JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1996, p. 2963–2967 Vol. 34, No. 12
0095-1137/96/$04.00+0

Characterization of an SAV Organism and Proposal of *Mycobacterium triplex* sp. nov.

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Received 10 May 1996/Returned for modification 3 August 1996/Accepted 16 September 1996

Polyphasic taxonomic methods were employed to characterize a new species of slowly growing, nonpigmented mycobacteria. We propose the name *Mycobacterium triplex* sp. nov. for this new taxon. Conventional identification testing demonstrated a group of similar organisms that were geographically widespread in the United States. Commercially available nucleic-acid probes specific for the *Mycobacterium avium* complex were unreactive for these strains. High-performance liquid chromatography analysis of the mycolic acids revealed mycolate profiles that closely resembled *Mycobacterium simiae*. Comparative 16S rRNA sequence data confirmed the phylogenetic relationship of the strains with the slowly growing mycobacteria. Representative-type strains have been deposited in the American Type Culture Collection as strain ATCC 70071.

For several years, the Mycobacteriology Laboratory at the Centers for Disease Control and Prevention (CDC) has routinely reported on an unusual group of organisms described as SAV. This group of slowly growing mycobacteria resembled *Mycobacterium avium* complex or *Mycobacterium simiae* by conventional biochemical tests. However, commercial genetic probes designed to detect species of the *M. avium* complex were negative. Analysis of mycolic acids with high-performance liquid chromatography (HPLC) demonstrated a mycolate pattern that was most consistent with *M. simiae* (9, 11). The HPLC analysis revealed several different chromatographic types for this SAV group. Since 1990, the CDC has used the term SAV in reporting this group of organisms. Mycolic-acid profiles, standard biochemical tests, and genetic probe results differentiated these organisms from *M. simiae* and *M. avium*. This study reports on the subgroup of SAV which is nonpigmented and nonphotochromogenic. The characteristics of these organisms indicated that they belonged to a new species for which we propose the name *Mycobacterium triplex.*

MATERIALS AND METHODS

Bacterial strains. Ten strains were studied. Sources of the strains and their designations are shown in Table 1. Authenticated mycobacterial strains from CDC and the California Department of Health Services, Microbial Diseases Laboratory, Berkeley, used for comparison were *M. simiae* (TMC 1226), *M. avium* (ATCC 25291), and *M. intracellulare* (ATCC 13950).

Growth, biochemical tests, and drug susceptibility. All mycobacterial strains were isolated by standard methods. These strains had been submitted to either CDC or the Microbial Diseases Laboratory for identification as clinical isolates. For testing, subcultures were prepared using Lowenstein-Jensen (LJ) slants and incubated at 35°C. Temperature studies were conducted at 42°C, 37°C, 35°C, 33°C, 28°C, and 25°C at 3 weeks of incubation on LJ slants. Colony morphology was determined on LJ slants and 7H10 agar plates at 37°C after 3 weeks of growth. Photochromogenicity and pigment production tests were conducted by standard methods at 1 and 3 weeks, respectively (15). The following tests were performed for differentiation: nitrate reduction, Tween 80 hydrolysis, semiquantitative catalase, 68°C heat-stable catalase, urease, arylsulfatase, sodium chloride tolerance, and niacin production. Drug susceptibility tests were performed on Middlebrook 7H10 agar using the proportion method (15). Drugs used and concentrations are shown in Table 2.

HPLC. Mycolic-acid pattern analysis was performed according to established procedure (3). Briefly, whole cells were saponified in methanolic potassium hydroxide solution. After acidification, chloroform extracts containing the mycolic acids were separated from the cellular materials. Mycolic acids were derivatized to bromophenacyl esters. Chromatography was performed using a Waters LCM1 chromatograph (Waters Chromatography, Milford, Mass.) controlled by Millennium software and equipped with a Nova-Pak C_{18} column (3.9 by 75 mm), a column heater (31 ± 1°C), and a variable wavelength UV detector adjusted to 260 nM. A 10-min methanol and methylene chloride gradient was used as previously described (10). Patterns generated with this method were visually matched to standard CDC HPLC patterns of authentic species using the relative retention time ratios as calculated by the software. These ratios were derived using a high-molecular-weight external standard (Ribi ImmunoChem Research, Inc., Hamilton, Mont.) as the reference peak (4).

16S rRNA amplification and sequencing. Mycobacterial cells were disrupted by shaking with siliconized glass minibeads in a Mickle apparatus as described previously (20). Aqueous cellular extracts were reacted with PCR to amplify the 16S gene with oligonucleotides 5′ GAGAGTTTGATCCTGGCTCAG 3′ and 5′ AAGGAGGTGATCCAGCCGCA 3′ as described previously (7). Thermocycling conditions included 39 cycles of denaturation at 94°C for 1 min, primer annealing at 68°C for 3 min, extension at 72°C for 2 min, and final extension at 72°C for 10 min. QIAquick PCR purification columns (Qiagen Inc., Chatsworth, Calif.) were used to remove unincorporated nucleotides and primers from the amplified DNA. Sequencing reactions were performed using the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Division, Perkin-Elmer Corp., Foster City, Calif.) following the manufacturer's specifications. The unincorporated dye terminators and primers were removed from the extension products using Centri-Sep spin columns (Princeton Separations Inc., Adelphia, N.J.). Samples were dried at ambient temperature and resuspended in 6.5 μl of loading buffer (5:1; deionized formamide and 30 mg of blue dextran per ml–50 mM EDTA [pH 8.0]). Resuspended products were denatured at 90°C for 2 min, and 5 μl of each sample was loaded onto a 6% acrylamide gel for analysis.

TABLE 1. Strains included in the study

Strain no.	Geographic location	Source
90-1019[Ta]	California	Lymph node
91-2146	California	Sputum
91-4993	California	Cervical lymph node
92-1281	California	Cerebrospinal fluid
94-0813	Georgia	Sputum
94-1001	Louisiana	Sputum
95-0575	Louisiana	Sputum
95-0667	Kentucky	Sputum
95-0915	California	Sputum
94-1006	Tennessee	Unknown

[a] T, type strain.

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TABLE 2. Characteristics of 10 *M. triplex* strains

Characteristic	% Positive reaction
Growth >7 days	100
Growth on LJ medium (21 days)	
25°C	10
30°C	80
35°C	100
42°C	20
52°C	0
Growth on LJ medium containing 5% NaCl	0
Pigment production in the dark	0
Photochromogenicity	0
Niacin	0
Arylsulfatase (3 days)	0
Arylsulfatase (14 days)	50
Semiquantitative catalase (>45-mm bubbles)	100
Catalase 68°C	100
Nitrate reductase	100
Urease	100
Tween 80 hydrolysis	0
Resistance to (7H10 test medium)	
Isoniazid (1.0 μg/ml)	100
Streptomycin (2.0 μg/ml)	100
Streptomycin (10.0 μg/ml)	80
Ethambutol (5.0 μg/ml)	90
Ethambutol (10.0 μg/ml)	10
Rifampin (6.0 μg/ml)	100
Kanamycin (5.0 μg/ml)	100
Ethionamide (5.0 μg/ml)	10
Capreomycin (10.0 μg/ml)	100
Growth on thiophen-2-carboxylic acid hydrazide (5.0 μg/ml)	100
M. avium complex DNA probe	0

Electrophoretic separation was with an automated ABI Model 373A DNA Sequencer (Applied Biosystems Division) operated at 30 W for 8 h. Sequencing data were generated by examination of almost 1,500 contiguous bases assembled from multiple regions of nucleotide overlap using DNASIS, version 7.0, software (Hitachi Software Engineering America, Ltd., San Bruno, Calif.). Selective 16S rRNA alignment, including the hypervariable region, was analyzed as described below.

Sequence analysis. The mycobacterial nucleotide sequences were aligned using the alignment program PILEUP, part of the Genetics Computer Group software package (6). The *M. triplex* sequence was aligned with published sequences of 16S rRNA from slowly growing mycobacterial species. Ambiguous positions within the alignment sequence were not analyzed. Regions of alignment uncertainty corresponding to *Escherichia coli* positions 30 to 47 and 75 to 84 were omitted. Additionally, positions corresponding to *E. coli* positions 1381 to 1525 were omitted because the contiguous sequence published for *M. xenopi* (GenBank accession number M61664) was incomplete. For the phylogenetic analysis, this reduced the number of positions used from 1,474 to 1,300. A phylogenetic tree was constructed using the software package PHYLIP (8). Similarity values were calculated by the Jukes and Cantor method (12). The neighbor-joining method of Saitou and Nei was used to construct the dendrogram (23).

Nucleotide sequence accession number. The GenBank nucleotide accession number for *M. triplex* is U57632.

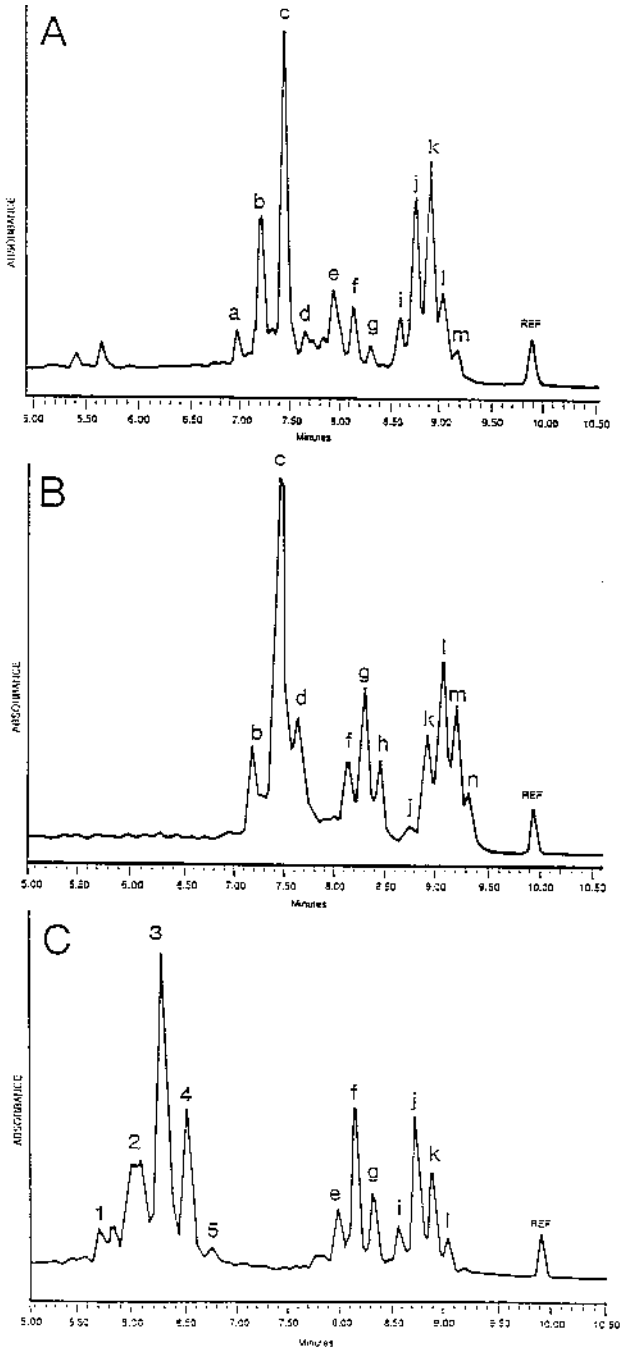

FIG. 1. Alignment of HPLC chromatograms of *M. triplex* (A), *M. simiae* (B), and *M. avium* (C) by the position of the reference peak (REF).

RESULTS AND DISCUSSION

As shown in Table 1, the geographic distribution of these strains in the United States was diverse. The isolates were recovered from sputum, lymph nodes, and cerebrospinal fluid.

Cells were acid-alcohol fast by Kinyoun stain. Microscopically, cells were short rods to coccoid. Colonies on LJ and 7H10 media were usually smooth, cream to buff, nonpigmented, and nonphotochromogenic. Visible growth was noted after 14 to 21 days at an optimum temperature of 35°C on LJ medium with no growth at 25°C, 42°C, and 52°C.

All of these isolates failed to hybridize with the commercially available genetic probe (Accuprobe, Gen-Probe, Inc., San Diego, Calif.) for *M. avium* complex.

The biochemical reactions of the isolates were compared with the known characteristics of other similar slowly growing strains of mycobacteria (15). Standard biochemical reactions placed the strains into a group. Strains were negative for niacin production, Tween 80 hydrolysis, and 3-day arylsulfatase and were variable for 14-day arylsulfatase. They did not grow on sodium chloride. Strains were positive for 68°C catalase, nitrate reduction, and urease. The semiquantitative catalase pro-

FIG. 2. Partial 16S rRNA sequence of type strain 90-1019^T (length, 1,474 nucleotides).

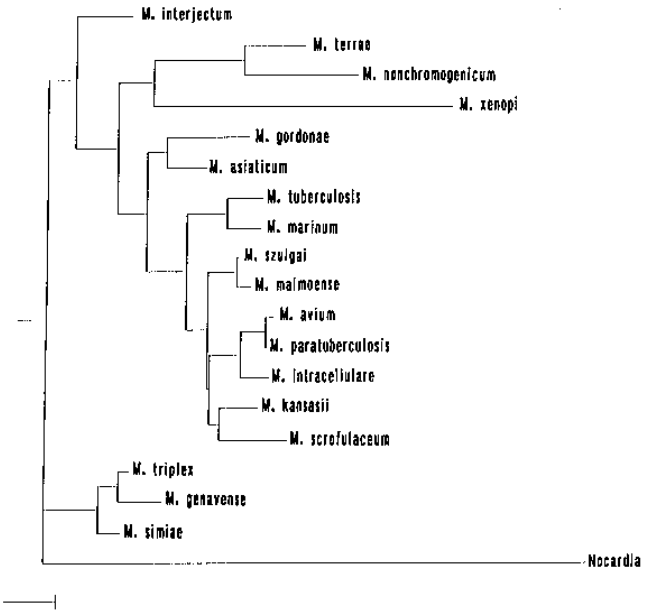

FIG. 4. Phylogenetic dendrogram indicating the relationship of *M. triplex* to species of slowly growing mycobacteria. Bar = 10-nucleotide difference, determined by measuring the length of the horizontal lines connecting two species. Sequence analysis is based on 1,300 of the 1,474 16S rRNA positions determined. The tree was rooted by using *Nocardia asteroides* as an outgroup.

duced a >45-mm column of bubbles. All strains grew on thiophen-2-carboxylic acid hydrazide at 5.0 μg/ml.

M. triplex strains were similar to *M. avium* complex in traditional susceptibility testing. All of the isolates were resistant to isoniazid at 1.0 μg/ml, streptomycin at 2 μg/ml, rifampin at 6.0 μg/ml, kanamycin at 5.0 μg/ml, and capreomycin at 10.0 μg/ml. Eight of the isolates were sensitive to ethambutol at 10.0 μg/ml, and nine were sensitive to ethionamide at 5.0 μg/ml. One of the isolates was resistant to both of these drugs. Generally, most strains were resistant to the commonly used antituberculosis drugs (Table 2).

Strains reported from CDC with the prior designation SAV had been shown to produce HPLC patterns that visually resembled *M. simiae* (9, 11). Identification by visual recognition, while somewhat subjective, has the advantage over computer methods because it does not force sample identification (3, 10). *M. avium*, *M. simiae*, and *M. triplex* produced an HPLC pattern with a triple mycolate cluster. The HPLC patterns for *M. triplex* and *M. simiae* were distinctly different from *M. avium* (Fig. 1). Major peaks were determined by the HPLC method used and labeled by the software according to relative retention time ratios by comparison with an added internal standard (10). For clarification, the primary mycolic-acid peaks have been labeled 1 to 5 and a to n (Fig. 1). Peaks with heights >2% of the total peak heights eluting between 5.5 min and 10.5 min were used for comparison. Peaks appearing between 4.5 min and 5.5 min for *M. triplex* (Fig. 1A) did not appear in all isolates and, therefore, were not considered for analysis. Peaks with the same relative retention time (±0.01 min) were used for alignment of chromatograms. *M. triplex* and *M. simiae* both produced a group of mycolates that eluted at 7.0 min. *M. avium* produced shorter carbon chain-length mycolic acids that emerged before 7.0 min. *M. simiae* produced a range of mycolates that started emerging with peak b at 7.2 min and terminated at 9.4 min with peak n. Most notable, *M. simiae* produced peak h and peak n. *M. triplex* does not have these peaks. Additional distinguishing features of *M. triplex* versus *M. simiae* include early eluting mycolates at 7.0 min (peak a), patterns terminating at 9.2 min (peak m), and exhibition of three peaks (a, e, and i) that are not common with *M. simiae.*

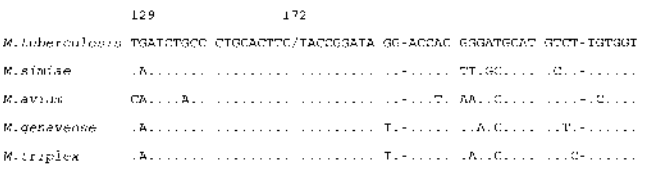

FIG. 3. Alignment of 16S rRNA variable region of selected strains of mycobacteria. *M. tuberculosis* was used as the reference sequence. Dots indicate nucleotide identity; dashes indicate deletions.

The 16S rRNA gene is highly conserved in certain regions and highly variable in others. Primers designed to the conserved regions have been used to amplify the 16S rRNA sequence for direct sequencing of almost the entire nucleotide sequence (7, 28). Although homology values demonstrated for the mycobacteria are >95% (27), a species-specific signature region for mycobacteria has been shown to exist in a hypervariable region corresponding to *E. coli* 16S rRNA positions 129 to 266 (16). This variable region has been used for identification and naming of a new species of mycobacteria (1, 2, 5, 13, 14, 17, 18, 24, 25). Analysis of the 16S rRNA hypervariable region revealed a unique sequence (Fig. 2 and 3). All 10 of the strains were identical in this region. Phylogenetic analysis showed a close relationship of *M. triplex* to *M. simiae* and the difficult-to-grow *M. genavense* (Fig. 4). Homologies of 99.4% were noted to similar organisms (Table 3). The similarity values revealed that *M. interjectum* is closer to *M. simiae* and *M. genavense* than to *M. avium* (Table 3). However, the HPLC pattern for *M. interjectum* is similar to the *M. avium* pattern and distinctly different from *M. simiae* and *M. genavense*. Phylogenetic relatedness schemes based on 16s rRNA also separate slow-growing from fast-growing species (26), demonstrating an intermediate position for *M. simiae* and *M. genavense*

TABLE 3. 16S rRNA similarity values for strains of slowly growing mycobacteria[a]

No.	Species	% Similarity with control no.																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*M. triplex*																		
2	*M. simiae*	99.5																	
3	*M. genavense*	99.5	99.2																
4	*M. interjectum*	98.5	98.8	98.2															
5	*M. terrae*	97.2	96.8	96.9	97.1														
6	*M. nonchromogenicum*	96.2	96.3	97.0	95.5	98.3													
7	*M. gordonae*	97.4	97.3	97.0	97.8	97.2	96.4												
8	*M. asiaticum*	97.8	97.7	97.5	98.2	97.6	96.6	98.8											
9	*M. tuberculosis*	96.6	96.6	96.4	97.1	97.1	96.3	97.6	98.5										
10	*M. marinum*	96.6	96.4	96.2	97.0	97.2	96.5	97.8	98.5	99.3									
11	*M. avium*	97.1	96.9	96.7	97.6	96.4	96.2	97.8	98.2	98.4	98.5								
12	*M. paratuberculosis*	97.1	97.0	96.8	97.7	96.5	96.3	97.8	98.2	98.5	98.6	99.9							
13	*M. intracellulare*	96.7	97.0	96.4	97.6	96.4	96.1	97.7	98.0	98.2	98.3	99.4	99.5						
14	*M. szulgai*	97.2	97.4	96.8	98.1	96.6	96.2	98.0	98.6	98.8	98.7	99.0	99.1	99.1					
15	*M. malmoense*	97.1	97.4	96.8	98.1	96.3	96.2	98.0	98.4	98.6	98.6	98.9	99.0	99.0	99.9				
16	*M. kansasii*	96.8	97.3	96.4	97.6	96.4	95.8	97.9	98.2	98.7	98.6	98.8	98.9	98.9	99.2	99.1			
17	*M. scrofulaceum*	96.9	97.4	96.6	97.9	96.5	96.1	97.6	97.8	98.1	98.2	98.5	98.6	98.8	98.8	98.7	98.9		
18	*M. xenopi*	95.0	95.0	94.7	95.1	95.6	95.1	95.2	95.5	95.7	95.6	95.2	95.2	95.4	95.5	95.3	95.2	95.2	
19	*Nocardia* sp.	93.9	93.8	93.9	93.6	93.1	92.6	93.1	93.0	92.7	92.5	92.5	92.6	92.6	92.6	92.4	92.4	92.4	91.6

[a] Values were derived from data shown in Fig. 4; 1,300 nucleotides were used.

(19, 21, 22). The homogeneous complex formed with *M. triplex* strains was in this same position, between rapidly and slowly growing mycobacteria.

Taxonomic description of *M. triplex* sp. nov. *M. triplex* (trip′leks. L. gen. adj. triplex referring to something consisting of three parts, specifically, the triple-cluster HPLC pattern produced by these isolates). Cells are acid-alcohol fast, short rods to coccoid. Colonies on LJ and 7H10 are cream to buff, nonpigmented, usually smooth, sometimes appearing rough with age, but always nonphotochromogenic. Growth occurs at both 30°C and 35°C but not at 25°C, 42°C, or 52°C. Strains have a similar drug pattern and are usually resistant to streptomycin, isoniazid, rifampin, ethambutol, ethionamide, kanamycin, thiopene-2-carboxylic acid hydrazide, and capreomycin. They do not react to the commercial genetic probe for *M. avium* complex. Mycolic acids analyzed by HPLC produce a three-cluster mycolate pattern, most closely resembling that of *M. simiae.*

Characteristics which differentiate *M. triplex* from *M. avium*, *M. simiae*, and *M. genavense.* Phenotypic differences were demonstrated among closely related species *M. simiae*, *M. avium*, and the new species, *M. triplex. M. triplex* can be distinguished by its HPLC-mycolic-acid pattern that resembles *M. simiae* but is clearly distinctive from *M. avium. M. triplex* is differentiated from *M. simiae* strains by lack of pigmentation, negative test for niacin, and positive test for nitrate. The abundant growth of *M. triplex* on LJ differentiates it from *M. genavense. M. triplex* produced a unique 16S rRNA sequence that is different from all described mycobacteria.

Combined results from HPLC, conventional biochemical testing, and commercial genetic probes were supportive of a new species. Conclusive genetic evidence of a novel species was demonstrated with 16S rRNA analysis.

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