Identification of *Mycobacterium fortuitum* and *Mycobacterium chelonae*

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The study of 52 strains of rapidly growing mycobacteria showed that *Mycobacterium fortuitum* and *M. chelonae* were clearly distinguished by the aid of seven key tests (nitrate reductase, iron uptake, β -glucosidase, penicillinase, growth on fructose, resistance to pipemidic acid, and resistance to capreomycin) and by analysis of their respective mycolic acids. However, the subdivision of these species into *M. fortuitum* var. *fortuitum* and *M. fortuitum* var. *peregrinum* and *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* was not satisfactorily accomplished.

Methods to discriminate *Mycobacterium fortuitum* and *M. chelonae* in clinical laboratories were recently proposed (1, 3, 4, 10, 11). In one report (11) the authors described biochemical methods for subdividing *M. fortuitum* into *M. fortuitum* var. *fortuitum* and *M. fortuitum* var. *peregrinum* and *M. chelonae* into *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* (5, 9). Thin-layer chromatography (TLC) of the complex lipids was also used for the same purpose (4, 10). Gas chromatography (GC) of the volatile lipids obtained by saponification of the bacteria and methylation (GC pattern) was proposed as a means of identification of most of the mycobacterial clinical isolates, but failed to discriminate between some species, especially *M. fortuitum* and *M. chelonae* (13). The above investigations were conducted independently, and we thought it would be of interest to evaluate them comparatively.

MATERIALS AND METHODS

Bacterial strains. The 52 strains of nonchromogenic rapidly growing mycobacteria used in this study are listed in Tables 1, 2, and 3. Thirty-eight of the strains were arylsulfatase positive, were resistant to 250 μ g of hydroxylamine in Lowenstein-Jensen medium, and grew on MacConkey agar medium (Difco Laboratories, Detroit, Mich.); they were thus included in the *M. fortuitum* complex (5, 9, 14). The remaining 14 strains were distinct from the above by their inability to grow on MacConkey agar and were included in the study for comparative purposes.

For comparison of mycolic acid contents, the following strains were also included: *M. smegmatis*

ATCC 607, *M. terrae* ATCC 15755, *M. tuberculosis* H37Rv, and *M. avium* CIPT 140310005.

Identification procedures. The identification procedures were those described by Casal and Rodriguez (1), David et al. (3), and Silcox et al. (11). The tests were performed exactly as described, except that the study of resistance to pipemidic acid was done at 30°C, using antibiotic medium no. 1 (Difco) instead of Mueller-Hinton agar.

Resistance to capreomycin was done on Lowenstein-Jensen medium containing 40 μ g of the drug per ml.

Hydrolysis of bacteria and extraction of lipids. The bacteria were grown as shaken cultures in Sauton medium at 33°C for several days and were then harvested by centrifugation. The wet bacteria were suspended in a methanol solution containing 5% potassium hydroxide, 5% water, and 20% benzene. The mixtures were heated at solvent reflux for 4 h. After cooling, the mixtures were acidified by the addition of 20% (vol/vol) sulfuric acid, and the lipids were extracted into ether. The ether extracts were washed with water until neutral and dried over sodium sulfate. The dried solutions were filtered, concentrated by evaporation, and then methylated with diazomethane.

TLC. TLC was performed by using glass plates (20 by 20 cm) coated with 0.3-mm-thick layers of Silica Gel G60 (E. Merck AG, Darmstadt, West Germany). Before use the plates were activated by heating at 110°C for 30 min.

The developing solvent used was a 9:1 (vol/vol) mixture of petroleum ether (50 to 60°C) and ether. Four runs were necessary to obtain good resolution of the different types of mycolates (Fig. 1), but the best resolution of type II and III mycolates was obtained after one or two runs only.

The lipid spots were revealed by charring after spraying the plates with 20% sulfuric acid.

FIG. 1. TLC of mycolic acid methyl esters from: 1 and 1', *M. smegmatis*; 2, *M. chelonae*; 3 and 3', *M. fortuitum*; 4, *M. tuberculosis*; 5, *M. terrae*; and 6, *M. avium*. The primed numbers represent mycolic acids extracted by acid methanolysis; the remaining numbers represent the mycolic acids methylated by the diazomethane method (for details, see text). The types of mycolic acids separated by TLC are as follows:

$n_1 = 15$ to 19 (odd values); $n_2 = 14$ to 19 ; $n_3 = 9$ to 19 ; $n_4 = 19$ to 23 (odd values). In two (types II and VI) of the six known types of mycolic acids the R group does not appear. Except for the type II which contains only the unsaturated species, the following structures were observed, as group B, in every one of the types of mycolic acid.

Group A, which mainly differentiates the mycolic acids, includes:

GC. GC of the methylated mixture of lipids was done with a 1.2-m-long nickel column with an inner diameter of 1.8 mm and filled with Chromosorb W containing 5% OV1. The nitrogen flow was 20 ml/min. The chromatograms were recorded from 100 to 320°C, first with the injector at 250°C and a second time with the injector at 320°C. Comparison of the two chromatograms showed the peaks arising from pyrolysis.

When the mixtures were treated by trimethylsilylation according to Sweeley et al. (12), the peaks corresponding to volatile hydroxylated compounds were recognized by their shifts in the chromatograms.

RESULTS

The properties of the 52 strains studied are depicted in Tables 1, 2, and 3. To facilitate analysis of the data, the properties examined were arranged as follows: the properties characteristic of the *M. fortuitum* complex are listed in column A; the biochemical properties reported useful to differentiate *M. fortuitum* and *M. chelonae* are listed in column B; the properties reported useful to separate variants or subspe-

TABLE 1. Test results of 23 strains of *M. fortuitum*

<i>M. fortuitum</i>			Biochemical and cultural properties							
Variant	Strain no. ^b	Source	A			B				
			Aryl-sulfatase	Hydroxyl-amine	McCon-key agar	NO ₃ reductase	Iron uptake	β-Glucosidase	Penicillinase	Trehalase
<i>M. fortuitum</i> var. <i>fortuitum</i>	211	Water	+	+	+	+	+	+	—	—
	810090	Urine	+	+	+	+	+	+	—	—
	123	Water	+	+	+	+	+	+	—	—
	810069	Sputum	+	+	+	+	+	+	—	—
	CIPT	Pleural fluid	+	+	+	+	+	+	—	—
	1404-10002									
	CIPT	ATCC 6841	+	+	+	+	+	+	—	—
	1404-10001									
	138	Water	+	+	+	+	+	+	—	—
<i>M. fortuitum</i> var. <i>peregrinum</i>	110	Water	+	+	+	+	+	+	—	—
	117	Water	+	+	+	+	+	+	—	—
	2	Water	+	+	+	+	+	+	—	—
	125	Water	+	+	+	+	+	+	—	—
	810031	Sputum	+	+	+	+	+	+	—	—
	148	Water	+	+	+	+	+	+	—	—
	111	Water	+	+	+	+	+	+	—	—
	810507	Sputum	+	+	+	+	+	+	—	—
	810539	Sputum	+	+	+	+	+	+	—	—
Miscellaneous strains	CIPT	ATCC 14467	+	+	+	+	+	+	—	—
	1404-10020									
	27	Water	+	+	+	+	+	+	—	—
	52	Water	+	+	+	+	+	+	—	—
	109	Water	+	+	+	+	—	+	—	—
	104	Water	+	+	+	—	+	—	—	+
	112	Water	+	+	—	+	+	+	—	—
	83	Water	+	+	+	+	+	+	+	—
Total strains or % positive characters	23		100.0	100.0	95.6	95.6	95.6	95.6	4.3	0
										4.3

^a See Fig. 1.^b CIPT, Collection Institut Pasteur Tuberculose.^c (+) indicates a positive but quantitatively light result.

cies are listed in column C; and the mycolic acids formed by each strain are listed in column D. For the 52 strains, the biochemical and mycolic acid studies were done completely blind.

The different types of mycolic acids and their TLC properties are shown in Fig. 1. Mycolates showing the same migration properties are arranged in the same type, in spite of the presence of cyclopropane groups instead of double bonds in several species. The sample process used was effective to obtain enough structural information to make a differentiation among the strains studied. It is also shown (Fig. 1) that methanolysis in the presence of sulfuric acid, according to Min-

nikin et al. (6), converted the epoxymycolate into two diolmycolates or into their methylated derivatives (2), which correspond to the non-identified polar lipids described by these authors (6).

All strains in this study were arylsulfatase positive, were resistant to 250 µg of hydroxylamine per ml, formed mycolic acids of type I, and were devoid of mycolic acids of types III and IV. Therefore, these properties were of little value for sorting out the strains of the *M. fortuitum* complex from all of the nonchromogenic rapidly growing mycobacteria in this study.

The ability to grow on MacConkey agar (95%

The subdivision of *M. fortuitum* or *M. chelonae* was not satisfactory because six strains of *M. fortuitum* (26%) and six strains of *M. chelonae* (33%) could not be subspeciated (Tables 1 and 2, column C). This may be expected because the subdivisions are based on the results of a single test character (11). An in-depth study including more tests would be necessary to characterize the miscellaneous strains shown in Tables 1 and 2 and to reach conclusions about

TABLE 2. Test results of 18 strains of *M. chelonae*

<i>M. chelonae</i>			Biochemical and cultural properties								
Variant	Strain no. ^b	Source	A			B					
			Aryl-sulfatase	Hydroxylamine	McConkey agar	NO ₃ reductase	Iron uptake	β-Glucosidase	Penicillinase	Trehalase	Pipemidic acid
<i>M. chelonae</i> subsp. <i>chelonae</i>	810071	Bronchic	+	+	+	—	—	—	+	—	+
	810099	Sputum	+	+	+	—	—	—	+	+	+
	801468	Endometer	+	+	+	—	—	—	+	+	+
	801322	Abcess	+	+	+	—	—	—	+	+	+
	801103	Sputum	+	+	+	—	—	—	+	+	+
	CIPT 1404-20003 ^c	NCTC 946 ^d	+	+	+	—	—	—	+	+	—
	CIPT 1404-20005	JS133	+	+	+	—	—	—	+	—	+
	CIPT 1404-20002	Abcess	+	+	+	—	—	—	+	—	+
	801200	Sputum	+	+	—	—	—	—	+	+	+
	810192 ^c	Sputum	+	+	+	—	—	—	+	+	+
<i>M. chelonae</i> subsp. <i>abscessus</i>	810402	Sputum	+	+	+	—	—	—	—	+	+
	801356	Urine	+	+	+	—	—	+	+	—	+
Miscellaneous strains	4 ^c	Water	+	+	+	—	—	—	+	+	—
	801159	Sputum	+	+	—	—	—	—	+	—	+
	810039	Gastric washing	+	+	+	—	—	—	+	—	+
	810244 ^c	Sputum	+	+	+	—	—	—	+	+	+
	810292	Sputum	+	+	+	—	—	—	+	+	+
	810787	Sputum	+	+	+	—	—	+	—	+	+
Total strains or % positive characters	18		100.0	100.0	88.9	0	0	11.2	88.9	66.7	88.9

^a See Fig. 1.^b CIPT, Collection Institut Pasteur Tuberculose.^c By GC, only these strains are devoid of an unidentified lipid thought to be specific for *M. chelonae* subsp. *chelonae* (13).^d NCTC, National Collection of Type Cultures (U.K.).

the reality of the subspecific separation or the occurrence of infrasubspecific variants.

The typical reaction patterns of the studied mycobacteria, relying on 14 test characters, are shown in Table 4. Concerning the mycolate composition, only the mycolates that play a role in differentiating the strains studied here were included.

Table 5 shows the degree of similarity of the strains classified as *M. fortuitum* or *M. chelonae* according to the key test patterns. According to the data in Table 4 the trehalase test ranked lower than the other tests in discrimination value, and therefore the results with this test

were deleted from the analysis of data depicted in Table 5. It is shown in Table 5 that 95% of the strains are either identical to or differ by only one character from the patterns established for *M. fortuitum* and *M. chelonae*. Only one strain, having the more remote pattern, differed by three test results (2.4% of the strains). These data contrasted distinctly from those obtained with the strains that were excluded from the *M. fortuitum* complex: the strain closer to *M. fortuitum* or *M. chelonae* differed from the key test pattern by five test results (35.7% of the tests), and half of the strains showed at least seven characters (50%) different from those of these

The TLC analysis of mycolic acids described

TABLE 3. Test results of miscellaneous nonchromogenic rapidly growing mycobacteria other than *M. fortuitum* complex

Unidentified species	Strain no. ^b	Source	Biochemical and cultural properties								
			A			B					
			Aryl-sulfatase	Hydroxylamine	McConkey agar	NO ₃ reductase	Iron uptake	β-Glucosidase	Penicillinase	Trehalase	Pipemidic acid
Strains unable to grow on McConkey agar medium	97	Water	+	+	—	+	—	—	—	—	—
	810100	Sputum	+	+	—	+	—	+	—	—	—
	82	Water	+	+	—	+	+	+	+	—	—
	810066	Sputum	+	+	—	—	—	—	—	—	—
	209	Water	+	+	—	—	—	—	—	—	—
	181	Water	+	+	—	—	—	—	+	—	—
	105	Water	+	+	—	—	—	—	—	+	—
	801481	Sputum	+	+	—	—	—	+	—	—	—
	118	Water	+	+	—	—	—	—	+	+	—
	810279 ^b	Gastric washing	+	+	—	—	—	+	—	+	—
	810502	Urine	+	+	—	—	+	—	—	—	—
Total strains or % positive characters	11		100.0	100.0	0	27.2	18.2	36.3	27.3	27.3	0

^a See Fig. 1.^b No growth on nutrient agar.

here does not discriminate between subspecies, but the method is very easy to perform and the results are very reproducible, whatever the growth conditions of the bacteria. Six types of mycolates were characterized and separated on TLC according to the nature of the oxygenated functions they contain or according to the length

of their carbon skeleton. Of course, the method does not give information about the fine molecular structure of the mycolic acids, as does mass spectrometry of isolated molecules. Therefore, this method must be completed by the study of other properties (see Table 4).

Although subdivisions within the two species

TABLE 4. Typical reaction patterns of 52 nonchromogenic rapidly growing mycobacteria^a

Test character	<i>M. fortuitum</i> (23 strains)		<i>M. chelonae</i> (18 strains)		Other (11 strains)	
	Result	Frequency (%)	Result	Frequency (%)	Result	Frequency (%)
Mycolic acid type II	+	100.0	+	100.0	—	0.0
Mycolic acid type VI	—	0.0	—	0.0	+	100.0
Hydroxylamine	+	100.0	+	100.0	+	100.0
MacConkey agar	+	95.6	+	88.9	—	0.0
Arylsulfatase	+	100.0	+	100.0	+	100.0
Mycolic acid type V	+	100.0	—	0.0	—	0.0
Fructose	+	100.0	—	0.0	+	81.9
Nitrate reductase	+	95.6	—	0.0	±	27.3
Iron uptake	+	95.6	—	0.0	—	18.2
β-Glucosidase	+	95.6	—	11.2	±	36.3
Penicillinase	—	4.3	+	88.9	±	27.3
Pipemidic acid	—	4.3	+	88.9	—	0.0
Capreomycin	—	17.0	+	100.0	+	81.9
Trehalase	—	0.0	±	66.7	±	27.3

^a The lower part gives the key tests for discrimination between *M. fortuitum* and *M. chelonae*.

TABLE 3—Continued

Biochemical and cultural properties						Mycolic acids ^a					
B		C				D					
Fructose	Capreomycin	Sodium chloride	Citrate	Mannitol	Inositol	Type I	Type II	Type III	Type IV	Type V	Type VI
+	+	—	—	+	—	+	—	—	—	—	+
—	+	—	—	+	—	+	—	—	—	—	+
+	—	+	—	+	—	+	—	—	—	—	+
+	+	—	—	+	—	+	—	—	—	—	+
+	+	—	—	+	—	+	—	—	—	—	+
+	+	—	—	—	—	+	—	—	—	—	+
+	—	—	+	—	—	+	—	—	—	—	+
+	+	—	—	—	—	+	—	—	—	—	+
+	+	—	—	+	—	+	—	—	—	—	+
—	+	—	—	—	—	+	—	—	—	—	+
+	+	—	+	—	—	+	—	—	—	—	+
81.9	81.9	9.0	18.2	54.6	0	100.0	0	0	0	0	100.0

TABLE 5. Number and percentage of strains identical to the key test reactions or differing by one, two, three, four, or more results

Organisms	Total strains	No. (%) with given no. of differences in test results				
		0	1	2	3	≥4
<i>M. fortuitum</i>	23	16 (69.5)	6 (26.0)	0	1 (4.3)	0
<i>M. chelonae</i>	18	12 (66.6)	5 (27.7)	1 (5.5)		0
Frequency	100.00		95.12	2.4	2.4	0

under consideration may be justifiable from a taxonomic point of view (4, 5, 8, 9, 14), this investigation showed that current methods may not be adequate to do so in the clinical laboratory. The procedures proposed by Silcox et al. (11) not only leave out a relatively high proportion of strains (about 30%), but also lead to the creation of additional variants or subspecies, as was also shown in their own report. In our opinion, and for the purpose of clinical microbiology, the biochemical variants should be considered occasional, may be useful for epidemiological investigations, and should not be subspecies in the clinical laboratory until further investigations are conducted. All of the tests mentioned in the lower part of Table 4 are useful for discriminating *M. chelonae* and *M. fortuitum*. In our laboratory we use association of the nitrate reductase, β -glucosidase, penicillinase, and trehalase tests because these tests give positive results for both species and are easily as well as readily performed. We think

that the trehalase test can be improved, and studies on this reaction are in progress in our laboratory.

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