Mycobacterium kubicae* sp. nov., a slowly growing, scotochromogenic *Mycobacterium

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A previously uncharacterized, slowly growing, scotochromogenic *Mycobacterium* species was detected by HPLC analysis of the cell-wall-bound mycolic acids. The mycolic acid pattern standard was shown to be a late-eluting, contiguous peak cluster occurring at approximately 8–9 min. The mycolic acid pattern was noted to be most similar in number of peaks and range of elution to that reported previously for *Mycobacterium asiaticum*. However, the relative distribution of peaks within the elution range demonstrated a pattern with prominent peaks that started to emerge later than the characteristic *M. asiaticum* pattern. Standard biochemical identification test results were similar to those of the photochromogenic species *M. asiaticum*. Comparative 16S rRNA gene sequence analysis confirmed the genetic uniqueness of the strains and demonstrated the unclassified mycobacteria to be in a unique, intermediate position between slow and rapid growers in the phylogenetic tree of *Mycobacterium*. The name *Mycobacterium kubicae* sp. nov. is proposed for this taxon. The type strain is CDC 941078^T (= ATCC 700732^T = CIP 106428^T).

Keywords: *Mycobacterium kubicae*, mycolic acids, HPLC

INTRODUCTION

Conventional biochemical tests for identification of mycobacteria were designed primarily to detect and identify *Mycobacterium tuberculosis* and clinically important nontuberculous mycobacteria (NTM) (Kent & Kubica, 1985). These tests have been extensively employed by clinical laboratories and have been shown to be valuable benchmarks for species identification. However, with the increasing number of newly defined taxa and the recognition of 'difficult-to-identify' variants of known species, the tests are proving insufficient for identification (Kirschner *et al.*, 1993).

In the United States, mycobacteriology reference laboratories with special expertise, like those at the

Centers for Disease Control and Prevention (CDC), frequently analyse such isolates. The standard method for identification is HPLC analysis of mycolic acids. This rapid and simple chromatographic method replaced standard biochemical tests as the primary tool for identification of *Mycobacterium* species at CDC in 1990. After using this method for several years, it was recognized that an extensive number of mycobacteria presented non-representative chromatographic patterns when compared to mycolic acid pattern standards (MAPS) of well-characterized species (Butler *et al.*, 1996, 1999). To facilitate communication of the occurrence of these unusual NTM to clinical laboratories the CDC Mycobacteriology Laboratory has used the following designations: (i) mycobacteria with unique mycolic acid patterns, reported as no common pattern (NCP); (ii) groups of 10 or more mycobacteria with matching patterns, reported as NCP with an assigned laboratory number; and (iii) mycobacteria with patterns that resembled known species, reported as NCP with an assigned laboratory number and as having a likeness to a known species. Described here are a group of NTM strains that initially were detected

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Abbreviations: NCP, no common pattern; NTM, nontuberculous mycobacteria; MAPS, mycolic acid pattern standards.

The GenBank accession number for the 16S rRNA gene sequence of *Mycobacterium kubicae* is AF133902.

by HPLC and reported as NCP 216 and *Mycobacterium asiaticum*-like.

METHODS

Strains. The strains used in the study were submitted to CDC as 'difficult-to-identify' isolates by state mycobacteriology laboratories from 1994 to 1997. They were recovered from -70°C storage by subculturing to Lowenstein-Jensen (L-J) slants and Middlebrook-Cohn 7H9 broths (Remel) incubated at 37°C . Characterization and reproducibility studies were done with 15 strains that exhibited a common HPLC pattern.

Phenotypic tests. Before comprehensive testing was initiated, strains were cultured in Middlebrook-Cohn 7H9 broth for 1 week. The 7H9 culture was streaked on Middlebrook-Cohn 7H10 and incubated for 3 weeks, and growth was examined at $\times 30$ magnification for purity. Growth studies were performed on L-J and Middlebrook-Cohn 7H11 media incubated at 28, 33, 37 and 42°C for 8 weeks. Colony morphology and pigment production were observed on both media types after 3 weeks incubation at 37°C . Cell morphology was determined from multiple smears prepared by the Kinyoun acid-fast method (Kinyoun, 1915) at $\times 1000$ magnification.

Previously described methods (Kent & Kubica, 1985) were used to determine the standard biochemical reactions for these strains. A representative strain was selected based on biochemical test results and deposited in the American Type Culture Collection as the type strain. Phenotypic tests were performed for scotochromogenic mycobacteria and included an initial description of morphology and pigment production, nitrate reduction and Tween hydrolysis. Other enzymic reactions included catalase production, aryl-sulfatase and urease.

Antimicrobial susceptibility testing was determined by the agar proportion method on 7H10 medium (Kent & Kubica, 1985).

Mycolic acid analysis. Mycolic acids were analysed by TLC and HPLC. The whole cells were saponified in 25% methanolic KOH for 1 h in an autoclave at 121°C . The saponified mixture was acidified and the mycolic acids were extracted by the addition of chloroform. For the TLC procedure, the mycolic acids were methylated with freshly prepared diazomethane and spotted onto two silica gel F-254 plates. One plate was developed with dichloromethane and the other with petroleum ether. Mycolic acids were visualized by spraying with 0.01% ethanolic rhodamine as described previously (Lévy-Frébault *et al.*, 1986). For the HPLC procedure, mycolic acids were derivatized to bromophenacyl esters with *para*-bromophenacyl-8 (Pierce). The derivatized samples were separated by reverse-phase HPLC with a C-18 column using a gradient elution of methanol and methylene chloride as described previously (Butler *et al.*, 1996).

16S rDNA sequencing. Cells suspended in buffer were disrupted with siliconized zirconium beads by vigorous shaking to obtain aqueous extracts containing chromosomal DNA. PCR amplification of the 16S rRNA gene region was done with oligonucleotides 5'-GAGAGTTTGATCCTGG-CTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' as described previously (Edwards *et al.*, 1989; Rogall *et al.*, 1990). Direct sequencing of the 16S rDNA amplicon was performed using a *Taq* DyeDeoxy terminator cycle sequen-

cing kit (Applied Biosystems) with an Applied Biosystems model 373A DNA sequencer, following the instructions of the manufacturer. Overlapping DNA segments from both the forward and reverse strands were analysed to determine a consensus sequence for the 16S rDNA. The analysis was repeated multiple times from single colony isolates. Consensus sequence data were compared to 16S rDNA sequence information in GenBank using the FASTA algorithm from the Wisconsin Package for Sequence Analysis [Genetics Computer Group (GCG)]. The GCG program PILEUP was used to create a multiple sequence alignment from related sequences in GenBank. To properly align the sequences it was necessary to truncate 4 nucleotides at the start of the consensus sequence and 11 at the end, leaving 1317 bases for analysis. Version 3.5c of the Phylogeny Inference Package (PHYLIP; Felsenstein, 1985, 1993) was used for evolutionary analysis. Programs used for the analysis included: DNADIST, for calculation of a distance matrix on the nucleotide substitution using the model of Jukes & Cantor (1969); NEIGHBOR, for construction of a tree using the neighbour-joining method; SEQBOOT, for bootstrap analysis of 100 replicates; and CONSENSE, for generation of a majority rule consensus tree representative of groups occurring in greater than 50% of all input trees.

RESULTS AND DISCUSSION

Geographically, the 15 isolates composing the study group originated from California, Florida, Georgia, Louisiana, Maryland, Oklahoma, Pennsylvania, Tennessee and Texas. The clinical source of four of the isolates were undetermined but eight were from sputum and three from bronchial washes. Additional clinical and strain history was not available.

Cells stained strongly acid-alcohol-fast, were rod-shaped and frequently bent, but did not form filaments or branches. Occasional coccoid forms were noted. The estimated width ranged from 0.25 to 0.5 μm and the length from 0.25 to 7.0 μm . The optimal growth temperature was 37°C . On Middlebrook-Cohn 7H11 medium, mature growth developed in 3 weeks at 28, 33 and 37°C . Microcolonies developed in 2 weeks at these same temperatures. On L-J medium, microcolonies developed in 3 weeks but mature colonies were not noted until after 4 weeks of incubation at 33 and 37°C . Thirteen of the fifteen isolates tested failed to grow on L-J at 28°C , and the remaining two isolates developed microcolonies after 3 weeks of incubation. None of the isolates grew on L-J at 42°C . Overall, colonies grown on 7H11 were generally larger. Mature colonies were usually smooth, occasionally rough and always yellow in appearance. Cells lightly inoculated on L-J medium grew film-like and produced a yellow pigment. The strains were negative in the 3 d arylsulfatase test and produced > 45 mm foam in the semiquantitative catalase test. They were negative for nitrate reduction and did not demonstrate enzymic hydrolysis of Tween 80 or urease activity (Table 1).

Standard biochemical reactions were homogeneous for the 15 strains, but were not completely distinctive when compared to other slow-growing mycobacteria.

Table 1. Characteristics of 15 slow-growing strains composing the study group

Characteristic	Positive reaction/ resistance (%)
Growth on Middlebrook–Cohn 7H11 (21 d):	
28, 33, 37 °C	100
42 °C	0
Growth on L–J (28 d):	
28 °C	13
33, 37 °C	100
42 °C	0
Arylsulfatase (3 d)	20
Nitrate reductase	0
Pigmented in the dark	100
Semiquantitative catalase (45 mm foam)	100
Tween 80 hydrolysis	100
Urease	0
Resistance to ($\mu\text{g ml}^{-1}$ in 7H10 test medium):	
Amikacin (6.0)	100
Ciprofloxacin (1.0, 2.0, 4.0, 8.0)	100, 93, 33, 6
Clarithromycin (3.0)	0
Clofazimine (1.0)	0
Cycloserine (25.0, 50.0)	66, 0
Ethambutol (10.0)	7
Ethionamide (5.0)	0
Isoniazid (0.2, 1.0)	100, 20
Kanamycin (6.0)	93
Rifabutin (1.0, 2.0)	100, 46
Rifampin (5.0)	100
Streptomycin (2.0, 10.0)	100, 86

Pigment production and growth rate were characteristic of Group II scotochromogens, by the classical Runyon Group Classification system. Overall, the biochemical results were most like the photochromogenic species *M. asiaticum* (Table 1). Results of drug susceptibility testing were unremarkable for differentiation of NTM and are shown in Table 1.

Alpha, keto and methoxy mycolates consistent with mycobacteria were detected by TLC (data not shown). This chemical association was confirmed with HPLC analysis by the elution of unique mycolic acids in the time range for mycobacteria. All 15 isolates analysed by HPLC produced a distinctive homogeneous mycolic acid group, for which a representative MAPS is shown in Fig. 1. The single cluster, seven-peak mycolate pattern was similar to the HPLC pattern reported previously with *M. asiaticum* (Butler *et al.*, 1999). However, a difference in the overall time range of elution, calculated by comparison with the late emerging internal standard, was 1.12 min for the study isolates and 1.16 min for *M. asiaticum*. Additionally,

Fig. 1. Representative MAPS for the study isolates (a) and the phenotypically similar *M. asiaticum* (b). ISTD, internal standard.

differences were reflected by the relative heights of corresponding peaks in the patterns (Fig. 1): peak 1, 2.3 and 19.2%; peak 2, 19.5 and 31.2%; peak 3, 17.2 and 31.2%; peak 4, 13.4 and 17.5%; peak 5, 28.1 and 14.6%; peak 6, 16.8 and 2.25%, for the study isolate and *M. asiaticum*, respectively. This disproportional amount of each peak detected by HPLC has previously been reported to be species-specific for mycobacteria (Butler *et al.*, 1996, 1999; Duffey *et al.*, 1996; Floyd *et al.*, 1996; Glickman *et al.*, 1994; Ramos, 1994; Thibert & LaPierre, 1993; Tortoli *et al.*, 1995; Wayne *et al.*, 1996).

Initially, all isolates were screened genetically by determination of the species-specific signature region A in the 16S rRNA gene, reported to be representative of slowly growing mycobacteria (Stackebrandt & Smida, 1988; Stahl & Urbance, 1990; Rogall *et al.*, 1990). This hypervariable region of approximately 139 nucleotides in the 5' end of the gene was found to be identical for the study isolates and divergent from other mycobacteria (Fig. 2). The distinctiveness of the sequence pattern for signature region A was further supported by the demonstration of a unique but short helix 18 for the signature region B (Fig. 2). Initial application of molecular sequencing for identification of *Mycobacterium* species had attributed the presence of a short helix 18 in region B to be a feature of rapidly growing mycobacteria. However, the short helix 18 has since been revealed in a number of slow-growing mycobacteria noted to be genetically similar to *Myco-*

A similarity search of GenBank for related sequences with 1321 continuous nucleotides of the consensus strand revealed 99.2% similarity to a deposited sequence determined from an uncharacterized mycobacterium isolate. The sequence originated from examination of a single isolate in a study of unusual slowly growing mycobacterium strains conducted by the International Working Group on Mycobacterial Taxonomy (IWGMT), strain number 90210, GenBank accession number X88913 (Wayne *et al.*, 1987). However, dissimilar nucleotides were shown for the IWGMT isolate compared to the study isolates, including eight significant nucleotides in the signature or hypervariable region A (Fig. 2). Moreover, this unavailable IWGMT isolate was biochemically distinct from the study isolates and had been reported to be non-pigmented, negative for catalase activity and positive for Tween hydrolysis. Further, genetic comparison with the phenotypically similar *M. asiaticum* demonstrated an overall difference of 49 nucleotides in the 16S rDNA strand with six occurring in signature region A. Further, phylogenetic analysis of the DNA consensus sequence was done by alignment with other representative mycobacteria sequences deposited in GenBank for a region of 1317 nucleotides. This sequence was used to construct the phylogenetic dendrogram shown in Fig. 3. The unique position of the study isolates was confirmed to be intermediate between slow- and rapidly growing mycobacteria on a branch with other slow-growing mycobacteria that

The *Mycobacterium* species found to be on this intermediate branch of the phylogenetic tree have been reported genetically similar. It was important to note that chemically each of these species produced a distinctive HPLC mycolic acid pattern that was complex and multiple-clustered. This was unlike the mycolic acid pattern of the study group, which was a simple, single-cluster MAPS (Fig. 1). The reliability of the tree branching pattern was tested by the construction of a majority-rule consensus tree for 100 replicates and indicated a moderate bootstrap value, 85% (Felsenstein, 1985; Hillis & Bull, 1993). Both the phylogenetic relationships and chemotaxonomical data were supportive of a novel *Mycobacterium* taxon.

Mycobacterium kubicæ (ku.bi'cae. N.L. gen. n. *kubicæ* of Kubica, to honour the contributions of George P. Kubica, an exceptional American mycobacteriologist, mentor and teacher).

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Fig. 3. Phylogenetic relationships of selected species belonging to the genus *Mycobacterium* based on 16S rRNA gene sequences. The tree was rooted with *Nocardia asteroides* as an outgroup. GenBank accession numbers are shown in parentheses.

coccoid forms, but without branches or aerial mycelia. Colonies grow smooth and domed on Middlebrook–Cohn 7H11, film-like on L–J and produce a yellow pigment in the dark. The organism produces > 45 mm foam in the semi-quantitative catalase test. It shows variable results for arylsulfatase at 3 d, and is negative for Tween 80 hydrolysis, nitrate reductase and urease. Isolates are resistant to amikacin and rifampin, partially resistant to ciprofloxacin, cycloserine, ethambutol, isoniazid, rifabutin and streptomycin, and susceptible to clarithromycin, clofazimine and ethionamide. Mycolic acids are long-carbon-chain-length, nonoxygenated alpha mycolates and oxygenated keto and methoxy mycolates, and produce a single-cluster MAPS by HPLC, typical of mycobacteria. Phylogenetically, the 16S rRNA gene sequence is unique and the species occupies a characteristic, intermediate position between slow- and rapidly growing myco-

bacteria. The GenBank accession number for the 16S rRNA gene sequence is AF133902. The type strain is CDC 941078^T from human sputum (= ATCC 700732^T = CIP 106428^T).

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