Phylogeny of the *Mycobacterium chelonae*-Like Organism Based on Partial Sequencing of the 16S rRNA Gene and Proposal of *Mycobacterium mucogenicum* sp. nov.

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The *Mycobacterium chelonae*-like organism (MCLO) is a recently described member of the *Mycobacterium fortuitum* complex which causes posttraumatic skin infections and catheter sepsis. This taxon is a distinct group biochemically and has a unique mycolic acid profile as determined by high-performance liquid chromatography. Its phylogenetic relationships to other mycobacteria, however, have not been studied previously. We sequenced 1,062 bp of the 16S rRNA genes from three MCLO strains obtained from the American Type Culture Collection and compared our results with the sequences of previously described taxa of rapidly growing and slowly growing mycobacteria. Two biochemically typical strains (ATCC 49650^T [T = type strain] and ATCC 49651) had identical sequences, while the sequence of a biochemically atypical strain (ATCC 49649) differed by 4 bp from the sequence of the two typical strains. The Hamming distances between these MCLO strains and related rapidly growing mycobacteria are comparable to the Hamming distances among taxa of rapidly growing mycobacteria established as species by DNA-DNA hybridization. We propose the name *Mycobacterium mucogenicum* sp. nov. for this new taxon because of the highly mucoid nature of most isolates on solid media.

Two closely related outbreaks of peritonitis associated with automated peritoneal dialysis machines occurred in 1976 and 1978 in the northwestern United States (2). The causative organism, which was recovered from peritoneal fluid, the automated dialysis machines, and tap water used to supply the machines, was a previously undescribed, nonpigmented, rapidly growing mycobacterium. This organism was identified as a member of the *Mycobacterium fortuitum* complex on the basis of its partial acid-fast staining (10 to 25%), a positive 3-day arylsulfatase reaction, and growth on MacConkey agar without crystal violet at 28°C (17). Because many other laboratory characteristics of the organism were similar to those of *Mycobacterium chelonae* (formerly *M. chelonae* subsp. *chelonae*) (17, 25), including failure to grow on medium containing 5% sodium chloride, inability to reduce nitrate, borderline or negative reaction in the iron uptake test, and utilization of citrate as a sole carbon source (Table 1), the organism was tentatively designated the *M. chelonae*-like organism (MCLO) by Silcox et al. (17).

In the subsequent 10 years, this organism was only rarely identified as a human pathogen; usually it was identified as a cause of nosocomial infections associated with water, such as the water in processed hemodialysis equipment (3). In 1993 Wallace et al. published a review of 87 clinical isolates of MCLO encountered sporadically in a mycobacterial laboratory in the southwestern United States (26). The majority of these isolates were respiratory isolates and had no clinical significance except in immunocompromised hosts. In contrast, there were 33 nonrespiratory isolates, approximately two-thirds of which were known to be clinically significant. The most common types of clinical disease associated with the MCLO were posttraumatic wound infections and catheter-related sepsis,

which are clinical diseases that are relatively commonly caused by members of other taxonomic groups of rapidly growing mycobacteria (3, 22–24, 27). Extensive growth and biochemical analyses of the 87 MCLO isolates revealed that they were fairly homogeneous and had characteristics similar to those described 10 years earlier (2, 17), except that they exhibited variable nitrate reduction reactions (45%) (Table 1). The MCLO produced an easily identified unique pattern consisting of two sets of mycolic acid ester peaks as determined by high-performance liquid chromatography (HPLC) (26) (Table 1). The uniqueness of the mycolic acids of the MCLO has also been revealed by gas-liquid chromatography with high-temperature thermal cleavage. As determined by this method, the MCLO strains are the only organisms among the members of the *M. fortuitum* complex which have a C₂₂ fatty acid methyl ester cleavage product in addition to the C₂₄ product which is always present (6).

The MCLO strains also exhibit drug susceptibility patterns that differ from those of other members of the *M. fortuitum* complex. In general, they are more susceptible to drugs than the members of any of the other groups are. They are the only organisms that are susceptible to cephalothin and ampicillin (20, 26). Like *M. fortuitum*, they are also susceptible to amikacin, imipenem, ciprofloxacin, and ofloxacin and the newer macrolide antibiotic clarithromycin (4, 20, 26).

Despite the fact that the MCLO strains have been established as human pathogens and as members of a unique group biochemically and on the basis of drug susceptibility data, the status of these organisms as a taxonomic group has not been determined previously genetically. Neither DNA-DNA hybridization experiments nor DNA and RNA comparative sequencing experiments have been performed previously with the MCLO. In this study we partially sequenced the 16S rRNA genes of three MCLO reference strains and compared the sequences which we obtained with sequences determined previously for other taxa of rapidly growing mycobacteria. Our

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TABLE 1. Biochemical characteristics of the pathogenic taxa of rapidly growing mycobacteria, including the MCLO strains^a

Characteristic	<i>M. fortuitum</i>	<i>M. peregrinum</i>	<i>M. fortuitum</i> third biovariant complex		<i>M. chelonae</i>	<i>M. abscessus</i>	MCLO
			Glucitol positive	Glucitol negative			
3-Day arylsulfatase activity	+ (97) ^b	+	+	+	+	+	+ (84)
Nitrate reduction	+ (100)	+	+	+	—	—	+/- (45)
Fe uptake	+ (100)	+	+	+	—	—	— (100)
Utilization of carbon sources							
Mannitol	— (0)	+	+ (92)	+ (92)	—	—	+ (94)
Inositol	— (0)	—	+ (90)	+ (90)	—	—	— (0)
Citrate	— (0)	—	— (11)	— (11)	+	—	+ (85)
Glucitol	— (0)	—	+ (100)	— (0)	—	—	— (2)
HPLC pattern ^c	F	F	F	F	C	C, A	M
Growth in the presence of 5% NaCl	+ (100)	+	+	+	+/-	+	— (0)
High catalase activity (>45 mm)	+ (100)	+	+ (100)	— (0)	+	+	— (15)

^a Data from references 5, 23, 24, and 26.^b +, ≥80% of the strains are positive; —, ≤20% of the strains are positive; +/-, 21 to 79% of the strains are positive. The numbers in parentheses are the percentages of positive strains.^c F, *M. fortuitum* pattern; C, *M. chelonae* pattern; A, *M. abscessus* pattern; M, MCLO pattern.

data strongly support the conclusion of previous phenotypic studies, namely, that the MCLO group is a unique taxonomic group that is distinct from the mycobacterial groups that have been characterized previously.

MATERIALS AND METHODS

Mycobacterial strains. MCLO strains ATCC 49649, ATCC 49650^T (T = type strain), and ATCC 49651 were obtained from frozen stock preparations which had been used for previous submissions of these three strains from our laboratory to the American Type Culture Collection (ATCC), Rockville, Md., in 1990 (26). The taxa used for comparison included *Mycobacterium abscessus* ATCC 19977^T, *M. chelonae* ATCC 35752^T, *M. fortuitum* third biovariant complex glucitol-positive (sorbitol-positive) strain ATCC 49403, *M. fortuitum* third biovariant complex glucitol-negative (sorbitol-negative) strain ATCC 49404, and *Mycobacterium peregrinum* ATCC 14467^T, which were obtained from the ATCC.

Two additional *M. fortuitum* third biovariant complex glucitol-positive (sorbitol-positive) strains, ATCC 49934 and ATCC 49936, as well as three additional *M. fortuitum* third biovariant complex glucitol-negative (sorbitol-negative) strains, ATCC 49935, ATCC 49937, and ATCC 49939, were obtained from frozen stock preparations that had been used to submit these strains to the ATCC. Each of the third biovariant complex strains was considered a typical example of the group that it represented.

Sequence determination. The 16S rRNA regions used for analysis were chosen on the basis of previously published sequence data available in the EMBL-GenBank data library (8–12, 15, 16, 18).

The methods used for DNA extraction, gene fragment amplification of 16S rRNA, and sequence determination have been described previously (10, 11). Briefly, to extract nucleic acids, a colony from a Löwenstein-Jensen slant was suspended in 500 µl of 10 mM Tris–1 mM EDTA (TE). The bacterial pellet was harvested by centrifugation in a microcentrifuge and then dispersed in 100 µl of TE containing acid-washed glass beads (diameter, 100 µm; Sigma, Deisenhofen, Germany). A tissue disintegrator (H. Mickle, Gomshall, Surrey, United Kingdom) was used to disrupt the cells (2 min at the maximum speed). After centrifugation for 5 min, 5 µl of the supernatant was used for PCR. The 16S rRNA gene was amplified by using two pairs of primers that yielded two overlapping DNA fragments. The sequences of the oligonucleotide primers were as follows: AGAGTTTGATCCTGGCTCAG (positions 8 to 28), used in combination with TGCACACAGGCCACAAGGGA (positions 1046 to 1026); and GTGTGGGTTCCTTCCTTGG (positions 830 to 847), used in combination with AAGGAGGTGATCAGCCGCA (positions 1542 to 1522) (the nucleotide positions correspond to the positions in the prokaryotic 16S rRNA sequence of *Escherichia coli*). Typically, one of the primers used in the amplification reaction was biotinylated so that single-stranded DNA sequencing could be performed (7). The single-stranded DNA template was prepared with Dynabeads (Dynal, Hamburg, Germany) as described by the manufacturer. The sequences which we obtained were aligned with selected 16S rRNA sequences as described previously (16).

For the phylogenetic analyses we used only those 16S rRNA regions that were available for the rapidly growing species included in the analysis (corresponding to *E. coli* positions 126 to 554, 894 to 1082, and 1101 to 1332). Pairwise distances (Hamming distances) were calculated by weighting nucleotide differences and

insertions-deletions equally. A phylogenetic tree was constructed by using the neighborliness method as described recently (16).

Nucleotide sequence accession numbers. The EMBL accession numbers for the MCLO strain ATCC 49649, ATCC 49650^T, and ATCC 49651 sequences which we determined are X80773, X80771, and X80772, respectively. The accession numbers for the *M. abscessus* ATCC 19977 and *M. chelonae* ATCC 35752 sequences are X82235 and X82236, respectively.

FIG. 1. Phylogenetic tree showing the relationships of the three MCLO strains to five other taxa belonging to the *M. fortuitum* complex and 19 other species of rapidly growing mycobacteria. The tree was rooted by using *Nocardia asteroides* as an outgroup. Bar = 5 nucleotide differences.

TABLE 3. Signature nucleotides at the 5' end of the 16S rRNA gene (the first nucleotide corresponds to *E. coli* position 175) suggested by Kirschner et al. for differentiating the pathogenic rapidly growing mycobacteria^a

Taxon(a)	Signature sequence ^a
<i>M. tuberculosis</i> - <i>M. bovis</i>	CGG ATA GGACCA CGG GAT GCA TGT CTT
<i>M. fortuitum</i>	••A ••• T••••• ••C •C• T•• ••G TG•
<i>M. peregrinum</i>	••A ••• T••••G ••C AC• T•C ••G TG•
<i>M. fortuitum</i> third biovariant complex glucitol-positive (sorbitol-positive) strains ATCC 49403, ATCC 49934, and ATCC 49936	••• ••• ••••• ••C •C• T•• ••G TG•
<i>M. fortuitum</i> third biovariant complex glucitol-negative (sorbitol-negative) strains ATCC 49404, ATCC 49935, ATCC 49937, and ATCC 49939	••A ••• •••••G ••C TC• T•• ••G GG•
<i>M. abscessus</i>	••• ••• ••••• •AC AC• T•• ••G TGA
<i>M. chelonae</i>	••• ••• ••••• •AC AC• T•• ••G TGA
MCLO strains ATCC 49649, ATCC 49650 ^T , and ATCC 49651	••A ••• ••••• ••C •C• T•• ••G TG•

^a See reference 9. A bullet (•) indicates that the base pair was identical to the *M. tuberculosis* base pair.

RESULTS AND DISCUSSION

We determined 1,062-bp sequences for each of the three MCLO strains, *M. abscessus*, and *M. chelonae*. The nucleotides examined corresponded to the *E. coli* nucleotides at positions 126 to 646, 894 to 1082, and 1101 to 1477. A phylogenetic tree based on equally weighted (Hamming) distances for members of the *M. fortuitum* complex and other rapidly growing mycobacteria is shown in Fig. 1, while the Hamming distances for these and previously described mycobacterial taxa are shown in Table 2.

The MCLO clearly occupy a separate taxonomic niche. These organisms appear to be most closely related to the *M. fortuitum* group; the sequences of strains ATCC 49650^T and ATCC 49651, which are identical, differ by 11 to 18 bp (1.0 to 1.7% of the 1,062 bp examined) from the sequences of five taxa belonging to the *M. fortuitum* group (*M. fortuitum*, *M. peregrinum*, *Mycobacterium senegalense*, and the two taxa of the *M. fortuitum* third biovariant complex). MCLO strains ATCC 49650^T and ATCC 49651 exhibited Hamming distances of more than 20 with all of the other mycobacterial taxa examined, including the two previously described species belonging to the *M. chelonae* group (*M. chelonae* and *M. abscessus*) (Table 2). The Hamming distances which we determined suggest that separate species status is appropriate for the MCLO, since except for the *M. chelonae* and *M. abscessus* sequences (which differed by only 4 bp [0.37%]), the sequences of the 34 previously described species of mycobacteria established by DNA-DNA hybridization studies exhibited differences of 8 bp or more ($\geq 0.75\%$) for the 1,062 bp studied (1, 13, 14). The sequences of *M. fortuitum* and *M. peregrinum*, for example, differed by 15 bp (1.4%), while the sequences of *M. fortuitum* and *M. abscessus* differed by 41 bp (3.9%). DNA-DNA hybridization studies have not been performed with the MCLO yet.

Previously, Kirschner et al. (9) identified a signature nucleotide region (*E. coli* positions 175 to 231) that allowed them to separate most of the six members of the *M. fortuitum* complex which they studied; the only two taxa that could not be separated were *M. chelonae* and *M. abscessus*. When the variable portion of this region (27 bp) was used as a signature sequence, the three MCLO strains had the same sequence, which differed by 4 bp from the *M. chelonae*-*M. abscessus* sequence. However, the MCLO sequence differed by only 1 bp from the sequences of members of the *M. fortuitum* third biovariant complex glucitol-positive group and *M. fortuitum* strains (Table 3).

A second, 26-bp variable region that allowed us to separate *M. chelonae*, *M. abscessus*, and most commonly encountered taxa of rapidly growing mycobacteria was identified between positions 1001 and 1027 (Table 4). This region could be used to differentiate *M. chelonae* and *M. abscessus*, whose sequences

differed by 2 bp. When this region was examined, the MCLO sequence differed by 4 bp from the sequences of *M. fortuitum*, *M. peregrinum*, and members of the *M. fortuitum* third biovariant complex glucitol-negative (sorbitol-negative) group. The sequences of most other taxa differed from each other by 2 bp or more; the exceptions were the sequences of *M. peregrinum* and members of the *M. fortuitum* third biovariant complex glucitol-negative group and the sequences of the MCLO and members of the *M. fortuitum* third biovariant complex glucitol-positive (sorbitol-positive) group, which were identical. We identified a third region (Table 5) that could be used to separate these four taxa easily.

Additional evidence that the MCLO and other species of rapidly growing mycobacteria differ genotypically was provided by the results of PCR amplification of a 439-bp sequence of the 65-kDa heat shock protein gene followed by restriction endonuclease analysis as described by Telenti et al. (21). Digestion of PCR products derived from 20 MCLO isolates, including reference strains ATCC 49649, ATCC 49650^T, and ATCC 49651, with *Hae*III, *Bst*EII, *Aci*I, and *Cfo*I (*Hha*I isoschizomer) yielded restriction fragment length polymorphism patterns which differed from the patterns obtained for members of all other taxonomic groups of nonpigmented rapidly growing mycobacteria (19). Digestion with *Hae*III yielded two similar restriction fragment length polymorphism patterns which differed in one of three fragment bands, while digestion with

TABLE 4. Second region in the 16S rRNA gene (corresponding to *E. coli* positions 1001 to 1027) that allowed us to differentiate the common clinical species belonging to the *M. fortuitum* complex and the MCLO

Taxon(a)	Sequence ^a
<i>M. tuberculosis</i> - <i>M. bovis</i>	ACAGGACGCGTCTAGAGATAGGCGTT
<i>M. fortuitum</i>	•••••••AC•GC•••••GT•GT••
<i>M. peregrinum</i>	•••••••CAG•••••TTG•••
<i>M. fortuitum</i> third biovariant complex, glucitol (sorbitol) negative	•••••••CAG•••••TTG•••
<i>M. abscessus</i>	•••••••TAC•••••••TA••
<i>M. chelonae</i>	G••••••TA•••••••TA••
<i>M. fortuitum</i> third biovariant complex, glucitol (sorbitol) positive	•••••••CGGC•••••GTGCG••
MCLO strains ATCC 49649, ATCC 49650 ^T , and ATCC 49651	•••••••CGGC•••••GTGCG••

^a A bullet (•) indicates that the base pair was identical to the *M. tuberculosis* base pair.

TABLE 5. Third variable region in the 16S rRNA gene (beginning with *E. coli* position 1117) which could be used to differentiate *M. peregrinum* from the *M. fortuitum* third biovariant complex glucitol-negative group and the MCLO from the closely related *M. fortuitum* third biovariant complex glucitol-positive (sorbitol-positive) group

Taxon	Sequence ^a
<i>M. tuberculosis</i>	GTCTCATGTTGCCAGCACGTAAGGTGGGACTCGTGAG
<i>M. fortuitum</i>	T.....
<i>M. peregrinum</i>	A...T.....G...C.....
<i>M. fortuitum</i> third biovariant complex, glucitol (sorbitol) positive	T.....
<i>M. fortuitum</i> third biovariant complex, glucitol (sorbitol) negative	
<i>M. abscessus</i>	...CT.....GG.....CC.....AG•
<i>M. chelonae</i>	...CT.....GG.....CC.....AG•
MCLO strain ATCC 49649	...CT.....GG.....CC.....AG•
MCLO strain ATCC 49650 ^T	...GG.....CC.....AG•
MCLO strain ATCC 49651	...CT.....GG.....CC.....AG•

^a A bullet (•) indicates that the base pair was identical to the *M. tuberculosis* base pair.

*Bst*EII, *Aci*I, and *Cfo*I yielded single, common restriction fragment length polymorphism patterns for all 20 MCLO isolates (19).

The first hypervariable regions of two new *M. fortuitum* third biovariant complex glucitol-positive (sorbitol-positive) strains (ATCC 49934 and ATCC 49936) and three new *M. fortuitum* third biovariant complex glucitol-negative (sorbitol-negative) strains (ATCC 49935, ATCC 49937, and ATCC 49939) were studied to confirm the results obtained with the single strain belonging to each group which Kirschner et al. studied previously (9). The new isolates gave results identical to the results obtained with the previously characterized strains (Table 3), confirming the validity of the sequences and the fact that they differed from the sequences of the MCLO and other members of the *M. fortuitum* complex.

We observed some differences between the sequence of MCLO strain ATCC 49649 and the sequences of the other two MCLO strains. We found four differences in the 1,062 16S rRNA nucleotides examined (C versus A, C versus T, G versus A, and G versus T at *E. coli* positions 441, 442, 491 and 492, respectively). Strain ATCC 49649 differs from most other MCLO strains in that it exhibits a high level of semiquantitative catalase activity, is cephalothin resistant, and is acetamidase negative (26). These characteristics were present in 15% or less of the 87 clinical isolates studied by Wallace et al. (26). In contrast, strains ATCC 49650^T and ATCC 49651 had identical 16S gene sequences and had phenotypic properties typical of the 87 isolates examined in the previous study (26). It is likely that the DNA sequences of the latter two strains are more representative of the sequences of common clinical MCLO isolates. Because the differences between the two groups were small and because only one phenotypically atypical strain was studied, we do not believe that these taxa should be considered separate species at the present time. Because of these differences, we recommend that typical strain ATCC 49650 rather than the previously recommended (26) but atypical strain ATCC 49649 should be the type strain of the new species described below.

The taxonomy of the *M. fortuitum* complex, especially its nomenclature, is in a rapid state of flux. Recently, the two subspecies of *M. chelonae* and the two named biovariants of *M. fortuitum* were shown to differ in their Hamming distances by 4 and 15 bp, respectively, for the 1,062 bp of the 16S rRNA gene which we studied (9, 15) and to have DNA-DNA complementarity differences that warrant separate species status (13, 14). Hence, these taxa are now recognized as *M. fortuitum*, *M. peregrinum*, *M. chelonae*, and *M. abscessus* (13, 25). Three additional clinically important taxonomic groups have been

shown to differ from these four species in their 16S rRNA gene sequences (9), but have not been described as new species previously. These groups are the *M. fortuitum* third biovariant complex glucitol-positive (sorbitol-positive) group (23), the *M. fortuitum* third biovariant complex glucitol-negative (sorbitol-negative) group (23), and the MCLO group. On the basis of previously determined phenotypic differences and the differences in rRNA gene sequences which we found, we propose the name *Mycobacterium mucogenicum* sp. nov. for the MCLO group. The specific epithet reflects the highly mucoid character of most clinical isolates on solid media (26).

Description of *Mycobacterium mucogenicum* sp. nov. *Mycobacterium mucogenicum* (mu.co.gen'i.cum. M.L. adj. *mucogenicum*, producing mucus, referring to the highly mucoid character of most strains on solid agar) is a gram-positive, acid- and alcohol-fast, curved bacillus that grows aerobically or microaerophilically (2). It does not form spores or aerial hyphae. Visible growth occurs in 2 to 4 days. Colonies on Middlebrook 7H10 agar or Trypticase soy agar are usually smooth, mucoid, and off-white. No pigment is produced. Growth occurs at 28 to 37°C but not at 42°C. Susceptible in vitro to amikacin, imipenem, cefoxitin, clarithromycin, and ciprofloxacin. Variable susceptibility is observed with doxycycline. Resistant to isoniazid and rifampin. Iron uptake negative (often the colonies are tan, and there is a rust-colored ring at the margin of the slant). Typically positive for arylsulfatase activity at 3 and 14 days, acetamidase activity, and Tween hydrolysis. Grows (poorly at times) on MacConkey agar and utilizes mannitol and citrate but not glucitol (sorbitol) or inositol as a sole carbon source. Does not grow in the presence of 5% NaCl. Nitrate reduction is variable. Strains produce catalase that is inactivated at 68°C and pH 7; the level of catalase activity is low. Reduces tellurite. Urease positive (2). Susceptible to cephalothin as determined by the disk method. Two widely separated sets of mycolic acid ester peaks occur on HPLC gels. The type strain is ATCC 49650, which was recovered from a human neck abscess.

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