Phylogeny of Rapidly Growing Members of the Genus *Mycobacterium*

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The 16S rRNAs from nine rapidly growing *Mycobacterium* species were partially sequenced by using the dideoxynucleotide-terminated, primer extension method with cDNA generated by reverse transcriptase. The sequences were aligned with 47 16S rRNA or DNA sequences that represented 30 previously described and 5 undescribed species of the genus *Mycobacterium*, and a dendrogram was constructed by using equally weighted distance values. Our results confirmed the phylogenetic separation of the rapidly and slowly growing mycobacteria and showed that the majority of the slowly growing members of the genus represent the most recently evolved organisms. The 24 strains which represented 21 rapidly growing species constituted several sublines, which were defined by the following taxa: (i) *Mycobacterium neoaurum* and *M. diernhoferi*, (ii) *M. gadium*, (iii) the *M. chubuense* cluster, (iv) the *M. fortuitum* cluster, (v) *M. kommosense*, (vi) *M. sphagni*, (vii) *M. fallax* and *M. chitae*, (viii) *M. aurum* and *M. vaccae*, (ix) the *M. flavescens* cluster, and (x) *M. chelonae* subsp. *abscessus*. Our phylogenetic analysis confirmed the validity of the phenotypically defined species mentioned above, but our conclusions disagree with most of the conclusions about intragenetic relationships derived from numerical phenetic analyses.

In the early 1980s, using 16S rRNA cataloging methods, workers determined that *Mycobacterium phlei*, one of the fast-growing *Mycobacterium* species, is a member of the phylogenetically defined order *Actinomycetales* (39). Together with other mycolic acid-containing organisms (i.e., members of the genera *Rhodococcus* and *Nocardia*), the members of the genus *Mycobacterium* form one of the several sublines of descent within the radiation of the actinomycetes. Later, the results of reverse transcriptase sequencing of 16S rRNAs from a larger number of strains confirmed the phylogenetic coherency of this subline, which was extended by the inclusion of the genera *Corynebacterium* (35), *Gordona* (36), and *Tsukamurella* (5). Within the genus *Mycobacterium*, species fall into one of two large groups which by and large correspond to the traditional groups containing the slowly growing and rapidly growing members of the genus (35).

With the introduction of even faster analytical methods (i.e., analysis of polymerase chain reaction-mediated DNA stretches [4, 28]), the number of actinomycetes analyzed and consequently the knowledge about the natural relationships of these organisms have increased considerably during the last 3 years. From a phylogenetic point of view, the genus *Mycobacterium* is one of the most thoroughly investigated bacterial taxa. There are 55 or so validly described species in this genus (51), and 45 strains of 34 species have been subjected to either 16S rRNA analysis (31, 35, 40), 16S rDNA analysis (4, 28), or 23S rDNA analysis (21). The bifurcation within the genus *Mycobacterium* has been confirmed, with the majority of the slowly growing strains forming a phylogenetically very shallow (i.e., recent) group. In addition to the phylogenetic aspect, sequence information from 16S and 23S rRNAs and rDNAs has been used to identify individual species and species complexes by using synthetic oligonucleotide probes (21, 37) and primers used in

the polymerase chain reaction-mediated amplification of specific regions (4). These techniques supplement the battery of rapid diagnostic tools used for the identification of pure cultures and of pathogens in biopsies, and they dramatically shorten the time-consuming process of phenotypic characterization.

Emphasis has been placed on the phylogenetic characterization of the slowly growing mycobacteria, many of which are human and animal pathogens. On the other hand, the 16S rRNA or DNA sequences of only 10 of the 29 validly described species of rapidly growing mycobacteria (16, 51) have been published previously (16, 28, 40).

In this paper we describe the primary structures of the 16S rRNAs from an additional 12 strains of eight mainly fast-growing *Mycobacterium* species and of an as-yet-undescribed *Mycobacterium* strain. The sequences were aligned with the existing data base of mycobacterial 16S rRNA and DNA sequences, and we generated the most comprehensive phylogenetic tree that has been generated to date.

MATERIALS AND METHODS

Bacterial strains. *Mycobacterium* strains (Table 1) were grown on Löwenstein-Jensen medium at the optimum growth temperatures; *Mycobacterium aichiense*, *M. chubuense*, *M. gilvum*, *M. obuense*, and *M. vaccae* strains and *Mycobacterium* sp. strain Madagascar were grown at 31°C, and all other strains were grown at 34°C. Cells were washed from the plates with a 0.9% NaCl solution at pH 7.2, harvested by centrifugation, washed twice in the same solution, and then stored at –70°C.

Sequence analysis. Crude RNA was isolated from 1 to 2 g (wet weight) of cells as described previously (33). The primary structure of the 16S rRNA was determined by sequencing cDNA generated by reverse transcription, using avian myeloblastosis virus reverse transcriptase (Promega) (18, 33) and terminal nucleotidyl transferase (Boehringer) (6). Oligonucleotide primers complementary to conserved

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TABLE 1. Strains analyzed in this study and reference strains used for constructing a rooted phylogenetic tree

Strain ^a	Nucleotide sequence accession no.	Reference and/or source ^a
<i>M. aichiense</i> ATCC 27280 ^T	X55598	This study
<i>M. asiaticum</i> ATCC 25276 ^T	M29556	40
<i>M. asiaticum</i> ATCC 25276 ^T	X55604	This study
<i>M. aurum</i> ATCC 23366 ^T	M29558	40
<i>M. aurum</i> ATCC 23366 ^T	X55595	This study
<i>M. avium</i> Chester (= DSM 43216)	X52918	28
<i>M. bovis</i> Vallee	X55589	31; FIB
<i>M. bovis</i> BCG	X52917	41
<i>M. chelonae</i> subsp. <i>abscessus</i> ATCC 19977 ^T	M29559	40
<i>M. chelonae</i> ATCC 14472	X52921	28
<i>M. chitae</i> ATCC 19627 ^T	M29560	40
<i>M. chitae</i> ATCC 19627 ^T	X55603	This study
<i>M. chubuense</i> ATCC 27278 ^T	X55596	This study
<i>M. cookii</i> ATCC 49103	X53896	16
<i>M. diernhoferi</i> ATCC 19340 ^T	X55593	This study
<i>M. fallax</i> ATCC 35219 ^T	M29562	40
<i>M. farcinogenes</i> DSM 43294 ^T	X55592	31
<i>M. flavescens</i> ATCC 14474 ^T	M29561	40
<i>M. flavescens</i> ATCC 14474 ^T	X52932	28
<i>M. fortuitum</i> ATCC 6841 ^T	X52933	28
<i>M. gadium</i> ATCC 27726 ^T	X55594	This study
<i>M. gilvum</i> ATCC 43909 ^T	X55599	This study
<i>M. gordonae</i> ATCC 14470 ^T	M29563	40
<i>M. gordonae</i> ATCC 14470 ^T	X52923	28
<i>M. intracellulare</i> ATCC 15985	X52927	28
<i>M. kansasii</i> DSM 43224	X15916	8
<i>M. kansasii</i> ATCC 12478 ^T	M29575	40
<i>M. komossense</i> ATCC 33013 ^T	X55591	31
<i>M. leprae</i> LTB	X55587	16
<i>M. malmoense</i> ATCC 29571 ^T	X52930	28
<i>M. marinum</i> FIB	X52920	28
<i>M. neoaurum</i> ATCC 25795 ^T	M29564	40
<i>M. nonchromogenicum</i> ATCC 19530 ^T	M29565	40
<i>M. nonchromogenicum</i> ATCC 19530 ^T	X52928	28
<i>M. obuense</i> ATCC 27023 ^T	X55597	This study
<i>M. paratuberculosis</i> 18	M29569	40; source unknown
<i>M. paratuberculosis</i> ATCC 19698 ^T	X52934	28
<i>M. phlei</i> ATCC 11758 ^T	M29566	40
<i>M. scrofulaceum</i> ATCC 19981 ^T	X52924	28
<i>M. senegalense</i> ATCC 35796 ^T	M29567	40
<i>M. simiae</i> ATCC 25275 ^T	X52931	28
<i>M. smegmatis</i> ATCC 14468	X52922	28
<i>M. sphagni</i> ATCC 33026 ^T	X55590	31
<i>M. szulgai</i> ATCC 25799 ^T	X52926	28
<i>M. terrae</i> ATCC 15755 ^T	M29568	40
<i>M. terrae</i> ATCC 15755 ^T	X52925	28
<i>M. thermoresistibile</i> ATCC 19527 ^T	M29570	40
<i>M. thermoresistibile</i> ATCC 19527 ^T	X55602	This study
<i>M. triviale</i> ATCC 23292 ^T	M29571	40
<i>M. tuberculosis</i> H37/Rv	X55588	31; FIB
<i>M. tuberculosis</i> H37/Rv	X52917	28; FIB
<i>M. vaccae</i> ATCC 15483 ^T	X55601	This study
<i>M. xenopi</i> ATCC 19250 ^T	X52929	28
Madagascar 3937	X55600	This study; FIB
MAIS 1	M29555	40; primate enteritis
MAIS 4	M29573	40; human
Chromogen	M29572	40; human
<i>Gordona terrae</i> ATCC 25594 ^T	M29554	40; bovine feces
<i>Rhodococcus equi</i> Equine lung	X53202	36
<i>R. erythropolis</i> DMS 43118	M29574	40
<i>R. fascians</i> DSM 20131	X53203	36
	X53204	36

^a ATCC, American Type Culture Collection, Rockville, Md.; TMC, National Institute of Allergy and Infectious Diseases, Bethesda, Md.; DSM, Deutsche Sammlung für Mikroorganismen und Zellkulturen, Stöckheim, Germany; FIB, Research Institute of Borstel, Borstel, Germany; LTB, Leprosy Tissue Bank, Research Institute of Borstel, Borstel, Germany.

TABLE 2. Percentages of similarity between partial 16S rDNA sequences of rapidly growing mycobacteria investigated in this study and equally weighted (Hamming) distances between the 16S rDNA sequences of these organisms and other rapidly growing mycobacteria whose sequences have been published previously

Organism ^a	% Similarity or Hamming distance ^b								Strain Madagascar
	<i>M. diernhoferi</i>	<i>M. gadium</i>	<i>M. vaccae</i>	<i>M. chubuense</i>	<i>M. obuense</i>	<i>M. aichiense</i>	<i>M. gilvum</i>	<i>M. chitae</i>	
<i>M. diernhoferi</i>		97.8	96.8	96.9	97.6	98.0	97.8	97.0	97.5
<i>M. gadium</i>	32		97.0	97.6	97.7	98.1	98.0	97.8	97.4
<i>M. vaccae</i>	46	43		97.2	97.2	97.2	97.7	96.3	97.7
<i>M. chubuense</i>	45	34	40		98.5	98.1	98.3	97.4	97.2
<i>M. obuense</i>	35	33	40	22		98.5	98.3	97.8	97.6
<i>M. aichiense</i>	29	27	40	27	21		98.9	98.4	97.5
<i>M. gilvum</i>	31	29	33	24	24	16		97.5	97.4
<i>M. chitae</i> ^c	43	31	53	37	32	23	36		97.3
Strain Madagascar	36	37	33	41	38	36	37	39	
<i>M. chelonae</i> ATCC 14472	26	28	35	36	30	23	22	33	40
<i>M. chelonae</i> ATCC 19977 ^T	44	49	58	54	50	49	48	58	62
<i>M. neoaurum</i>	15	35	41	43	31	28	33	34	35
<i>M. aurum</i>	40	31	29	27	31	35	29	51	44
<i>M. phlei</i>	53	41	40	38	43	37	39	37	32
<i>M. smegmatis</i>	36	32	32	38	37	28	29	33	17
<i>M. flavescens</i>	49	34	34	37	36	29	33	28	25
Strain Chromogen	44	33	42	40	46	39	40	29	23
<i>M. thermoresistibile</i>	43	27	40	50	51	43	44	36	32
<i>M. fallax</i>	37	28	52	34	36	26	30	18	38
<i>M. farcinigenes</i>	37	26	40	30	27	24	24	32	35
<i>M. senegalense</i>	33	27	42	29	21	19	20	28	38
<i>M. fortuitum</i>	33	28	44	31	27	18	18	26	41
<i>M. komossense</i>	42	38	48	36	30	25	34	35	40
<i>M. sphagni</i>	34	33	48	25	28	17	24	27	41

^a Table 1 gives the strain designations for organisms for which no strain designations are included.

^b The values above the diagonal are percentages of similarity, and the values below the diagonal are Hamming distances.

^c Investigated in this study.

RNA regions were synthesized with an Applied Biosystems model 381A DNA synthesizer by using the phosphoramidite method (3) and were purified by fast performance liquid chromatography (LKB-Pharmacia). Electrophoresis of preparations from sequence assays of cDNA was carried out as described previously (33).

Phylogenetic analysis. The sequence data for 58 mycobacterial strains, including the strains investigated in this study, were aligned with a universal alignment of more than 450 small-subunit rRNAs by using the alignment editor ALMA (43). The alignment was manually corrected by using common secondary-structure features. The variable regions between positions 79 and 90, 838 and 848, and 1450 and 1455 (*Escherichia coli* nomenclature [52]) were removed prior to the determination of pairwise distances by weighting nucleotide differences and insertions-deletions equally (Hamming distances). A rooted phylogenetic tree containing the mycobacterial strains and four reference actinomycetes was constructed by using the neighborliness method (9, 30).

Nucleotide sequence accession numbers. Sequence data are available from EMBL or Genbank; the accession numbers are shown in Table 1.

RESULTS AND DISCUSSION

As compared with the complete 16S rRNA sequence of *M. bovis* BCG (41), about 98% of the 16S rRNA sequences of the organisms investigated in this study were analyzed. The sequences of *M. cookii* ATCC 49103 (16), *M. farcinigenes* DSM 43294 (31), *M. komossense* ATCC 33103 (31), and *M. sphagni* ATCC 23026 (31) were extended by performing an analysis of the 5'-terminal 100 nucleotides. The sequences of

M. asiaticum ATCC 25276^T (T = type strain), *M. aurum* ATCC 29558, and *M. thermoresistibile* ATCC 19527^T have been published previously by Stahl and Urbanc (40). In order to determine the influence of sequences generated by different methods and in different laboratories on the tree topology, the data were nevertheless included in the comparison. The root of the tree was determined by using sequences from a group of organisms that are known to be closely related to mycobacteria (i.e., one member of the genus *Rhodococcus* and one member of the genus *Gordona*) (36). The size of the matrix of pairwise percentages of similarity and distance values (3,782 values from 62 organisms) is too great to be presented here in total. Therefore, the number of binary distance values is restricted to those values that were calculated with the mycobacterial species that were investigated in this study, and we include similarity values only for these organisms (Table 2). Likewise, the discussion of the phylogenetic positions of strains (Fig. 1) is by and large restricted to the fast-growing organisms, and readers are referred to previous publications (34, 28, 40) for information on phylogenetic and taxonomic aspects of the slow-growing members of the genus.

As described previously (4, 40), a highly variable region between positions 175 and 238 (*E. coli* numbering system) contains possible species-specific regions. Table 3 shows an alignment of this sequence stretch for the rapidly growing mycobacteria for which this information has not been published yet. Unique sequence stretches are found for all species, although some of these sequences differ in only a single nucleotide. The finding that the phylogenetically distantly related species *M. aurum* and *M. gadium* have nearly identical sequences in this region demonstrates the fact that

FIG. 1. Phylogenetic structure of the genus *Mycobacterium*. The evolutionary distance between two species is the sum of the branch lengths between them. The distance values for rapidly growing strains are shown in Table 2; the distance values for slowly growing strains are not shown. The numbers in brackets refer to the sources of 16S rRNA sequences, as follows: 1, reference 31; 2, reference 36; 3, reference 41; 4, reference 8; 5, reference 40; 6, this study; 7, reference 28; and 8, reference 16.

hypervariable stretches cannot be used as a sole basis for deriving phylogenies (34). Nevertheless, this 16S rRNA region is extremely valuable for species identification and for the development of oligonucleotide probes and polymerase chain reaction primers (4, 27, 38).

As reported previously (28, 32, 35, 40), the fast-growing mycobacteria (ranging from *M. chelonae* subsp. *abscessus* to *M. thermoresistibile* in Fig. 1) and the slow-growing mycobacteria (ranging from *M. triviale* to *M. paratuberculosis* in Fig. 1) are phylogenetically separated. The branching point of the rapidly growing members close to the root of the mycobacterial subline indicates that these organisms are phylogenetically older than their slow-growing relatives. Most slow growers are defined by an extended 16S rRNA helix at positions 451 to 482 (*E. coli* numbering) (35). The exceptions are *M. simiae* (28) and *M. triviale* (40), which

branch off at the root of the slow-growing strains but exhibit the short-helix version which is the signature of the rapidly growing mycobacteria.

The rapidly growing organisms investigated in this study (including the organisms for which the data base was extended) significantly enlarge the phylogenetic subcluster defined previously by *M. neoaurum*, *M. aurum*, *M. chelonae* subsp. *abscessus*, *M. chelonae* subsp. *chelonae*, *M. senegalense*, *M. fortuitum*, *M. phlei*, *M. thermoresistibile*, *M. flavescens*, *M. smegmatis*, and *M. fallax* (28, 40). Except for *M. chitae* ATCC 19627 rRNA, the reanalyzed 16S rRNAs of all of the strains that had identical origins were phylogenetically almost indistinguishable (>99.6%) if not identical. The small differences could be accounted for by the slightly inferior quality of the rRNA sequencing approach. The unexpected position of *M. chitae* ATCC 19627

TABLE 3. Sequences of the hypervariable regions of 16S rRNAs (positions 175 through 238) from certain rapidly growing members of the genus *Mycobacterium*^a

Sequence	Organism
CCGAAUUG-ACCGCGCUCUUAUGGG-GUGUGGUGGAAA	<i>M. diernhoferi</i>
...G...GG-ACUACGCGAUG...GUC-GU.UG.U.G...	<i>M. gadium</i>
...G...GG-ACCACGGCAUG...GUG-CU.UG.U.G...	<i>M. chubuense</i>
...G...GG-ACCACGGCAU...GUG-CU.UG.U.G...	<i>M. obuense</i>
...A...GG-AUCAUGGCCUU...GGG-UU.UG.U.G...	<i>M. aichiense</i>
...A...GG-ACCGCAUGCUU...AGU-GU.UG.U.G...	<i>M. gilvum</i>
...A...UG-AUCAUGGCCUU...GGG-UU.UG.U.G...	<i>M. chitae</i>
...G...UG-AUCAUGCGCU...GGU-GU.UG.U.G...	<i>M. fallax</i>
...A...UG-AUCAUGCGCU...GGU-CG.UG.U.G...	<i>M. neoaurum</i>
...A...GG-ACUACGCGAUG...GUC-GU.UG.U.G...	<i>M. aurum</i>
...G...GG-ACCACGCGCU...GGU-GU.UG.U.G...	<i>M. senegalense</i>
...nn...CACCCUGCUAGUAG...UGCCUG.UG.U.G...	Strain Madagascar
...A...CACCCUUCUGGUG...nGUCUG.GA.G.G...	<i>M. vaccae</i>
...A...UCCCCUAUUGGUCG...GGCCUG.UA.G.G...	<i>M. flavescens</i>
...A...GUCCUGCUGGUCG...GGCCUG.UG.G.n...	Strain Chromogen
...A...CACCCUGCUGGUCG...GGCCUG.UG.G.G...	<i>M. thermoresistibile</i>
...G...GG-ACCACACACUU...GGU-GA.UG.U.C...	<i>M. chelonae</i> ATCC 19977

^a Additional sequences have been published by Böddinghaus et al. (4). Strain designations are given in Table 1. Each dot indicates a nucleotide which is identical to the nucleotide found in the sequence of *M. diernhoferi*.

(40), which apparently is a member of the most ancient branch within the genus *Mycobacterium*, could not be verified (Fig. 1). A reexamination of the same culture obtained from the American Type Culture Collection showed that this organism is closely related to *M. fallax*. Using a different selection of reference actinomycetes, Stahl and Urbance (40) found that *M. chitae* ATCC 19627 clusters deeply among the more ancient representatives of the order. As mentioned previously by these authors, this culture of *M. chitae* should be checked for authenticity.

All of the rapidly growing mycobacteria are closely related to each other, although the levels of relationship are in general lower than the levels found among their slow-growing relatives. Most rapidly growing strains are well separated from each other, although in most cases the distances between branching points are small. The main problem with the arrangement of branches in a fan- or bushlike topography is that there is a certain degree of instability in the branching order which may change slightly when new sequences are compared (11, 34). Nevertheless, the following recognizable clusters emerged from our analysis: (i) *M. neoaurum* and *M. diernhoferi*; (ii) *M. gadium*; (iii) the *M. chubuense* cluster; (iv) the *M. fortuitum* cluster; (v) *M. komossense*; (vi) *M. sphagni*; (vii) *M. fallax* and *M. chitae*; (viii) *M. aurum* and *M. vaccae*; (ix) the *M. flavescens* cluster; and *M. chelonae* subsp. *abscessus*.

Rapidly growing mycobacteria have been studied extensively by using numerical analyses (17, 25, 29, 44, 47, 49), comparative immunology (23, 24, 26, 29), and certain chemotaxonomic properties (7, 13, 14, 22, 42, 48), while genetic analyses, such as DNA-DNA pairing, have been restricted to a few species (1, 10, 15, 19, 20). Until the early 1980s overview articles dealing with the taxonomy and nomenclature of the fast-growing mycobacteria impressively demonstrated the problem concerning the taxonomy of mycobacteria (12, 45, 46, 50). The results of chemotaxonomic studies, DNA pairing studies, and 16S rRNA-based investigations have confirmed the validity of most of the described species. However, support for the intrageneric relationships of the rapidly growing mycobacteria from numerical phenetic and chemotaxonomic studies has been sparse. Most of the chemotaxonomic studies have included

only selected groups of organisms, and not a single study has included all of the rapidly growing mycobacteria. The following species were identified as individual species in the first broad cooperative numerical phenetic study of rapidly growing mycobacteria (17): *M. fortuitum*, *M. vaccae*, *M. flavescens*, *M. chelonae*, *M. phlei*, *M. smegmatis*, and *M. diernhoferi*. This list was later extended in a second cooperative study by the addition of *M. chitae* and *M. thermoresistibile* (29). The different resolution powers of selected phenotypic and immunological markers to unravel taxonomic relationships among rapidly growing members resulted in a rather complex and ambiguous picture. Results from these studies were contradictory and sometimes not able to resolve relationships even between those species that were later shown to be phylogenetically closely related. No single phenetic approach that convincingly clusters organisms according to phylogeny has been described. Furthermore, except for *M. diernhoferi*, most of the species dealt with in this study have been included in phenetic analyses only rarely.

M. parafortuitum was found to share high levels of phenetic similarity with *M. vaccae* by Kubica et al. (17). In a later study, *M. parafortuitum*, *M. diernhoferi*, *M. aurum*, and *M. neoaurum* were described as being closely associated (the *M. fortuitum* complex) but *M. vaccae* was excluded (29). The coherency of this complex has been contradictory in most subsequent studies, indicating that this group is a superficial assembly of unrelated species. While the close relationship between *M. diernhoferi* and *M. neoaurum* has now been confirmed by the results of 16S rRNA analyses, the relationship between *M. diernhoferi* and the other species of this complex has not been supported by the results of bacteriocin typing (23), by the results of serogrouping (29), or by the results of a phylogenetic analysis (note that *M. parafortuitum* has not been included in 16S rRNA studies yet). On the other hand, in contrast to the findings of numerical analyses, the results of cytoplasmic antigen serogrouping (29) have shown that *M. aurum* is related to *M. vaccae* at the serospecies level. This close relationship is in accord with the results of bacteriocin typing (42) and 16S rRNA similarity data.

Another group of species that exhibit apparent phenotypic

similarity comprises *M. fortuitum*, *M. chelonae* subsp. *chelonae*, and *M. chelonae* subsp. *abscessus*, the *M. fortuitum*-*M. chelonae* complex (2). When the S1 nuclease method for determining DNA similarities was used, *M. fortuitum* and the two subspecies of *M. chelonae* were found to be only remotely related (5 to 12% similarity), while the two subspecies were found to be moderately related to each other (26 to 40% similarity) (20). The latter finding contradicted results of the spectrophotometric DNA hybridization method, which did not detect any DNA differences between the two subspecies (99% similarity) (1). The results of both of these studies are in obvious contrast to the results of the 16S rRNA analysis. When the same strains that were included in the latter hybridization study (strains ATCC 19235 and ATCC 19977) were used, the levels of rRNA sequence similarity were so low that the possibility of any significant DNA similarity could be excluded. Apparently, the authenticities of these *M. chelonae* strains, which have been deposited in different culture collections under American Type Culture Collection numbers, need to be investigated. The inclusion of sequences from new organisms in the existing data base for mycobacteria causes *M. chelonae* subsp. *abscessus* to branch off in a significantly different position than the positions determined by Stahl and Urbance (40). While *M. chelonae* subsp. *abscessus* exhibited a remote relationship to *M. aurum* in a previous phylogenetic survey (40), its new position at the root of mycobacteria is unexpected and has not been confirmed by additional phylogenetic and phenotypic evidence. On the basis of DNA similarities, *M. fortuitum* has been found to be more closely related to *M. senegalense* (47% similarity) than to *M. chelonae* subsp. *chelonae* (7% similarity) (1). This finding is not in accord with the results of this study, in which the three organisms appeared to be equidistantly related. Early studies included neither *M. farcinogenes* nor *M. senegalense*, the causative agents of bovine farcy. Both of these species possess α -mycolates and polar mycolates (22, 26); these organisms exhibit significant levels of DNA similarity (47%) (15), and they cannot be separated serologically (26). The serological analysis also confirmed the similarity between the farcy strains and *M. fortuitum*.

The grouping of the type strains of *M. chitae* and *M. fallax* in an individual subcluster is not supported by the levels of DNA relatedness (19), which revealed that *M. fallax* is about equidistantly related to *M. chitae*, *M. phlei*, and *M. duvalii* (20 to 25% DNA similarity). *M. chubuense*, *M. obuense*, *M. aichiense*, and *M. gilvum* form a coherent group within the radiation of the rapidly growing mycobacteria, and this group is adjacent to the *M. farcinogenes*-*M. fortuitum* cluster. Phylogenetic coherency of *M. chubuense* and its relatives has not been proven yet, and support for such clustering from phenetic analyses is lacking. The phylogenetic distinctness of the strains that have been investigated supports the validity of allocation of these strains into different species. *M. gilvum* has been found to be unrelated to any of the other rapidly growing mycobacteria tested for DNA similarity (10), and the same organism formed an individual line in a numerical phenetic analysis (47). Different mycolic acid patterns have been found for *M. aichiense* and *M. chubuense*-*M. gilvum*. These patterns are not species specific but were found to be identical in groups of strains containing both rapidly and slowly growing mycobacteria (7). Certain species of this cluster differ in their carotenoid pigments, and *M. aichiense*, *M. chubuense*, and *M. obuense* can be distinguished clearly from each other (14).

The thermotolerant species *M. phlei*, *M. flavescens*, *M.*

thermoresistibile, and *M. smegmatis* cluster together on the basis of 16S rRNA similarity. This agrees with the results of phenotypic analyses, such as enzyme typing, phage and bacteriocin typing, and sensitivity testing (12, 50). In a numerical phenetic analysis in which 38 characters were used, *M. smegmatis* and similar isolates shared less than 76% similarity with any of the 22 rapidly growing mycobacteria included in the study (15a). There is a high probability that strain Madagascar represents a new *Mycobacterium* species. All of the species belonging to this cluster are distinct entities, a finding which for *M. smegmatis* and *M. phlei* is supported by the low levels of DNA similarity (1); all of these organisms share an idiosyncratic 16S rRNA feature, the insertion of a base pair in the hypervariable region shown in Fig. 1. It has been speculated (40) that this insertion could be a molecular signature for the phylogenetic definition of thermotolerant strains. However, thermotolerance has not been observed in isolate Chromogen (40) and in strain Madagascar.

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