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Two Different 16S rRNA Genes in a Mycobacterial Strain

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Sequencing of the gene coding for 16S rRNA (16S rDNA) is a well-established method used to identify bacteria, particularly mycobacteria. Unique sequences allow identification of a particular genus and species. If more than one 16S rDNA is present on one mycobacterial genome, their sequences are assumed to be strictly or almost identical. We have isolated a slowly growing *Mycobacterium* strain, "*X*", identified by conventional biochemical tests as *Mycobacterium terrae*. Identification by amplification and direct sequencing of 16S rDNA yielded ambiguous results in two variable regions, suggesting the presence of different copies of the sequenced gene. Total DNA was digested by restriction enzymes and hybridized after Southern blotting to a probe representing about two-thirds of the 16S rDNA. Two copies of 16S rDNA were identified and cloned. By sequencing, the clones were of two different types, A and B, differing in 18 positions. Oligonucleotides specific to each copy of the 16S rDNA were used to distinguish the positions of the two genes observed in the Southern blot. We conclude that *Mycobacterium* strain "*X*" has two different copies of 16S rDNA. Variations in the sequence between two copies of 16S rDNA gene have been described in archaeobacteria, but not in mycobacteria. When placed in a phylogenetic tree together with other slowly growing mycobacteria, gene A shows a common root with *M. terrae*, whereas gene B is placed separately.

Genes coding for the small ribosomal subunit (henceforth called 16S rDNA) have great significance in the phylogenetic analysis of bacteria because of their universal distribution and because mutations occur at a slow and constant rate (25). At the species level, 16S rDNA is assumed to be stable and specific (19). The numbers of the 16S rRNA genes depend on the species of bacteria studied, which contain, for example, four to six operons (designated *rrn* operons) in enterococci (21) or seven operons in *Escherichia coli* (1). 16S rDNAs generally differ between species or subspecies of bacteria (23), but when multiple 16S rRNA genes of the same isolate have been sequenced, they are identical or show only minor differences (5, 10).

For the genus *Mycobacterium*, direct 16S rDNA sequencing is extensively used to identify and classify different species (19), because more traditional methods involving culture and biochemical reactions are slow and may yield ambiguous results (22). Fast-growing and slowly growing mycobacteria reportedly differ in the number of *rrn* operons. Fast growers such as *Mycobacterium smegmatis* generally have two *rrn* operons (7), whereas slow growers, including *Mycobacterium tuberculosis* and *Mycobacterium avium*, have one operon (10). In general, one particular rDNA sequence is associated with one species. Different isolates of some species such as *Mycobacterium gordonae* may show different rDNA sequences (12), but to our knowledge, no difference has ever been shown between different copies of the 16S rRNA genes of a single mycobacterium isolate. For this paper, we investigated a slowly growing mycobacterium (*Mycobacterium* strain "*X*") belonging to the *Mycobacterium terrae* complex according to biochemical criteria. 16S rDNA sequencing yielded ambiguous results because of the presence of two different 16S rDNAs. These two 16S rDNAs were cloned and sequenced, and their expression was investigated.

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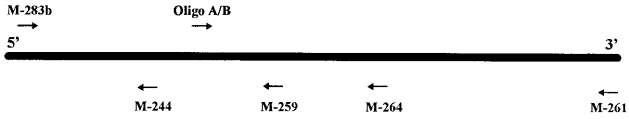
MATERIALS AND METHODS

Mycobacterial isolates and cultures. Four strains of *M. terrae* were used for this study: three isolates of clinical origin ("*X*," "*Y*," and "*Z*") and one isolate obtained from the American Type Culture Collection, *M. terrae* ATCC 15755. The isolates were grown on Coletsos medium (Biomérieux) and examined for growth rate, colony morphology, and pigmentation. Phenotypic identification tests were performed as described by Vincent Lévy-Frébault and al. (24), and all strains were identified by biochemical tests as *M. terrae*.

PCR and direct sequencing. For each strain, four subcultures were analyzed. An isolated colony was suspended in 1 ml of Tris-EDTA (pH 7.5) (TE) with acid-washed glass beads (Sigma), and nucleic acids were extracted by mechanical lysis with a tissue disintegrator (Mickle Laboratories, Gomshall, United Kingdom) according to the method described by Kirschner and al. (13). Disrupted cells in 100 μl of TE were centrifuged for 20 min at 15,000 × *g*. With 5 μl of the supernatant, PCR was performed in a 100-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 500 μM deoxynucleoside triphosphate, 2.5 U of *Taq* LD polymerase (Perkin-Elmer Cetus), 20 pmol of biotinylated primer M-283 (corresponding to *E. coli* 16S rDNA, positions 9 to 30), and 75 pmol of primer M-264 (corresponding to *E. coli* 16S rDNA, positions 1046 to 1027) (Fig. 1) (13). All primers were synthesized by a commercial supplier (Microsynth GmbH, Balgach, Switzerland). The amplification products were sequenced directly according to the method of Kirschner and al. (13). The biotinylated single-stranded DNA template was prepared by use of streptavidin-coated magnetic beads (Dynabeads M-280–streptavidin; Dynal, Milan, Italy). Sequencing was performed with primers M-244 (corresponding to *E. coli* 16S rRNA from positions 341 to 361) and M-259 (corresponding to *E. coli* 16S rRNA from positions 590 to 609) (Fig. 1).

Southern blotting. For each strain of mycobacterium, two Coletsos media were inoculated, and all cells were harvested. DNA was extracted by the standard method of phenol-chloroform-isoamyl alcohol after the cells had been lysed in a solution of lysozyme, sodium dodecyl sulfate (SDS), and proteinase K. DNA (1.5 to 2 μg) was digested by the restriction enzymes *Eco*RI, *Pvu*II, *Sal*I, and *Pst*I (Life Technologies).

Digested DNA (0.5 μg) was loaded on a 0.8% agarose gel and then transferred onto a Zetaprobe (Bio-Rad) nylon membrane by capillary blotting with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) solution. The amplicons representing two-thirds of the 16S rDNA of the isolate "*X*" were labeled with 50 μCi of [^{32}P]dCTP with the Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemica) and used as a probe. They were hybridized to the total DNA under high-stringency conditions as described by Monod and al. (16). The membrane was prehybridized at 65°C for 5 min in a solution containing 7% SDS, 0.5 M NaH_2PO_4 (pH 7.2), and 1 mM EDTA. Hybridization was then performed at 65°C overnight in the same solution containing ^{32}P random primed labeled probe. The membrane was exposed to X-ray film after two washes in 2× SSC–0.1% SDS at 55°C.



M-283b: 5' GAGTTTGATCCTGGCTCAGGA 3' (position 9-30)
M-244: 5' CCCACTGCTGCCTCCCGTAG 3' (position 341-361)
Oligo A: 5' CTTGCGGGTTTTCTCGCAGGT 3' (position 457-478)
Oligo B: 5' CTCCCGGCTTTTGTTGGGGGT 3' (position 457-478)
M-259: 5' TTTCACGAACAACGCGACAA 3' (position 590-609)
M-264: 5' TGCACACAGGCCACAAGGGA 3' (position 1027-1046)
M-261: 5' AAGGAGGTGATCCAGCCGCA 3' (position 1523-1542)

FIG. 1. List and position of the primers and/or probes used to analyze the 16S rDNA.

Cloning and sequencing of 16S rDNA. The gene was amplified and cloned in two fragments with the following primers shown in Fig. 1: M-283 and M-264 for the first cloning and oligonucleotide A or B and M-261 for the second cloning. Oligonucleotides A and B correspond to positions 457 to 478 (*E. coli* alignment) on the 16S rDNA and differed between the two copies of the 16S rDNA. One microliter of the PCR product was cloned with a direct insertion into a plasmid vector (pCR II) with a size of 3.9 kb (TA Cloning Kit; Invitrogen). Competent cells (*E. coli* DH5) were then transformed and selected on Luria-Bertani medium with ampicillin in the presence of X-Gal–IPTG (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside–isopropyl-β-D-thiogalactopyranoside). Plasmid DNA was prepared with a Qiagen Plasmid Kit (Qiagen) and sequenced.

Hybridization with oligonucleotides. Oligonucleotide A and oligonucleotide B labeled with [^{32}P]dATP were used as probes to confirm the presence of two copies of the 16S rDNA gene. Restriction digests of mycobacterial DNA were transferred to a membrane cut in half (Zetaprobe; Bio-Rad). After prehybridization for 1 h at 50°C in a solution containing 5× SSC, 7% SDS, 10× Denhardt's solution, 20 mM NaH_2PO_4 (pH 7.0), and 100 μg of denatured salmon sperm DNA per ml, one-half was hybridized in the same solution with oligonucleotide A and the other half was hybridized with oligonucleotide B at 50°C overnight. The membranes were exposed to X-ray film after two washes at 37°C and at 40°C in 3× SSC–1% SDS.

RNA extraction and Northern (RNA) blotting. *Mycobacterium* strain "*X*," *M. terrae* ATCC 15755, and one strain of *E. coli* containing the pCR II plasmid with the insert oligoB-M261 (positions 457 to 1523 of the rRNA gene) were used for the RNA extraction. For the two *Mycobacterium* strains, cells of two Coletsos media were harvested in 1 ml of water. After one centrifugation (5 min at 9,000 × *g*), the pellet was resuspended in 100 μl of TE in the presence of acid-washed glass beads. The samples were placed for 2 min in a tissue disintegrator and centrifuged for 10 min at 15,000 × *g* to recover the supernatant. The supernatant was used directly in the Rneasy kit (Qiagen) according to standard procedures. Samples were brought to 20 μl with loading buffer (with 0.01% ethidium bromide) and heated at 60°C for 15 min in order to denature the RNA. Gel electrophoresis of RNA and Northern blotting were performed according to standard protocols (15). After the RNA transfer was complete, the membrane filters (Zetaprobe; Bio-Rad) were air dried and exposed to UV radiation from a UV illuminator for 2 min. The 16S and 23S RNA bands were visualized at this stage and marked on the membrane with a pencil. Hybridization was performed with labeled oligonucleotides as previously described. Oligonucleotide A′ (corresponding to the inverse and complementary sequence of oligonucleotide A) and oligonucleotide B′ (corresponding to the inverse and complementary sequence of oligonucleotide B) were labeled by [^{32}P]dATP. The amounts of radioactivity were controlled after labeling and were the same for each probe. We compared the two RNA-DNA hybridizations with two oligonucleotides with approximately the same melting temperature (66°C for A′ and 68°C for B′). The membrane was cut, and one-half was hybridized with oligonucleotide A′; the other half was hybridized with oligonucleotide B′. After exposure of X-ray film, the two membranes were washed two times at 95°C in 0.1× SSC–0.5% SDS and rehybridized with oligonucleotide A′ and oligonucleotide B′, contrary to the previous experiment. The level of radioactivity for each band was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Phylogenetic analysis. The sequences were aligned with other mycobacterial 16S rDNA sequences with Genetic Data Environment software, version 2.2 (14). A phylogenetic tree was built by the neighbor-joining (NJ) method (20) applied to distances corrected for multiple hits and for unequal transition and transversion rates according to Kimura's two-parameter model (11). All analyses were based on 1,385 unambiguously aligned sites, excluding short (about 50-bp) regions at both extremities of the gene for which a reliable alignment was not possible. Furthermore, all pairs of sites containing gaps were excluded. The reliability of internal branches in the NJ tree was assessed by the bootstrap method (9) with 1,000 replicates. The Phylo-Win program was used for distance computations, tree building, and bootstrapping. A phylogenetic tree was plotted with the NJplot program.

RESULTS

Evidence for two 16S rRNA genes in three strains of *M. terrae* and in *Mycobacterium* strain "*X*." *Mycobacterium* strain "*X*" was isolated from the sputum of one human immunodeficiency virus-positive woman known to be infected by *Mycobacterium kansasii. Mycobacterium* strain "*X*" hybridized with a commercial probe for *M. tuberculosis* complex (Accuprobe; Gen-Probe, San Diego, Calif.) but was identified as *M. terrae* by biochemical tests. Several attempts at direct sequencing of 16S rDNA of *Mycobacterium* strain "*X*" yielded ambiguities in two regions used to identify mycobacterial species (Fig. 2). In the region sequenced with M-244 primer, double bands were evident in three positions (positions 177, 201, and 202 according to the *E. coli* alignment). In the region sequenced with M-259 primer, seven positions were ambiguous (positions 459, 460, 464, 469, 471, 473, and 474 according to the *E. coli* alignment). *M. terrae* ATCC 15755 and clinical isolates of *M. terrae* ("*Y*" and "*Z*") did not show these double bands. The ambiguities lead us to suspect that *Mycobacterium* strain "*X*" had two different 16S rRNA genes. To verify this hypothesis, we performed Southern blotting experiments with mycobacterial DNAs digested by *Eco*RI, *Pvu*II, *Sal*I, and *Pst*I. The membrane was hybridized to the PCR product of *Mycobacterium* strain "*X*" obtained by amplification of positions 9 to 1039 of the 16S rDNA (Fig. 3). The *M. terrae* strains tested gave four hybridization fragments with *Eco*RI, which cuts once within the gene. With the three other restriction enzymes, which have no cutting site in the gene, one double or two separate hybridization fragments were apparent. We concluded that two copies of the 16S rDNA were present in all *Mycobacterium* isolates tested, including the *M. terrae* reference strain, ATCC 15755.

Evidence for two different sequences of the 16S rDNA of *Mycobacterium* strain "*X*." In order to analyze the differences between the genes encoding 16S rRNA, molecules obtained by PCR amplification (positions 9 to 1039) were cloned separately into the PCR II plasmid (Invitrogen, NV Leek, The Netherlands). Fifteen clones were sequenced, and two types (A and B) were found: 8 clones were type A, and 7 were type B. The two variable regions of this part of the gene are presented in Fig. 2. Three differences occurred in the first hypervariable region of the 16S rDNA (comprising helix 10), but only two of these three differences (positions 201 and 202, *E. coli* alignment) are displayed in Fig. 2. The third difference was at a position more distant from the sequencing primer M-244. Seven differences are shown in the second hypervariable region of the 16S rDNA comprising helix 18. These differences corresponded to the ambiguities observed in direct manual sequencing of *Mycobacterium* strain "*X*" (Fig. 2). Two oligonucleotides were synthesized from position 457 to position 478 of the 16S rDNA, with oligonucleotide A complementary to clones of type A and oligonucleotide B complementary to clones of type B. These oligonucleotides were used to amplify the second part of the 16S rDNA (positions 457 to 1542). This region, which was sequenced several times, consistently revealed eight additional modifications. All were at positions which show variations between mycobacterial species. The sequences of genes A and B were identical to X93027 and X93031 of the EMBL database sequences, respectively, as described by Springer and al. (22).

As expected, oligonucleotides A and B hybridized to only one of the two copies of rDNA, resulting in one band after digestion with *Eco*RI or *Pst*I (Fig. 4). The positions of the bands corresponded to the position of one of the multiple bands observed when the probe was the total PCR product.

Expression of rRNA genes of *Mycobacterium* strain "*X*". Two

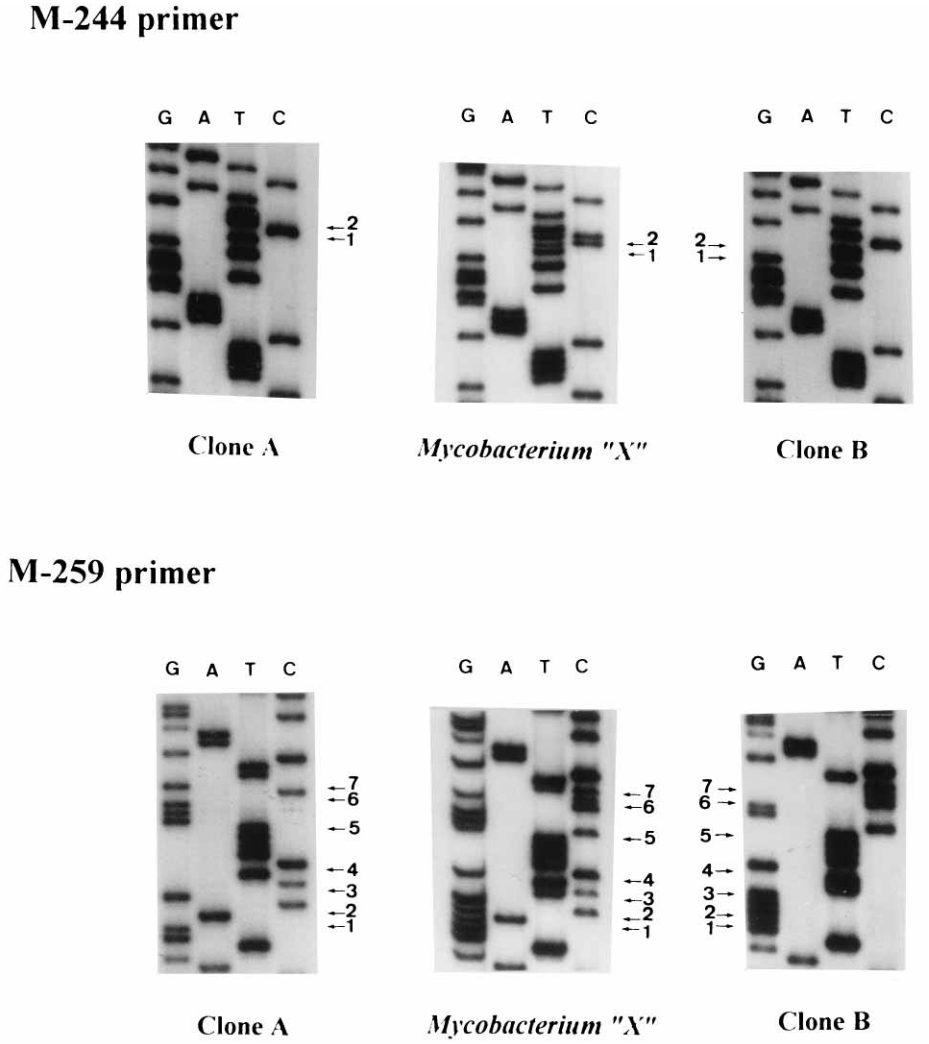


FIG. 2. Sequencing gels of the 16S rDNA of *Mycobacterium* strain "*X*" and clones A and B. Two parts of variable regions were sequenced by the M-244 primer (positions 341 to 361 in the *E. coli* alignment) and M-259 primer (positions 590 to 609 in the *E. coli* alignment). The arrows and numbers mark the positions of ambiguities in the sequencing of 16S rDNA.

blots of RNA isolated from *Mycobacterium* strain "*X*" were probed with oligonucleotide A′ and oligonucleotide B′ (Fig. 5), respectively. Both bands appeared at the level of 16S rRNA, but with a higher intensity for A′ compared with that for B′. The membranes were washed, and hybridization was repeated by inverting the probes. Again, A′ showed a stronger signal than B′. Quantified by a PhosphorImager, the hybridizations with oligonucleotide A′ were 6- and 10-fold more important than those with oligonucleotide B′. Hybridization was not affected by washing at 50°C in 2× SSC–1% SDS, indicating a high degree of specificity. We conclude that both genes were transcribed.

Phylogenetic analysis. The 16S rDNA sequences of genes A and B of *Mycobacterium* strain "*X*" were aligned with 23 other 16S rDNA sequences of mycobacteria, including 21 species of slowly growing mycobacteria and 2 species of rapidly growing mycobacteria (*M. fortuitum* and *M. flavescens*). The latter were added as out-groups together with *Nocardia asteroides.*

The comparison of the 16S rDNA sequences of the genes A and B found in *Mycobacterium* strain "*X*" shows that they differ in 18 single substitutions, accounting for about 1.2% sequence divergence between both copies. Each copy differs from the reference sequence of *M. terrae* by 1%. For comparison, the sequence divergence between *M. terrae* and other slowly growing mycobacteria ranges from 2.3% to 4.4%. A phylogenetic tree of slowly growing mycobacteria, inferred from 16S rDNA sequences by the NJ method, is presented in Fig. 6. The tree reveals different subgroups of slowly growing mycobacteria. One of these groups is composed of *M. nonchromogenicum*, *M. haemophilum*, and the *M. terrae* complex, including the sequences of *M. terrae* and genes A and B from *Mycobacterium* strain "*X*."

DISCUSSION

Information regarding the number of rRNA genes present in the members of the genus *Mycobacterium* has been published. Southern blots with a probe complementary to 16S rDNA have demonstrated two copies of 16S rDNA in the chromosome of most fast-growing mycobacterial species, except *M. chelonae* and *M. chelonae* subsp. *abscessus* (7). On the other hand, all slowly growing mycobacteria investigated so far

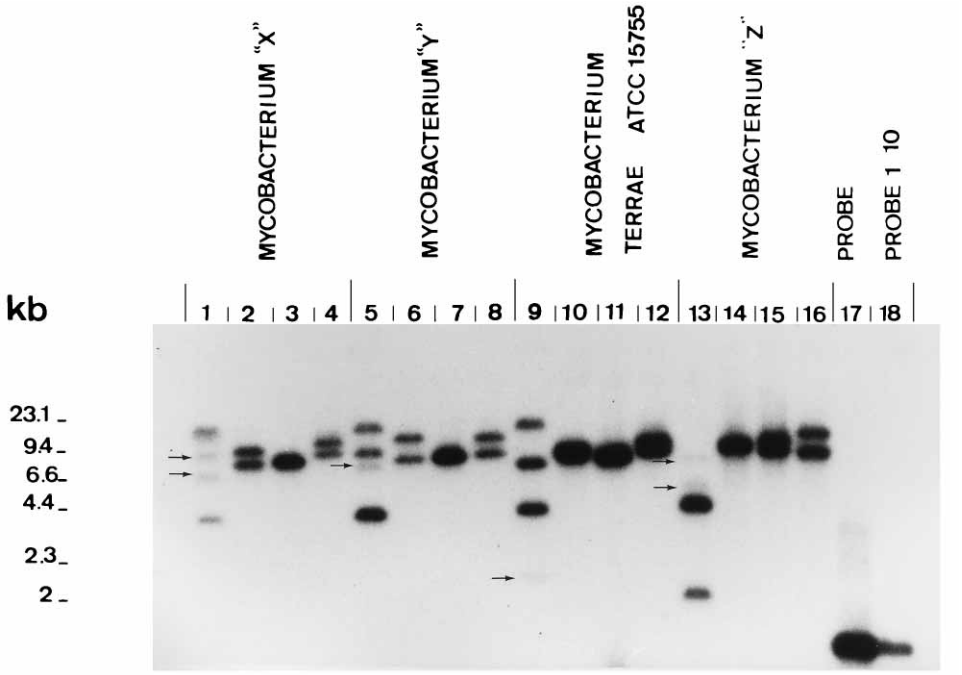


FIG. 3. Southern blot of different mycobacterial DNAs (*Mycobacterium* strains "*X*", "*Y*", and "*Z*" and *M. terrae* ATCC 15755) digested by *Eco*RI (lanes 1, 5, 9, and 13), *Pvu*II (lanes 2, 6, 10, and 14), *Sal*I (lanes 3, 7, 11, and 15), and *Pst*I (lanes 4, 8, 12, and 16). Results from hybridization with PCR products of *Mycobacterium* strain "*X*" (positions 9 to 1046 of *E. coli* 16S rDNA) are shown. Some bands which were not clearly visible (particularly with DNAs digested by *Eco*RI) are pointed out by arrows: two bands in lane 1, one band in lane 5, one band in lane 9, and two bands in lane 13. All of these lanes (1, 5, 9, and 13) have four bands.

had only one copy of 16S rDNA. (2, 10). Our data show that the *M. terrae* group is an exception to this rule: *M. terrae* ATCC 15755, two *M. terrae* strains from our laboratory ("*Y*" and "*Z*"), and *Mycobacterium* strain "*X*" are slow growers but have two copies of 16S rDNA. Our data suggest that *Mycobacterium* strain "*X*" not only has two copies of the 16S rDNA, but also that these copies differ from each other. The two rRNA genes of *Mycobacterium* strain "*X*" are about 1,540 nucleotides in length and differ by 18 nucleotide substitutions (1.2%). This amount of diversity attains or exceeds the difference found

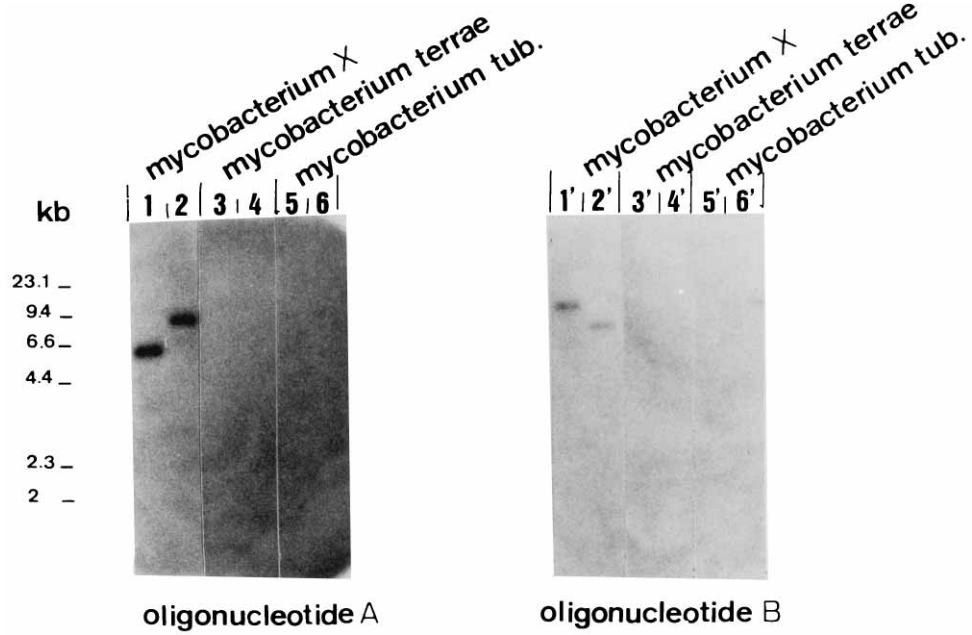


FIG. 4. Southern blots of mycobacterial DNAs (*Mycobacterium* strain "*X*," *M. terrae* ATCC 15755, and *M. tuberculosis* [tub.]) digested by *Eco*RI (lanes 1, 3, 5, 1′, 3′, and 5′) and *Pst*I (lanes 2, 4, 6, 2′, 4′, and 6′). Results were obtained by hybridization with oligonucleotide A as a probe for the first membrane and oligonucleotide B as a probe for the second membrane.

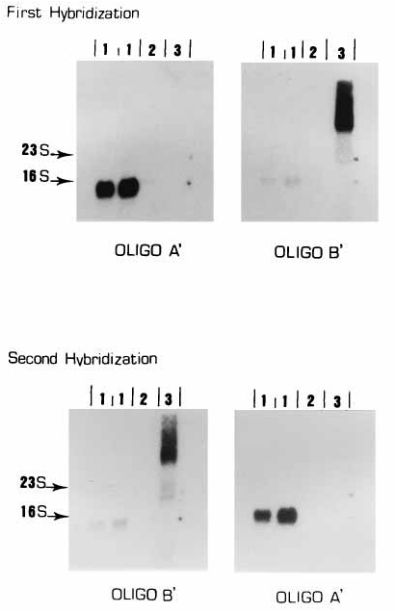


FIG. 5. Northern blots of RNA from *Mycobacterium* strain "*X*" (lanes 1), *M. terrae* ATCC 15755 (lanes 2), and *E. coli* with the plasmid pCR II (insert oligonucleotide B-M-261) (lanes 3).

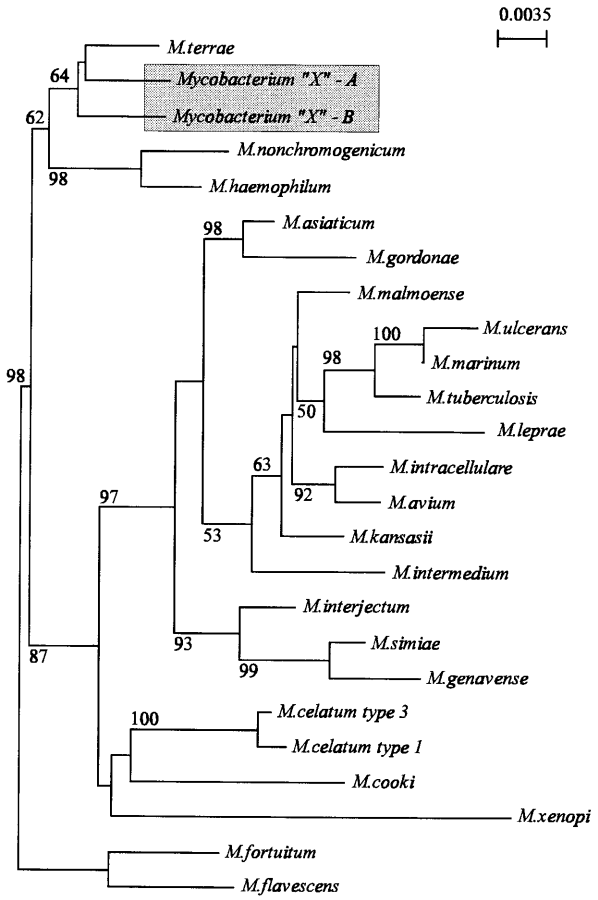


FIG. 6. Phylogenetic tree of the slowly growing mycobacteria. The tree is based on 16S rRNA gene sequences and illustrates the position of genes A and B of *Mycobacterium* strain "*X*." The tree was rooted with rapidly growing mycobacteria (*M. fortuitum* and *M. flavescens*) as the out-group.

between the 16S rDNA sequences of some well-established species (18). Sequencing errors were excluded, because the two cloned 16S rDNAs were sequenced from several clones, and the same positions were always divergent. Ten of these substitutions were concentrated in two specific domains which are highly variable among mycobacteria (positions 177 to 200 and positions 450 to 480) and which therefore establish the identity of a particular *Mycobacterium* species (13). The other eight substitutions are dispersed along the gene, but six of these eight positions vary among other strains of mycobacteria. Surprisingly, the sequences of genes A and B were identical to X93027 and X93031 of the EMBL database, respectively. These sequences were described under the names MCRO 16 and MCRO 24 by Springer and al. (22) and originated in two isolates resembling *M. terrae* by biochemical tests. This raises the possibility that the ambiguities in the sequencing we observed were in fact due to a mixture of two strains being analyzed. However, we took care to eliminate this possibility by isolating single colonies, inoculating fresh plates, and repeating the process three times. DNA was prepared from single colonies. The ambiguities (Fig. 1) were always evident, whether the DNA was isolated from the original culture or from one of the four successive subcultures.

False-positive hybridization of *Mycobacterium* strain "*X*" with the Gen-Probe test for the *M. tuberculosis* complex occurred, because one of the sequences (sequence A) obtained was identical to the *M. tuberculosis* sequence in the region of the commercial probe (positions 188 to 207). These false-positive reactions have already been described for strains of *M. celatum* (4) and strains of *M. terrae* (8).

Examination of all 16S rDNA sequences published in the GenBank database for several strains of the same species of bacteria surprisingly revealed considerable variation (6). As a matter of fact, the differences between two sequences deposited as being typical of the same species were often as large as the differences between sequences belonging to different species. It is likely that record keeping and sequencing errors, as well as real differences between different isolates of one species (3), are in large part responsible for the discrepancies. In rare cases, as noted by Clayton et al. (6), variations between multiple copies of rDNA genes may explain the discrepancies. The most spectacular example is found in the archaeobacterium *Halobacterium marismortui*, in which the two 16S rDNAs differ by nucleotide substitutions in 74 positions (17), contrary to most organisms, which exhibited perfect or nearly perfect sequence identity between operons (10).

The phylogenetic position of two 16S rDNA sequences from *Mycobacterium* strain "*X*" close to *M. terrae* in the NJ tree correlates with the identification of this strain as *M. terrae* by conventional biochemical tests. The tree topology indicates that gene A of *Mycobacterium* strain "*X*" is more closely related to *M. terrae* than is gene B. Similar results were obtained by phylogenetic analysis with maximum-parsimony and maximum-likelihood methods (data not shown). Although this branching order is associated with very low bootstrap scores, we can suppose that gene B of *Mycobacterium* strain "*X*" may have evolved more rapidly than gene A. The gene B product seems to be present in amounts that are 10 times lower than that of gene A, as indicated by our Northern blot experiment. This difference could be interpreted as representing the different evolutionary rates of both genes, in agreement with the theory that rapid evolution of a gene is correlated with instability and loss of activity (25).

The presence of two different 16S rDNA sequences in the same strain of a mycobacterium has obvious implications for the use of 16S rDNA sequencing in the identification of species. However, the identification of the most important pathogenic mycobacteria, such as *M. tuberculosis* complex, *M. leprae*, and *M. avium*, should not be affected because they have only one *rrn* operon.

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REFERENCES

1. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54:**130–197.
2. **Bercovier, H., O. Kafri, and S. Sela.** 1986. Mycobacteria possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochem. Biophys. Res. Commun. **136:**1136–1142.
3. **Bull, T. J., D. C. Shanson, L. C. Archard, M. D. Yates, M. E. Hamid, and D. E. Minnikin.** 1995. A new group (type 3) of *Mycobacterium celatum* isolated from AIDS patients in the London area. Int. J. Syst. Bacteriol. **45:**861–862.
4. **Butler, W. R., S. P. O'Connor, M. A. Yakrus, and W. M. Gross.** 1994. Cross-reactivity of genetic probe for detection of *Mycobacterium tuberculosis* with newly described species *Mycobacterium celatum*. J. Clin. Microbiol. **32:**536–538.
5. **Cilia, V., B. Lafay, and R. Christen.** 1996. Sequence heterogeneities among 16S ribosomal sequences, and their effect on phylogenetic analyses at the species level. Mol. Biol. Evol. **13:**451–461.
6. **Clayton, R. A., G. Sutton, P. S. Hinkle, Jr., C. Bult, and C. Fields.** 1995. Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. Int. J. Syst. Bacteriol. **45:**595–599.
7. **Domenech, P., M. C. Menendez, and M. J. Garcia.** 1994. Restriction fragment length polymorphisms of 16S rRNA genes in the differentiation of fast-growing mycobacterial species. FEMS Microbiol. Lett. **116:**19–24.
8. **Emler, S., B. Ninet, P. Rohner, R. Auckenthaler, D. Jäger, and B. Hirschel.** 1996. Molecular basis for cross-reactivity between a strain of *Mycobacterium terrae* and DNA probes for *Mycobacterium tuberculosis* complex. Eur. J. Clin. Microbiol. Infect. Dis. **14:**627–629.
9. **Felsenstein, J.** 1988. Phylogenies from molecular sequences: inference and reliability. Annu. Rev. Genet. **22:**521–565.
10. **Ji, Y.-E., M. J. Colston, and R. A. Cox.** 1994. The ribosomal RNA (rrn) operons of fast growing mycobacteria: primary and secondary structures and their relation to rrn operons of pathogenic slow-growers. Microbiology **140:** 2829–2840.
11. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
12. **Kirschner, P., and E. C. Böttger.** 1992. Microheterogeneity within rRNA of *Mycobacterium gordonae*. J. Clin. Microbiol. **30:**1049.
13. **Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F. C. Bange, and E. C. Böttger.** 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. J. Clin. Microbiol. **31:**2882–2889.
14. **Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese.** 1993. The ribosomal database project. Nucleic Acids Res. **21:**3021–3023.
15. **Monod, M., and L. A. Applegate.** 1994. RNA extraction and Northern hybridization of *Aspergillus fumigatus* for the detection of alkaline metalloprotease gene expression, p. 19–27. *In* B. Maresca and G. S. Kobayashi (ed.), Molecular biology of pathogenic fungi: a laboratory manual. Telos Press, New York.
16. **Monod, M., G. Togni, B. Hube, and D. Sanglard.** 1994. Multiplicity of genes encoding secreted aspartic proteinases in Candida species. Mol. Microbiol. **13:**357–367.
17. **Mylvaganam, S., and P. P. Dennis.** 1992. Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. Genetics **130:**399–410.
18. **Portaels, F., P.-A. Fonteyne, H. De Beenhouwer, P. de Rijk, A. Guédénon, J. Hayman, and W. M. Meyers.** 1996. Variability in 3′ end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to geographic origin of isolates. J. Clin. Microbiol. **34:**962–965.
19. **Rogall, T., J. Wolters, T. Flohr, and E. C. Böttger.** 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. Int. J. Syst. Bacteriol. **40:**323–330.
20. **Saitou, N., and M. Nei.** 1987. The neighbor joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
21. **Sechi, L. A., F. M. Zuccon, J. E. Mortensen, and L. Daneo-Moore.** 1994. Ribosomal RNA gene (rrn) organization in enterococci. FEMS Microbiol. Lett. **116:**307–314.
22. **Springer, B., L. Stockman, K. Teschner, G. D. Roberts, and E. C. Böttger.** 1996. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J. Clin. Microbiol. **34:**296–303.
23. **Stahl, D. A., and J. W. Urbance.** 1990. The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. J. Bacteriol. **172:**116–124.
24. **Vincent Lévy-Frébault, V., and F. Portaels.** 1992. Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. Int. J. Syst. Bacteriol. **42:**315–323.
25. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.