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Genetic Heterogeneity within *Mycobacterium fortuitum* Complex Species: Genotypic Criteria for Identification

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A 1.5-kb segment of the DNA that encodes 16S rRNA was amplified by polymerase chain reaction, and 880 nucleotide positions were determined from each of the described biovariants of *Mycobacterium fortuitum*. Signature sequences which allow rapid identification of *M. fortuitum* strains at the biovariant level are described. Our data demonstrate a close phylogenetic relationship between *Mycobacterium senegalense* and *M. fortuitum* and indicate that the described biovariants of *M. fortuitum* represent genetically distinct taxa.

Only 2 of the 28 rapidly growing *Mycobacterium* species are commonly encountered as human pathogens (22). These two species, *Mycobacterium fortuitum* and *Mycobacterium chelonae*, are included in the *M. fortuitum* complex, which is identified at the complex level by degradation of *p*-aminosalicylic acid, growth on MacConkey agar without crystal violet, growth in the presence of 500 μg of hydroxylamine HCl per ml, and a positive 3-day arylsulfatase test (23). Although *M. fortuitum* complex isolates are relatively rare pathogens that account for approximately 6% of all mycobacterial isolates (8), recent evidence indicates that the reported numbers of *M. fortuitum* complex isolates have steadily increased during the last decade (22). *M. fortuitum* and *M. chelonae* are resistant to most of the commonly used antituberculous drugs. However, differences in drug susceptibilities have been noted. Isolates of *M. fortuitum* have been shown to be more susceptible to antimicrobial agents than those of *M. chelonae* (17, 21). It is important, therefore, both clinically and epidemiologically, to separate and correctly identify isolates of the *M. fortuitum* complex.

The species *M. chelonae* consists of two subspecies, namely, *Mycobacterium chelonae* subsp. *abscessus* and *Mycobacterium chelonae* subsp. *chelonae* (23). With respect to *M. fortuitum*, the situation is more confusing. Although *M. fortuitum* is the correct name for a well-defined species, the taxa are not homogeneous (23). On the basis of acid production from various carbohydrates, three subgroups of *M. fortuitum* have been described (2). Studies analyzing carbohydrate usage patterns, whole-cell agglutination, immunodiffusion, lipid chromatography, intradermal skin testing, and standard biochemical reactions characterized two groups, identified as *M. fortuitum* subsp. *fortuitum* and *M. fortuitum* subsp. *peregrinum*, which gave a uniform and unique pattern (12). A third group, the so-called unnamed third biovariant, which was the least homogeneous of the groups, was identified. These isolates were unique in their immunodiffusion pattern and production of acid from various carbohydrates (9, 12, 17). More recently, Wallace and colleagues undertook a detailed and extensive study to further characterize the unnamed third biovariant of *M. fortuitum*. The ability to use sorbitol as a carbon source was used to define two subgroups of the third-biovariant complex. This division correlated with the semiquantitative catalase reaction height, the presence of the pI 5.2 β-lactamase, and cefoxitin resistance (20).

The present study was an effort to characterize the genotypic relationships among the described biovariants of *M. fortuitum* by using comparative sequencing of genes coding for 16S rRNA as well as an effort to define genotypic approaches for rapid identification of isolates belonging to the *M. fortuitum* complex.

MATERIALS AND METHODS

Bacterial strains. *M. fortuitum* subsp. *peregrinum* (ATCC 14467), *M. fortuitum* sorbitol-positive third biovariant (ATCC 49403), *M. fortuitum* sorbitol-negative third biovariant (ATCC 49404), *M. chelonae* subsp. *abscessus* (ATCC 19977), and *M. chelonae* subsp. *chelonae* (ATCC 35752) were obtained from the American Type Culture Collection (Rockville, Md.).

A comparison of the biochemical patterns of *M. fortuitum* subsp. *fortuitum*, *M. fortuitum* subsp. *peregrinum*, *M. fortuitum* third-biovariant complex, *M. chelonae* subsp. *chelonae*, *M. chelonae* subsp. *abscessus*, and *Mycobacterium senegalense* is shown in Table 1.

Determination of sequences and data analysis. The 16S rRNA regions for sequence determination were chosen by careful inspection of the sequences available in the EMBL-GenBank data library (i.e., *M. fortuitum* subsp. *fortuitum* and *M. chelonae* subsp. *abscessus*; note that the sequence X52921 deposited in the EMBL data base as *M. chelonae* actually represents the sequence of *M. fortuitum* subsp. *peregrinum*; in retrospect, we have found this misidentification to be due to mislabeling of the strain at the culture collection from which it was obtained). The sequence positions chosen contain the variable parts of the molecule and thus have a high density of information, especially for comparisons of closely related organisms.

Extraction of nucleic acids, amplification of gene fragments coding for 16S rRNA, and sequence determination were performed as described previously (3, 4, 15). In brief, a bacterial colony from a Löwenstein-Jensen slant was dissolved in 0.5 ml of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA (TE); the solution was heated at 80°C for 20 min to inactivate the mycobacteria and centrifuged. The pellet was redissolved in 100 μl of TE, a loopful of glass beads with a diameter of 100 μm (Sigma, Munich, Germany) was added, and the sample was placed for 2 min in a tissue disintegrator

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TABLE 1. Comparison of biochemical patterns of selected *Mycobacterium* species, subspecies, and biovariants[a]

Characteristic	Result for:						
	M. fortuitum subsp. *peregrinum*	*M. fortuitum* subsp. *fortuitum*	*M. fortuitum* third-biovariant complex[b]		*M. chelonae* subsp. *chelonae*	*M. chelonae* subsp. *abscessus*	*M. senegalense*
			ATCC 49403	ATCC 49404			
Growth							
<7 days	+	+	+	+	+	+	+
At 45°C	−	−	+	−	−	−	−
At 52°C	−	−	−	−	−	−	−
Pigmentation	−	−	−	−	−	−	−
Arylsulfatase (3-day test)	+	+	+	+	+	+	+
Iron uptake	+	+	+	+	−	−	−
Nitrate reduction	+	+	+	+	+/−	−	−
Semiquantitative catalase values							
<45 mm		−	+	−			
45–88 mm		+	−	−			
>80 mm		−	−	+			
Growth at 28°C on:							
5% NaCl	+	+	+	+	−	+	+
MacConkey agar without crystal violet	+	+	+	+	+	+	
Growth on single carbon source							
Citrate	−	−	−	−	+	−	+
Mannitol	+	−	+	+	−	−	
Inositol	−	−	+	+	−	−	
Sorbitol	−	−	+	−			
Trehalose	−	−	+	−			
Resistance to:							
Polymyxin B	−	−	−	−	+	+	
Pipemidic acid	−	−	+	+	+	+	
Ofloxacin							
1 μg/ml	−	−	+	−			
5 μg/ml	−	−	−	−			

[a] Data were tabulated from references 8, 20, and 23. +, positive; +/−, intermediate; −, negative.
[b] ATCC 49403, sorbitol-positive subgroup; ATCC 49404, sorbitol-negative subgroup.

at maximum speed (Mickle Laboratory, Gomshall, Surrey, United Kingdom). After centrifugation, 5 μl of the supernatant was used in polymerase chain reaction. The 16S rRNA gene fragment was amplified in two overlapping parts by using primers 285 GAG AGT TTG ATC CTG GCT CAG (9 to 29) and 264 TGC ACA CAG GCC ACA AGG GA (1046 to 1027) for synthesis of the 5′ part of the gene and primers 248 GTG TGG GTT TCC TTC CTT GG (831 to 850) and 261 AAG GAG GTG ATC CAG CCG CA (1542 to 1523) for synthesis of the 3′ part of the gene (the numbers in parentheses correspond to the respective *Escherichia coli* 16S rRNA nucleotide positions). The amplified DNA fragment was purified, and the nucleotide sequences of the relevant regions were determined by direct sequencing of the amplified fragment. Pairwise distances were calculated by weighting nucleotide differences and insertions-deletions equally (Hamming distance). The phylogenetic tree was constructed by using the neighborliness method (5) as described previously (16).

RESULTS AND DISCUSSION

Sequence determination resulted in 880 nucleotide positions corresponding to *E. coli* positions 119 to 555 and 887 to 1347 (EMBL sequence accession no. X65528 and X65529 for *M. fortuitum* ATCC 49404 and *M. fortuitum* ATCC

TABLE 2. Hamming distances derived from 16S rRNA sequences[a]

Organism	Hamming distance						
	M. fortuitum ATCC 49403	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum* subsp. *fortuitum*	*M. senegalense*	*M. fallax*	*M. neoaurum*	*M. chelonae* subsp. *chelonae*
M. fortuitum ATCC 49404	9	9	11	6	24	34	41
M. fortuitum ATCC 49403		15	6	5	17	31	41
M. fortuitum subsp. *peregrinum*			15	12	28	32	40
M. fortuitum subsp. *fortuitum*				9	21	33	42
M. senegalense					22	32	40
M. fallax						37	47
M. neoaurum							43

[a] Values are based on 880 nucleotides. The sequences of *M. fortuitum* subsp. *fortuitum*, *M. fortuitum* subsp. *peregrinum*, *M. senegalense*, *M. neoaurum*, *M. fallax*, and *M. chelonae* subsp. *abscessus* were obtained from references 16 and 18.

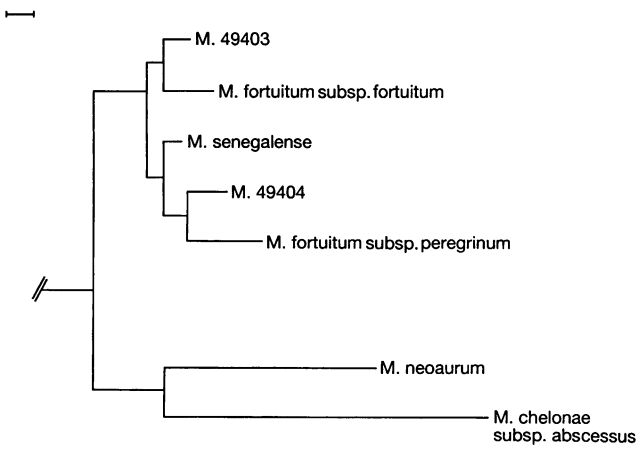

FIG. 1. Phylogenetic tree showing the relationships of the different biovariants of *M. fortuitum*. *M. fortuitum* ATCC 49403 represents the sorbitol-positive unnamed third biovariant; *M. fortuitum* ATCC 49404 represents the sorbitol-negative unnamed third biovariant. The tree has been rooted by using *Mycobacterium fallax* as the outgroup. Bar, 2 nucleotide differences.

49403, respectively). A phylogenetic tree displaying the natural relationships among the described biovariants of *M. fortuitum* was constructed from equally weighted (Hamming) distances (Table 2).

The relationships determined by comparative 16S rRNA sequencing (Fig. 1) demonstrate that *M. senegalense* clearly falls within the confines of the described biovariants of *M. fortuitum*, suggesting a close phylogenetic relationship between these species. This result is in good agreement with DNA-DNA homology studies which had indicated a moderate relatedness between *M. senegalense* and *M. fortuitum* subsp. *fortuitum* and *M. fortuitum* subsp. *peregrinum* (1) and with numerical classification, which had revealed a high overall similarity of *M. senegalense* to *M. fortuitum* and *M. chelonae* (13). Likewise, comparative immunodiffusion studies have shown that *M. senegalense* and *M. fortuitum* are very similar serologically (14).

The determined distances support the results of DNA-DNA homology studies (1, 10), showing that *M. fortuitum* subsp. *peregrinum* should be separated from *M. fortuitum* subsp. *fortuitum* at the species level. Although, because of the short branch length, the branching pattern cannot be established with certainty, the two subgroups of the unnamed third biovariant appear genetically more distinct from

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1 CGG ATA GGA CCA CGG GAT GCA TGT CTT GTG GTG GAA AGC GCT TTA GCG GTG TGG GAT GA
2 ..A ... T.. ..G ..C AC. T.C ..G TG. ... ... ... .-- ... ..T ... ... ... ... .G
3 ..A ... T.. ... ..C .C. T.. ..G TG. ... ... ... .-- ... ..T ... ... ... ... .G
4 ..A ... ... ..G ..C TC. T.. ..G GG. ... ... ... .-- ... ..T ... ... ... ... .G
5 ... ... ... ... ..C .C. T.. ..G TG. ... ... ... .-- ... ..T ... ... ... ... .G
6 ... ... ... ... .AC AC. T.. ..G TGA ... ... C.. .-- ... ..T ... ... ... ... ..
7 ... ... ... ... .AC AC. T.. ..G TGA ... ... C.. .-- ... ..T ... ... ... ... ..
```

FIG. 2. Signature nucleotides in the 5′ end of the 16S rRNA gene (corresponding to *E. coli* positions 175 to 231) for differentiating among the described biovariants of *M. fortuitum*. *Mycobacterium tuberculosis* (row 1) was used as the reference sequence. Dots, identical nucleotides; dashes, deletions. Nucleotides different from those of *M. tuberculosis* are indicated. Within the region shown, the sequence of *M. senegalense* is identical to that of *M. fortuitum* ATCC 49403. Rows: 1, *M. tuberculosis*; 2, *M. fortuitum* subsp. *peregrinum*; 3, *M. fortuitum* subsp. *fortuitum*; 4, *M. fortuitum* ATCC 49404; 5, *M. fortuitum* ATCC 49403; 6, *M. chelonae* subsp. *chelonae*; 7, *M. chelonae* subsp. *abscessus*.

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1 AAA CCT CTT TCA ATA GGG ACG AAG CGC AAG TGA CGG TAC CTA TAG AA
2 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
3 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
4 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
5 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
6 ... ... ... ... G.. ... ... ... ..A ... ... ... ... ... C.. ..
7 ... ... ... ... G.. ... ... ... ..A ... ... ... ... ... C.. ..
```

FIG. 3. Signature nucleotides in the 16S rRNA gene (corresponding to *E. coli* positions 430 to 496) for differentiating *M. fortuitum* isolates from *M. chelonae* isolates. Rows: 1, *M. fortuitum* ATCC 49403; 2, *M. fortuitum* ATCC 49404; 3, *M. fortuitum* subsp. *fortuitum*; 4, *M. fortuitum* subsp. *peregrinum*; 5, *M. senegalense*; 6, *M. chelonae* subsp. *chelonae*; 7, *M. chelonae* subsp. *abscessus*.

one another than from *M. fortuitum* subsp. *fortuitum* and *M. fortuitum* subsp. *peregrinum*. Interestingly, when using thin-layer chromatography on two isolates of the unnamed third biovariant of *M. fortuitum*, Tsang and coworkers noted that the lipid profile of one isolate was identical to that of *M. fortuitum* subsp. *fortuitum* and that of the other was identical to that of *M. fortuitum* subsp. *peregrinum* (19). This observation supports our interpretation that the two subgroups of the unnamed third biovariant represent genetically distinct species.

Drug sensitivity differs among the various subspecies and biovars within the *M. fortuitum* complex (17, 20–22). Although the first reports on the third biovariant commented that most isolates were environmental (6), the detailed investigation by Wallace and colleagues clearly identified isolates of the third biovariant as human pathogens and indicated that the types of trauma and penetrating injuries that result in infections are similar to those described for the other subgroups of *M. fortuitum* and *M. chelonae* (20). Inspection of the aligned 16S rRNA sequences revealed a region where specific signature sequences identify each of the described biovariants of *M. fortuitum* (Fig. 2). Note that in this region *M. senegalense* possesses a sequence identical to that of the sorbitol-positive subgroup of the third biovariant of *M. fortuitum*. In practical terms, however, *M. senegalense*, the causative agent of bovine farcy, is not encountered in human samples (23).

The closely related species *M. chelonae* and *M. fortuitum* can be distinguished by a number of growth and physiological properties (Table 1) and by more elaborative techniques such as determination of the mycolic acid pattern (11), polyacrylamide gel electrophoresis of cell proteins (7), and immunodiffusion (12). Whereas the most variable regions within the 16S rRNA molecule differ among species of bacteria (Fig. 2), regions of intermediate conservation can serve to mark higher-order assemblage. As shown in Fig. 3, the analysis of the aligned sequences identified a region within the 16S rRNA molecule where all described biovariants of *M. fortuitum*, including *M. senegalense*, show a common yet unique sequence. This sequence differs markedly from that of *M. chelonae*, thus allowing a clear identification of an unknown isolate.

The recognition of effective therapeutic drugs for the various subspecies and biovars within the *M. fortuitum* complex makes subspecific and biovar recognition important. Identification of *M. fortuitum* complex isolates at the biovariant level by conventional methods is difficult and often not possible even in highly specialized laboratories. Direct sequencing of polymerase chain reaction-amplified 16S rRNA gene fragments has been introduced recently for rapid identification of cultural isolates (15). The identification of 16S rRNA sequences specific for the described

biovariants of *M. fortuitum* reported here meets the necessity for prompt recognition of an *M. fortuitum* complex isolate at the biovariant level.

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