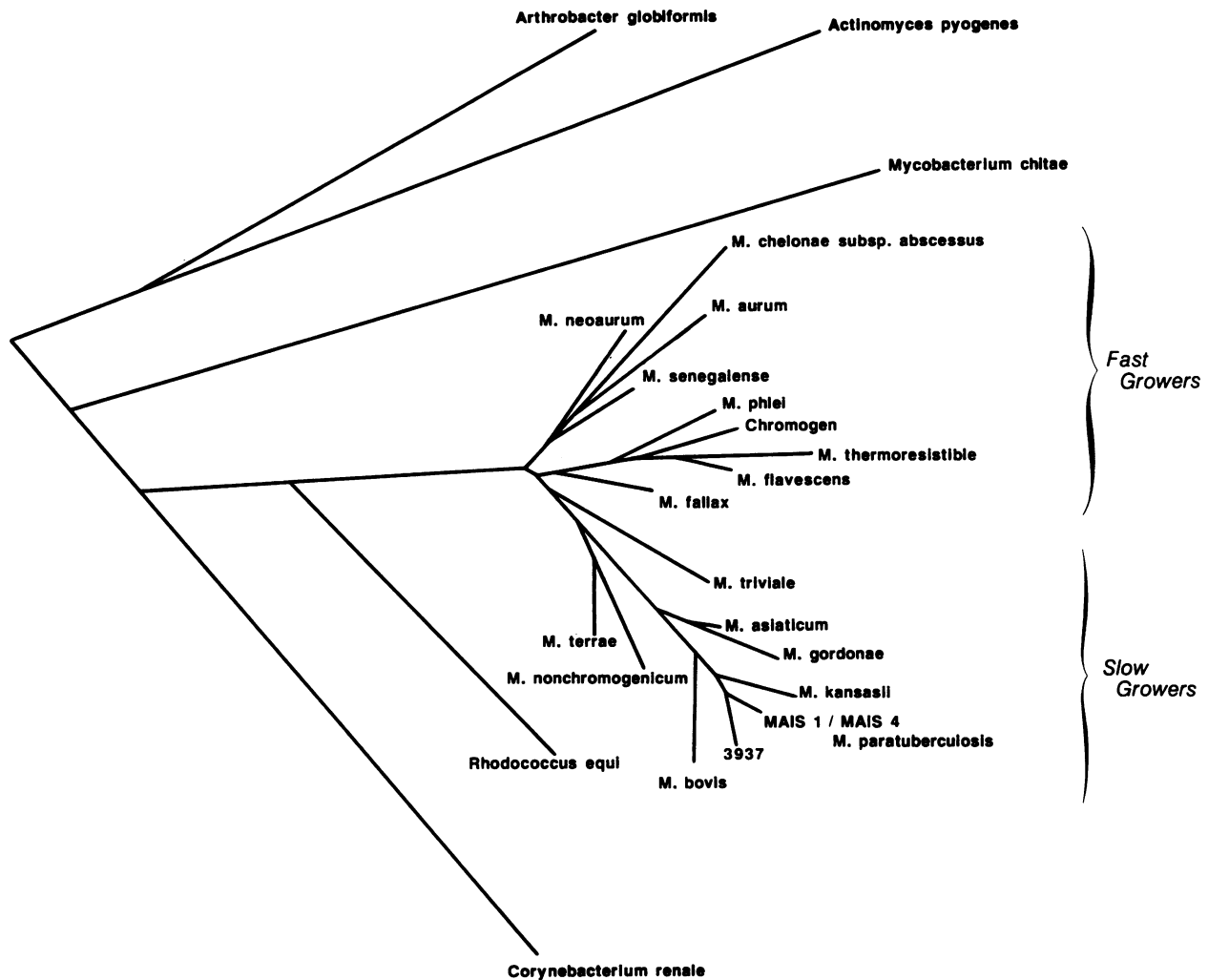
FIG. 1. Phylogeny of 16S rRNAs of characterized mycobacteria and related organisms. The tree was derived by the distance matrix method (Materials and Methods). MAIS, *M. avium-M. intracellulare-M. scrofulaceum*.

referred to here as the chromogen. The chromogen is a rapidly growing, saprophytic *Mycobacterium* species isolated from bovine feces and is yet to be formally identified. *M. phlei*, *M. flavescens*, and *M. thermoresistible* have often been grouped together in numerical classification schemes, showing 75 to 84% similarity (35, 44, 47). They represent thermotolerant scotochromogens. All grow at 45°C, and *M. phlei* and *M. thermoresistible* are the only mycobacteria known to grow at 52°C. *M. flavescens* has an intermediate growth rate and has been classified with both the slow and the rapid growers, but its metabolism and physiological activities are most like those of the rapid growers (14). This is in accordance with the inferred phylogenetic position of *M. flavescens*. *M. fallax* falls outside the thermotolerant cluster and in contrast to the thermotolerant species grows rapidly at 30°C and slowly at 37°C. Phenotypic similarity (47 characters compared) between *M. fallax* and *M. flavescens* (55.32%) or *M. thermoresistible* (55.32%) was less than the similarity to other fast- and slow-growing species (e.g., 72.21% similarity to *M. triviale*). Thus, no overt phenotypic characteristics unite *M. fallax* with the thermotolerant species (24).

The second branch of the rapid growers consists of two nonpigmented species, *M. chelonei* subsp. *abscessus* and *M.*

senegalense, and two scotochromogens, *M. aurum* and *M. neoaurum*. Except for their growth rates and pigmentation, there is little in the literature to group these organisms together. A numerical classification of *M. farcinogenes* and related taxa based on 69 characters showed a high overall similarity between *M. senegalense* and *M. chelonei* clusters (31).

Within the slow-growing line of descent, *M. terrae* and *M. nonchromogenicum* diverge from a common line. These two organisms, along with *M. triviale*, make up what has been referred to as the *M. terrae* complex (14, 55). All three are nonpigmented slow growers. Numerical classification studies linked *M. nonchromogenicum* and *M. terrae* so closely that species distinction between them has been questioned (27,46). Based on the results of this study (98% similarity), the species distinction appears justified relative to other species of mycobacteria. The evolutionary distance separating them is approximately equal to that separating *M. avium-M. intracellulare-M. scrofulaceum* serovar 4 and *M. kansasii* or *M. asiaticum* and *M. gordonae*. *M. triviale* shares substantially less sequence similarity to the other two members of the complex (96 to 97%). Phenotypically, however, it differs very little from *M. terrae* and *M. nonchromogenicum* except that it is the only slow grower known to

TABLE 2. 16S rRNA similarity values for some mycobacteria and related organisms

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. A. globiformis	0.896																								
2. A. pyogenes	0.897	0.878																							
3. C. renale	0.901	0.875	0.892																						
4. R. equi	0.867	0.865	0.874	0.878																					
5. M. chitae	0.885	0.872	0.905	0.934	0.878																				
6. M. phlei	0.881	0.872	0.929	0.873	0.873	0.987																			
7. Chromogen	0.883	0.868	0.902	0.932	0.873	0.981	0.981																		
8. M. flavescens	0.878	0.865	0.895	0.923	0.868	0.975	0.977	0.974																	
9. M. thermoresistible	0.890	0.877	0.907	0.934	0.878	0.968	0.983	0.974	0.970																
10. M. fallax	0.883	0.853	0.899	0.930	0.875	0.959	0.957	0.965	0.952	0.962															
11. M. aurum	0.886	0.865	0.901	0.934	0.875	0.954	0.949	0.953	0.952	0.963	0.966														
12. M. chelonei subsp. abscessus	0.881	0.869	0.899	0.934	0.876	0.966	0.967	0.971	0.967	0.976	0.968	0.969													
13. M. neoaurum	0.888	0.868	0.907	0.933	0.879	0.974	0.976	0.962	0.981	0.975	0.969	0.978	0.978												
14. M. senegalense	0.869	0.868	0.902	0.936	0.881	0.963	0.971	0.965	0.960	0.971	0.957	0.953	0.966	0.970											
15. M. triviale	0.893	0.869	0.902	0.936	0.881	0.963	0.971	0.965	0.960	0.971	0.957	0.953	0.966	0.972	0.970										
16. M. terrae	0.878	0.866	0.903	0.925	0.872	0.974	0.971	0.966	0.959	0.980	0.957	0.952	0.966	0.972	0.966	0.964									
17. M. nonchromogenicum	0.881	0.872	0.902	0.927	0.878	0.968	0.968	0.964	0.956	0.975	0.955	0.954	0.964	0.971	0.966	0.979	0.983								
18. M. asiaticum	0.873	0.857	0.897	0.925	0.871	0.963	0.966	0.959	0.958	0.968	0.963	0.949	0.965	0.964	0.972	0.979	0.964	0.968							
19. M. gordonae	0.873	0.857	0.893	0.924	0.868	0.955	0.961	0.955	0.954	0.963	0.957	0.960	0.960	0.957	0.959	0.972	0.964	0.968	0.988						
20. M. bovis	0.869	0.854	0.897	0.918	0.870	0.954	0.951	0.948	0.947	0.959	0.943	0.945	0.953	0.959	0.972	0.968	0.957	0.981	0.975	0.984					
21. M. kansasii	0.869	0.853	0.889	0.920	0.867	0.953	0.953	0.947	0.946	0.957	0.963	0.946	0.960	0.955	0.955	0.963	0.957	0.981	0.975	0.981	0.986				
22. Mycobacterium sp. strain 3937	0.873	0.865	0.895	0.924	0.874	0.954	0.955	0.947	0.947	0.957	0.955	0.949	0.956	0.955	0.956	0.964	0.961	0.980	0.976	0.978	0.986				
23. MAIS[a] 4	0.873	0.859	0.896	0.922	0.875	0.954	0.956	0.948	0.949	0.956	0.958	0.950	0.955	0.957	0.959	0.965	0.963	0.981	0.975	0.982	0.987	0.992			
24. MAIS[a] 1	0.874	0.859	0.895	0.923	0.875	0.954	0.955	0.949	0.949	0.957	0.958	0.951	0.955	0.957	0.958	0.965	0.963	0.982	0.976	0.983	0.988	0.993	0.999		
25. M. paratuberculosis	0.872	0.858	0.893	0.921	0.872	0.953	0.954	0.948	0.948	0.957	0.958	0.951	0.955	0.956	0.956	0.964	0.962	0.982	0.977	0.981	0.988	0.993	0.999	0.999	1.000

[a] MAIS, M. avium-M. intracellulare-M. scrofulaceum.

TABLE 3. Phenotypic characteristics corresponding to phylogenetic relationships among the mycobacteria (24, 28, 34, 55)

Organism	Pigmentation		Growth at 45°C	Growth at 52°C	Catalase	Mycolic acids present					
	Photochro-mogenic	Scotochro-mogenic				α	α'	Methoxy	Keto	Wax-ester	Epoxy
Fast growers											
M. chelonei subsp. abscessus	−	−	−	−	M	+	+	−	−	−	−
M. senegalense	−	−	−	−		+	+	−	−	−	+
M. aurum	+	−	−	−		+	−	−	+	+	−
M. neoaurum	+	−	−	−		+	−	−	+	+	−
M. thermoresistible	−	+	+	+		+	+	+	+	−	−
M. flavescens	−	+	d^a	−	M	+	−	−	+	+	−
M. phlei	−	+	+	+	M	+	−	−	+	+	−
M. fallax	−	−	−	−	T	+	−	−	−	−	−
Slow growers											
M. triviale	−	−	−	−	M	+	−	−	−	−	−
M. terrae	−	−	−	−	M	+	−	−	+	+	−
M. nonchromogenicum	−	−	−	−	M	+	−	−	+	+	−
M. asiaticum	d	d	−	−	M and T	+	−	+	+	−	−
M. gordonae	−	+	−	−	M and T	+	−	+	+	−	−
M. kansasii	+	−	−	−	M and T	+	−	+	+	−	−
M. bovis	−	−	−	−	T	+	−	+	+	−	−
M. avium	−	−	±	−	T	+	−	−	+	+	−
M. paratuberculosis	−	−	−	−		+	−	−	+	+	−

^{a} d, Variable.

grow in the presence of 5% NaCl, a trait it shares with the rapid growers (55).

The species distinction between M. asiaticum, a photochromogen, and M. gordonae, a scotochromogen, has also been questioned (14). They cannot be distinguished by intradermal skin tests (3) and differ little biochemically (14, 50), yet the 16S rRNA similarity (98%) is comparable to that between M. kansasii and the M. avium-M. intracellulare-M. scrofulaceum complex or M. terrae and M. nonchromogenicum.

M. bovis, a nonphotochromogen, and M. kansasii, a photochromogen, are closely related to the M. avium-M. intracellulare-M. scrofulaceum complex (98.8%). These and the remaining members of the slow-growing branch (M. avium-M. intracellulare-M. scrofulaceum complex, M. paratuberculosis 18, and the M. paratuberculosis-like isolate 3937) form a closely related collection of mycobacteria. The 16S rRNA sequence of the M. paratuberculosis strain used in this study is identical to that of M. avium-M. intracellulare-M. scrofulaceum serovar 1 and differs from M. avium-M. intracellulare-M. scrofulaceum serovar 4 at only one nucleotide position. Strain 3937 is a mycobactin-dependent mycobacterium isolated from a stump tail macaque with chronic gastric enteritic disease. Although growth rate, mycobactin dependence, and site of isolation affiliate it with M. paratuberculosis, it is more distantly related to M. paratuberculosis than are the M. avium-M. intracellulare-M. scrofulaceum serovars (10 to 11 mismatches).
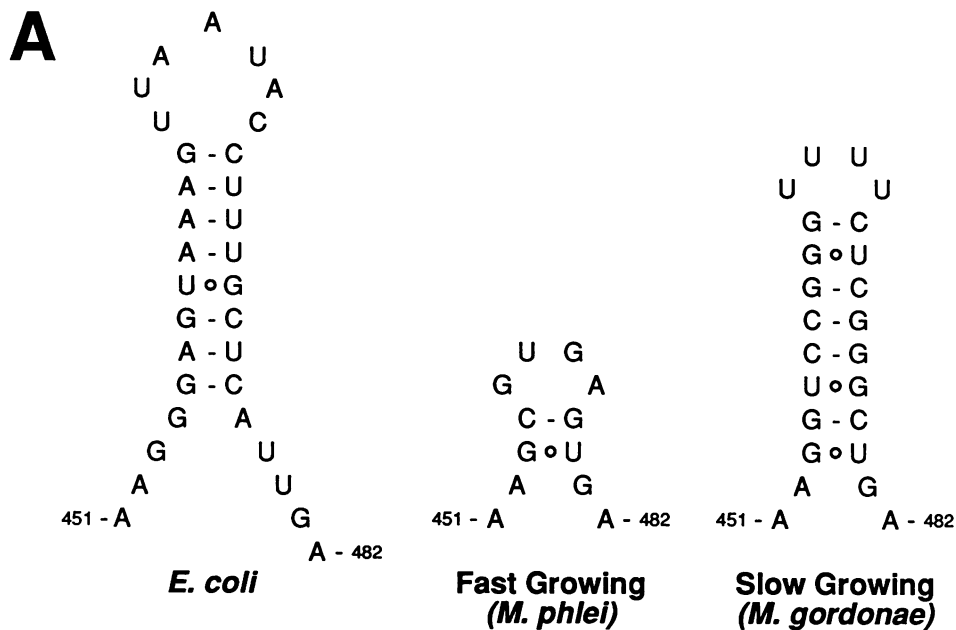
This study and others strongly suggest that M. paratuberculosis should be placed within the M. avium complex. For example, antigenic and DNA homology studies have shown strains of M. paratuberculosis to be similar to M. avium (5, 25, 36, 37, 48). Continuing comparative 16S rRNA sequencing studies have shown most field isolates of M. paratuberculosis to be indistinguishable from M. avium-M. intracellulare-M. scrofulaceum serovar 1 and strain 18 (unpublished data). This contrasts with the observation of marked differences between the restriction endonuclease fingerprints of strain 18 and wild-type strains (7, 56). Also, a type-specific

antigen from strain 18 is missing from many wild-type strains but is present in various M. avium-M. intracellulare-M. scrofulaceum complex organisms (5). Thus, additional comparative analyses are required to resolve the relationships within this closely related group of organisms. Preliminary sequencing of the more variable 23S rRNA from these isolates suggests that this will provide additional discerning sequence attributes (unpublished observations).

The distribution of mycolic acids and T- or M-class catalases are additional characteristics that correspond with the phylogeny inferred by 16S rRNA sequence comparisons (51–54). For example, of the species characterized, M. terrae, M. nonchromogenicum, and M. triviale failed to produce T-class catalase; M. gordonae, M. asiaticum, and M. kansasii produced both T-class and M-class catalase; the overt pathogens M. avium, M. intracellulare, and M. tuberculosis produced only M-class catalase (Table 3).

Primary and secondary rRNA structures corresponding to major phylogenetic divisions within the mycobacterial line of descent. Positional sequence divergence among 16S rRNAs is not random. Certain regions of the molecule are invariant or highly conserved, while others are highly variable (58). The most variable regions generally differ between species or subspecies of bacteria (38, 40), whereas regions of intermediate conservation can serve to mark higher-order assemblages. Members of the same genus (or higher natural groupings) generally share sequence elements unique to that group. Thus, although the tree representation of relationship is derived from complete sequence comparisons, the inferred relationships are also reflected in common regional sequence or structure among members of a common line of descent. In general, bacteria that compose a coherent phylogenetic assemblage can be circumscribed by one or several defining structural elements. Two examples of such signature structures are presented in Fig. 2 and 3. These examples demonstrate both length (insertion or deletion) and sequence variation as defining elements of a signature.

The division between the fast- and slow-growing mycobacteria is marked by length and sequence variation within
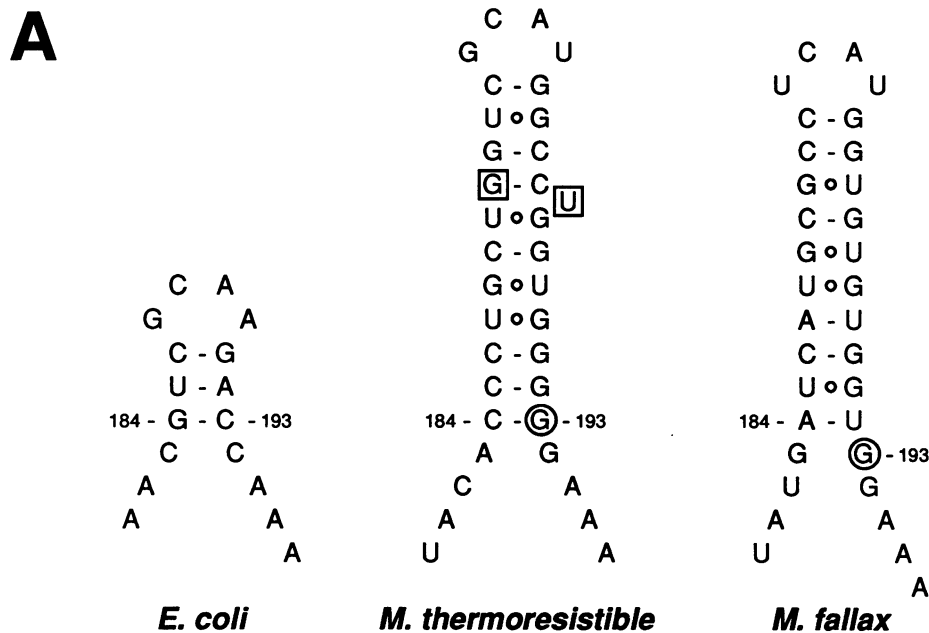
A

```
              A
         A         U
      U               A
      U               C
         G - C                              U     U
         A - U                            U           U
         A - U                              G - C
         A - U                              G o U
         U o G                              G - C
         G - C                              C - G
         A - U           U   G              C - G
         G - C         G       A            U o G
       G     A         C - G                G - C
     G         U         G o U              G o U
   A             U     A       G          A       G
 451 - A           G  451 - A     A - 482  451 - A     A - 482
              A - 482

      E. coli          Fast Growing        Slow Growing
                        (M. phlei)          (M. gordonae)
```

B

Sequence
identity:

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 1 E. coli    GAGGAAGG GA GUAAAG UUAA  UAC CUUUGCU C AUUGACGUU ACCCGCAGAA
 2 Arth. glo  GAAGAA GC G- ---------- ---------A AA GU GACGGU ACCUGCAGAA
 3 A. pyogen  GAAGAA GCAu ---------- ---------uU UU GU GACGGU AUGUGCAGAA
 4 R. equi    GACGAA GC G- ---------- ---------A GA GU GACGGU ACCUGCAGAA
 5 M. phlei   GACGAA GC G- ---------- ---------U GA GU GACGGU ACCUACAGAA
 6 Chromogen  GACGAA GC G- ---------- ---------U AA GU GACGGU AGGUACAGAA
 7 M. flaves  GACGAA GC G- ---------- ---------C AA GU GACGGU ACCGACAGAA
 8 M. thermo  GACGAA GC G- ---------- ---------G AA GU GACGGU AGGCACAGAA
 9 M. fallax  GACGAA GC G- ---------- ---------Y AA GU GACGGU AGGUACAGAA
10 M. aurum   GACGAA GC G- ---------- ---------C AA GU GACGGU ACCUgGAGAA
11 M. chelon  GACGAA GC G- ---------- ---------A AA GU GACGGU ACCUNCANAA
12 M. neoaur  GACGAA GC G- ---------- ---------C AA GU GACGGU AUGUGCAGAA
13 M. senega  GACGAA GC G- ---------- ---------C AA GU GACGGU ACCUAUAGAA
14 M. trivia  GACGAA GC G- ---------- ---------C AA GU GACGGU AGUGACAGAA
15 M. terrae  GGCGAA GCUC CGUNG UU--- ---UU CUGCG GGGU GACGGU AGGUACAGAA
16 M. nonchr  GGCGAA GCUC CAAGG UN--- ---UU CUUUG GGGU GACGGU AGGUACAGAA
17 M. asiati  GACGAA GGUC CG-GG UU--- ---UU CU-CG GAUU GACGGU AGGUGGAGAA
18 M. gordon  GACGAA GGUC CG-GG UU--- ---UU CU-CG GGCU GACGGU AGGUGGAGAA
19 M. bovis   GACGAA GGUC CG-GG UU--- ---CU CU-CG GAUU GACGGU AGGUGGAGAA
20 M. kansas  GACGAA GGUC CG-GG UU--- ---CU CU-CG GAUU GACGGU AGGUGGAGAA
21 Myco 3937  GACGAA GGUC CG-GG UU--- ---UU CU-CG GAUU GACGGU AGGUGGAGAA
22 M. paratb  GACGAA GGUC CG-GG UU--- ---UU CU-CG GAUU GACGGU AGGUGGAGAA
23 MAIS 4     GACGAA GGUC Cg-gG UU--- ---UU CU-CG GAUU GACGGU AGGUNGAGAA
24 MAIS 1     GACGAA GGUC CG-GG UU--- ---UU CU-CG GAUU GACGGU AGGUNgAGAA
25 M. chitae  GACGAA GC G- ---------- ---------U GA GU GACGGU AAUGGGUAAA
26 C. renale  GACGAA GCU- ---------- ---------U UU GU GACGGU ACCUaGAGAA
```

FIG. 2. Signature for slow-growing mycobacteria. Length variation in mycobacterial helices homologous to the helix in *E. coli* bounded by positions 455 to 477 (*E. coli* numbering). The extended helix is present only among mycobacteria composing the slow-growing line of descent. The structure for a representative fast-growing mycobacterium is for *M. phlei*. *M. gordonae* is displayed as a representative slow-growing species. MAIS, *M. avium-M. intracellulare-M. scrofulaceum*.

the helix flanked by positions 451 to 482 in the *E. coli* 16S rRNA numbering (Fig. 2). An extended helix is found only among members of the slow-growing mycobacteria (with the exception of *M. triviale*) so far characterized (Fig. 2). The natural division between fast- and slow-growing species is also reported to be reflected by rRNA gene copy number: one genomic copy among the slow-growing strains (*M. tuberculosis, M. bovis, M. intracellulare*) and two copies among the fast-growing species (*M. phlei, M. smegmatis*) (4, 42).

The thermotolerant fast-growing species (*M. phlei, M. flavescens, M. thermoresistible*, and the chromogen) are united by sequence and length variation within the helix defined by positions 184 to 193 in the *E. coli* 16S rRNA numbering (Fig. 3). *M. fallax* lacks this signature and thus is distinct from the thermotolerant species by this criterion as well as by the other phenotypic attributes described above. The use of such signature structures in microbial systematics, diagnosis, and studies of microbial ecology has been discussed previously (38–40, 58).

A

```
                    C  A
                 G      U
                 C - G
                 U o G
                 G - C
                 [G]- C
                 U o G [U]
                 C - G
          C  A   G o U
       G     A   U o G
       C - G     C - G          C  A
       U - A     C - G       U     U
184 - G - C -193 184 - C -(G)-193  C - G
       C   C     A    G            C - G
      A     A   C      A           G o U
     A       A  A       A          C - G
      A         U        A         G o U
                                   U o G
                                   A - U
                                   C - G
                                   U o G
                          184 - A - U
                                 G   (G)-193
                                 U   G
                                A     A
                                U      A
                                        A
    E. coli      M. thermoresistible   M. fallax
```

B

```
Sequence
identity:

 1 E. coli    GCAUAA|GU- C|GC------ ---------- AA|GAC|CAAAG AGGG
 2 Arth.glo   GGAUAUG|AC- UCCUC-AUC|G CAU|GGUG-GG GGGU|GGAAAG CUU-
 3 A. pyogn   GGAUAUU|CUG CUUUU-GCC|G CAU|GGUG-GG gguU|GGNaAG AUu-
 4 R. equi    GGAUAUG|AG- CUCCU-GUC|g CAU|GGCG-GG GGUU|GGAAAG GUU-
 5 M. phlei   GGAUACA|CC- CUUCUGGUU|G CAU|GGCU-GG GAGG|GgAAAG CUu-
 6 Chromogen  GAAUAGU|CC- CUgCUGGUC|G CAU|GGCCUGG UGGG|GNAAAG CUU-
 7 M. flaves  GAAUAUU|CC- CUAUUGGUC|G CAU|GGCCUGG UAGG|GGAAAG CUN-
 8 M. thermo  GAAUACA|CC- CUGCUGGUC|G CAU|GGCCUGG UGGG|GGAAAG CGU-
 9 M. fallax  GGAUAUG|AU- CAUGC-GCC|U CAU|GGUG-UG UGGU|GGAAAG CUU-
10 M. aurum   GAAUAGG|AC- UACGC-GAU|G CAU|GUCG-UG UGGU|GGAAAG CUU-
11 M. chelon  GGAUAGG|AC- CACAC-ACU|U CAU|GGUG-AG UGGU|GCAAAG CUu-
12 M. neoaur  GAAUAUG|AU- CAUCG-GCU|U CAU|GGUC-GG UGGU|GGAAAG CUU-
13 M. senega  GGAUAGG|AC- CACGC-GCU|U CAU|GGUG-UG UGGU|GGAAAG CUU-
14 M. trivia  GGAUAGG|AC- CACAU-GUC|g CAU|GGUG-UG UGGU|GGAAAG CUN-
15 M. terrae  GGAUAGG|AC- CAUGG-GAU|G CAU|gUUC-UG UGGU|GGAAAG CUU-
16 M. nonchr  GAAUAGG|AC- CGCAU-GCU|G CAU|GGUG-UG UGGU|GGAAAG CUU-
17 M. asiati  GGAUAGG|AC- CACGG-GAU|G CAU|gUCC-UG UGGU|gGAAAG CUU-
18 M. gordon  GAAUAGG|AC- CACAG-GAC|A CAU|gUCC-UN UGGU|GGAAAG CUU-
19 M. bovis   GGAUAGG|AC- CACGG-GAU|G CAU|GUCU-UG UGGU|GGAAAG CGC-
20 M. kansas  GGAUAGG|AC- CACUU-GGC|G CAU|GCCU-UG UGGU|GGAAAG CUU-
21 Myco 3937  GGAUAGG|AC- CUUUA-GGC|G CAU|GUCU-UU AGGU|GGAAAG CUN-
22 M. paratb  GGAUAGG|AC- CUCAA-GAC|G CAU|GUCU-UC UGGU|GGAAAG CUU-
23 MAIS 4     GGAUAGG|AC- CUCAA-GAC|G cAU|GUCU-UC UGGU|GGAAAG CUu-
24 MAIS 1     GGAUAGG|AC- CUCAA-GAC|G CAU|GUCU-UC UgGU|GGAAAG CUN-
25 M. chitae  GGAUAGG|AG- CUCCU-GCU|G CAU|GGUG-GG GGUU|GGAAAG UUU-
26 C. renale  GGAUAGG|AC- CAUCG-UU|UA G--U|GUC-GG UGGU|GGAAAG CUU-
```

FIG. 3. Signature for thermotolerant fast-growing mycobacteria. Boxed nucleotides correspond to inserted nucleotides relative to other mycobacteria characterized. The mycobacterial helix corresponding to the *E. coli* helix (3 base pairs bounded by nucleotides 184 to 193) is 10 base pairs in all mycobacteria except the thermotolerant fast-growing species (*M. phlei, M. flavescens, M. thermoresistible*). The helix is 11 base pairs in the thermotolerant mycobacteria as a consequence of a G insertion (and inclusion of the universal G [circled] in base pairing). The bulged U is absent in *M. phlei* but present in the remaining thermotolerant species. MAIS, *M. avium-M. intracellulare-M. scrofulaceum*.

Relationship between nucleic acid hybridization studies and RNA sequence similarity. The use of DNA-DNA, rDNA-DNA, and rRNA-DNA homology measures to establish natural affiliations among microorganisms is well accepted. Table 4 lists the comparisons relevant to the present study. Although numerous studies have applied these measures to the classification of the mycobacteria, no clear pattern of natural relationships within the genus has emerged (49).

Some generalities can be made, however, with the caveat of very limited sample and the experimental variation among independent measures of DNA-DNA homology: homology within strains of the same species, 69 to 100%; between slow-growing species, 20 to 55%; between rapid-growing species, 10 to 20%; between slow and rapid growers, <20%; between mycobacteria and other genera, <1% (50).

The comparative sequencing of mycobacterial 16S rRNAs

TABLE 4. Comparison of DNA-DNA homologies and 16S rRNA percent similarity for selected mycobacteria

Organism	Strain	M. fallax[a]		M. kansasii[b]	
		% DNA homology	rRNA similarity	% DNA homology	rRNA similarity
M. chelonei	NCTC 946	7	0.952		
M. chitae	ATCC 19627[c]	22	0.867		
M. flavescens	ATCC 14474[c]	14	0.972	13	0.941
M. neoaurum	ATCC 25795[c]	19	0.974		
M. phlei	ATCC 11758[c]	22	0.969	15	0.947
M. senegalense	NCTC 10956	21	0.979		
M. thermoresistible	ATCC 19527[c]	13–20	0.961		
M. gordonae	ATCC 19277			36	0.974
M. aurum	ATCC 23366[c]	18			

[a] DNA homology values from Levy-Frebault et al. (22).
[b] DNA homology values from Gross and Wayne (15).
[c] Strains used for comparative 16S rRNA sequencing.

suggested the outlines of a natural classification of the mycobacteria. In general, it is in good correspondence with the existing classification. Comparative rRNA sequencing should therefore serve to unify mycobacterial systematics by placing available phenotypic measures of identity within a phylogenetic framework. Finally, elements contributing to virulence might be underscored by comparisons between members of the pathogenic group and closely related saprophytes.

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