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Mycobactin Analysis as an Aid for the Identification of *Mycobacterium fortuitum* and *Mycobacterium chelonae* Subspecies

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Mycobactin patterns from 65 *Mycobacterium fortuitum* and *Mycobacterium chelonae* strains have been determined by thin-layer chromatography. By use of a rich liquid medium containing an iron chelator (ethylenediamine-di-*o*-hydroxyphenylacetic acid [EDDA]) to ensure iron starvation, all strains were able to form mycobactins. The method developed here allows sensitive detection of mycobactin by thin-layer chromatography from as little as 5 ml of culture after a 2-week incubation. Within *M. fortuitum* two mycobactin patterns were identified, whereas within *M. chelonae* four were recognized. Comparisons with the subspecific identification performed by using biochemical tests showed that 73% of the *M. fortuitum* subsp. *fortuitum* strains shared the same mycobactin pattern (designated F), whereas 75% of the *M. fortuitum* subsp. *peregrinum* strains shared the other mycobactin pattern (designated P). Within the *M. fortuitum* strains that cannot be assigned to a subspecies on the basis of their biochemical features, only F and P patterns were determined. Similarly, 93% of the *M. chelonae* subsp. *chelonae* strains produced the so-called C1 and C2 patterns and 86% of the *M. chelonae* subsp. *abscessus* strains produced A1 and A2 patterns. C2 and A2 were the patterns most frequently encountered; they were represented by 65 and 50% of the *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* strains, respectively. Within the biochemically *M. chelonae* strains that did not fit any subspecies on the basis of biochemical test results, C1, C2, and A1 patterns were found. Whereas about 30% of both *M. fortuitum* and *M. chelonae* strains cannot be characterized to the subspecies level on the basis of biochemical tests, 100% of the strains of both species can be characterized on the basis of mycobactin patterns.

Mycobacterium fortuitum and *Mycobacterium chelonae* are the most frequently isolated rapidly growing mycobacteria of clinical importance. They are rare agents of pulmonary infections, but owing to their presence in the environment and their peculiar ecological parameters (19, 42) they are frequently involved in cutaneous and soft tissue infections following surgical procedures or accidental trauma and in disseminated diseases occurring mainly in patients under dialysis or after cardiac surgery or transplantation (45, 46).

These two nonchromogenic species have been referred to as the *M. fortuitum* complex, but their differential identification is achieved by the use of several phenotypic features, including biochemical tests (8, 18, 21, 33), mycolate (5, 8, 26) or mycobactin (11) analysis, lipid thin-layer chromatography (TLC) patterns (16, 40), antibiotic susceptibilities (4, 44), polyacrylamide gel electrophoresis of proteins (9), mycobacteriocin typing (39), and delayed hypersensitivity testing (25, 38). The validity of the two species has been confirmed at the genomic level by DNA-DNA hybridization studies (1, 23) and rRNA sequencing (31, 36).

Moreover, DNA-DNA hybridization studies confirmed the presence of subspecies within each species: subspecies *fortuitum* and *peregrinum* (not formally described) within *M. fortuitum* (1, 23) and subspecies *abscessus* and *chelonae* within *M. chelonae* (23). However, good agreement between the various methods for subspecies identification could not be found. Several methods did not detect subgroups in one or both species, e.g., mycobacteriocin typing (39), mycolic acid patterns (21), and glycopeptidolipid analysis (40). In contrast, the detection of two or more subgroups in both species was reported by different investigators using biochemical tests and antibiotic susceptibility schemes (21, 33, 37, 41, 43), and Pattyn et al. recognized two agglutination types, four sensitin types, five immunodiffusion types, three lipid types, and three biotypes (28). Plasmid profiles did not relate to phenotypic properties of strains (15, 20).

In the present study, we reexamined the use of mycobactins as taxonomic markers for *M. fortuitum* and *M. chelonae*.

MATERIALS AND METHODS

Bacterial strains. The 65 strains used in this study are listed in Tables 1 and 2. Strains consisted of a set of strains included in either a previous study (21) or an international cooperative study coordinated by P. A. Jenkins. These latter strains are referred to with their Open-Ended Study (OES) numbers. Strains were environmental isolates from natural waters (French rivers), human isolates, or collection strains from the American Type Culture Collection (ATCC), the National Collection of Type Cultures (NCTC), the Trudeau Mycobacterial Culture Collection (TMC), or the Collection Institut Pasteur Tuberculose (CIPT).

For comparative purposes, four other rapidly growing *Mycobacterium* reference strains were also examined for mycobactin content.

Strain identification. Strains were identified as *M. fortuitum* or *M. chelonae* on the basis of tests previously described, including mycolic acid content (21, 33).

Culture conditions. Liquid medium containing (per 1,000 ml) 5 g of nutrient broth (Difco, Detroit, Mich.), 5.2 g of Middlebrook 7H9 broth (Difco), and 10 ml of glycerol was rendered iron deficient by the addition of ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDA; 100 μg/ml), as described below. Five to ten milliliters of medium was inocu-

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TABLE 1. Biochemical properties and mycobactin profiles of *M. fortuitum* strains

Strain designation		Source	Reaction in the following test[a]:															Mycobactin
CIPT	Other		A			B								C				
			Aryl	Hydr	MCon	Nit	CFA	βglu	Peni	Tre	Pip	Fru	Cap	NaCl	Citr	Mann	Inos	
M. fortuitum subsp. *fortuitum*																		
140410001	ATCC 6841[Tb]		+	+	+	+	+	+	−	−	−	+	−	+	−	−	−	F
140420002			+	+	+	+	+	+	−	−	−	+	−	+	−	−	−	F
110		Water	+	+	+	+	+	+	−	−	n	+	−	+	−	−	−	F
117		Water	+	+	+	+	+	+	−	−	−	+	−	+	−	−	−	F
123		Water	+	+	+	+	+	+	−	−	−	+	−	+	−	−	−	F
832306	OES 67	Lymph node	+	+	+	+	+	+	−	−	−	+	+	+	−	−	−	F
832315	OES 77	Lymph node	+	+	+	+	+	+	−	−	−	+	+	+	−	−	−	F
832317	OES 79	Abscess	+	+	+	+	+	+	−	−	+	+	+	+	−	−	−	F
832304	OES 65	Abscess	+	+	+	+	+	+	−	−	−	+	−	+	−	−	−	P
832316	OES 78	Abscess	+	+	+	−	+	+	−	−	−	+	+	+	−	−	−	P
832314	OES 76	Abscess	+	+	+	+	−	+	−	−	−	+	+	+	−	−	−	P
M. fortuitum subsp. *peregrinum*																		
140410020	ATCC 19420[c]		+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	P
810031		Sputum	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	P
111		Water	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	P
831804	OES 51	Sputum	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	P
831125	TMC 1545		+	+	+	+	+	+	−	−	n	+	−	+	−	+	−	P
831112	OES 24	Leg	+	+	+	+	+	+	+	−	n	+	−	+	−	+	−	P
831127	TMC 1547		+	+	+	+	+	+	−	−	n	+	+	+	−	+	−	P
832302	OES 63	Abscess	+	+	+	+	−	+	−	−	+	+	−	+	−	+	−	P
810539		Sputum	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	P
125		Water	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	F
832307	OES 68	Abscess	+	+	+	+	+	+	−	−	−	+	−	+	−	+	−	F
810507		Sputum	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	F
Miscellaneous strains[d]																		
840771	OES 91	Abscess	+	+	+	+	−	+	−	−	+	−	−	+	−	−	+	P
831115	OES 27	Wound	+	+	+	+	+	+	−	−	n	+	+	+	−	+	+	P
821887	OES 07	Sputum	+	+	+	+	+	+	−	−	n	+	−	+	−	+	+	P
831116	OES 28	Ulcer	+	+	+	+	+	+	−	−	n	+	−	+	−	+	+	P
104		Water	+	+	+	−	+	−	−	−	+	+	−	+	−	−	+	P
840791	OES 112	Water	+	+	+	+	+	+	−	−	+	+	−	+	−	+	+	P
112		Water	+	+	−	+	+	+	−	−	−	+	−	−	−	+	−	P
821886	OES 06	Sputum	+	+	+	+	+	+	+	−	n	+	−	+	−	+	+	F
109		Water	+	+	+	+	−	+	−	−	−	+	−	−	−	+	+	F

[a] Abbreviations used: Aryl, arylsulfatase test; Hydr, growth on hydroxylamine; MCon, growth on MacConkey medium; Nit, nitrate reductase test; CFA, iron uptake; βglu, β-glucosidase test; Peni, penicillinase test; Tre, trehalase test; Pip, growth on pipemidic acid; Fru, fructose as a sole carbon source; Cap, growth on capreomycin; NaCl, growth on sodium chloride; Citr, citrate as a sole carbon source; Mann, mannitol as a sole carbon source; Inos, inositol as a sole carbon source; n, not done.

[b] *M. fortuitum* type strain.

[c] *M. fortuitum* subsp. *peregrinum* reference strain.

[d] *M. fortuitum* strains that could not be assigned to a subspecies on the basis of biochemical test results (21).

lated per strain. Cultures were incubated at 30°C or 37°C, as required for different strains, for 15 to 20 days to allow sufficient growth before mycobactin extraction.

Chelator preparation. EDDA was obtained from Sigma Chemical Co., St. Louis, Mo. It was prepared, freed from contaminating iron, by the method of Rogers (32). Briefly, a solution containing 10 g of EDDA in 190 ml of 1 N HCl was boiled and stirred for 1 h. After being cooled and filtered, it was diluted with 1,500 ml of pure acetone and the pH was raised to 6.0 by adding 1 N NaOH. After standing overnight at 4°C, the precipitate was collected by filtration and washed with cold acetone. Dilution in acetone and filtration were repeated twice. The precipitate was dried overnight at 40°C and then collected into a plastic container for storage. The deferrated EDDA was incorporated into the growth medium by the procedure of Ong et al. (27). To prepare a stock solution, 1 g of deferrated EDDA was dissolved in 15 ml of 1 N NaOH. The pH was adjusted to 9 with concentrated hydrochloric acid, and the total volume was brought to 20 ml/g of EDDA with distilled water. A concentration of 100 μg of EDDA per ml was then added to the medium for growth and mycobactin production.

Mycobactin extraction. Bacteria were harvested by centrifugation and suspended in pure ethanol. After centrifugation, the ethanol extract was recovered and the ethanol-soluble mycobactins were revealed by the addition of a 0.1 M $FeCl_3$-ethanol solution. The colorless nonferric mycobactin solution takes on a deep rusty color upon fixation of the iron furnished by the yellow $FeCl_3$-ethanol solution. The extract was evaporated to dryness, dissolved in chloroform, and washed three times in distilled water to eliminate excess iron. The final chloroform extract was evaporated to dryness and then spotted in chloroform.

Determination of mycobactin content by TLC. TLC was

TABLE 2. Biochemical properties and mycobactin patterns of *M. chelonae* strains

Strain designation		Source	Reaction in the following test[a]:															Mycobactin
CIPT	Other		A			B								C				
			Aryl	Hydr	MCon	Nit	CFA	βglu	Peni	Tre	Pip	Fru	Cap	NaCl	Citr	Mann	Inos	
M. chelonae subsp. *chelonae*																		
801468		Endometer	+	+	+	−	−	−	+	+	+	−	+	−	+	−	−	C1
801103		Sputum	+	+	+	−	−	−	+	+	+	−	+	−	+	−	−	C1
810071		Bronchial	+	+	+	−	−	−	+	−	+	−	+	−	+	−	−	C1
840765	OES 84	Abscess	+	+	+	−	−	+	+	+	+	−	+	−	+	−	−	C1
840775	OES 95	Abscess	+	+	+	−	−	−	+	+	+	−	+	−	+	−	−	C2
840774	OES 94	Arm	+	+	+	−	−	−	+	+	+	−	+	−	+	−	−	C2
831801	TMC 1537		+	+	+	−	−	−	+	+	+	−	+	−	+	−	−	C2
420006[b]	ATCC 19236[c]		+	+	+	−	−	−	+	+	+	−	+	−	+	−	−	C2
420003[b]	NCTC 946[Td]		+	+	+	−	−	−	+	+	−	−	+	−	+	−	−	C2
420004[b]		Abscess	+	+	+	−	−	−	+	+	+	−	+	−	+	−	−	C2
840784	OES 104	Heart valve	+	+	+	−	−	−	−	+	+	−	+	−	+	−	−	C2
420005[b]		Abscess	+	+	+	−	−	−	+	−	+	−	+	−	+	−	−	C2
801200		Sputum	+	+	−	−	−	−	+	+	+	−	+	−	+	−	−	C2
810099		Sputum	+	+	+	−	−	−	+	+	+	−	+	−	+	−	−	A2
M. chelonae subsp. *abscessus*																		
821890	OES 10	Lung	+	+	+	−	−	−	+	+	n	−	+	+	−	−	−	A1
821892	OES 12	Lung	+	+	+	−	−	−	+	−	n	−	+	+	−	−	−	A1
821891	OES 11	Lung	+	+	+	−	−	−	+	−	n	−	+	+	−	−	−	A1
821894	OES 15	Lung	+	+	+	−	−	−	+	−	n	−	+	+	−	−	−	A1
810402[e]		Sputum	+	+	+	−	−	−	−	+	+	−	+	+	−	−	−	A1
420009[e]	ATCC 14472[f]		+	+	+	−	−	−	+	+	+	−	+	+	−	−	−	A2
420023[e]	ATCC 19977[Tg]		+	+	+	−	−	−	+	+	+	−	+	+	−	−	−	A2
810192[e]		Sputum	+	+	+	−	−	−	+	+	+	−	+	+	−	−	−	A2
832318	OES 80	Abscess	+	+	+	−	−	−	+	+	+	−	+	+	−	−	−	A2
831898	OES 19	Abscess	+	+	+	−	−	−	+	+	n	−	+	+	−	−	−	A2
831117	OES 29	Ulcer	+	+	+	−	−	−	+	−	n	−	+	+	−	−	−	A2
831109	OES 21	Sputum	+	+	+	−	−	−	+	−	n	−	+	+	−	−	−	A2
832319	OES 81	Adenopathy	+	+	+	−	−	−	+	+	+	−	+	+	−	−	−	C2
821881	OES 01	Ankle	+	+	+	−	−	−	−	−	n	−	+	+	−	−	−	C2
Miscellaneous strains[h]																		
810039		Gastric washing	+	+	+	−	−	−	+	+	+	−	+	+	+	−	−	C1
810292[b]		Sputum	+	+	+	−	−	−	+	+	+	−	+	+	+	−	−	C2
821899	OES 20	Hand	+	+	+	−	−	−	+	−	n	−	+	+	+	−	−	C2
832301	OES 62	Adenopathy	+	+	+	−	−	−	+	+	+	−	+	+	+	−	−	C2
821895	OES 16	Lung	+	+	+	−	−	−	+	−	n	−	+	−	−	−	−	A1

[a] For test result abbreviations, see Table 1, footnote *a*.
[b] Belongs to the genomic group of *M. chelonae* subsp. *chelonae* (22).
[c] "*M. borstelense*" reference strain.
[d] *M. chelonae* subsp. *chelonae* type strain.
[e] Belongs to the DNA genomic group of *M. chelonae* subsp. *abscessus* (22).
[f] "*M. runyonii*" reference strain.
[g] *M. chelonae* subsp. *abscessus* type strain.
[h] *M. chelonae* strains that could not be assigned to a subspecies on the basis of biochemical test results (21).

performed by using glass plates (10 by 20 cm) coated with a 200-μm-thick layer of silica gel (HP-K High Performance Silica Gel; Whatman, Clifton, N.J.). The developing solvent used was ethanol-petroleum ether-ethyl acetate (1:4:6, vol/vol/vol), as described by Barclay et al. (2).

RESULTS

Conditions of mycobactin production. Mycobactins have been obtained from cultures on basal solid asparagine-glycerol medium by other investigators (10). However, mycobactins failed to be produced in liquid medium (10). The addition of an exogenous iron chelator, EDDA, allowed us to ensure mycobactin synthesis in such a medium. The concentration of EDDA required for iron limitation was dependent on the medium composition. When basal liquid asparagine-glycerol medium (10) was used, growth was fully inhibited at 50 μg of EDDA per ml. In contrast, in a rich medium combining nutrient broth, Middlebrook 7H9 broth, and glycerol, EDDA at a final concentration of 100 μg/ml allowed both satisfactory rates of growth and mycobactin production. Under these conditions, mycobactin extracted from a 5-ml mycobacterial culture after 2 weeks of incubation could be detected by TLC.

Biochemical properties and mycolic acid content. Characteristics of the 65 *M. fortuitum* and *M. chelonae* strains studied are listed in Tables 1 and 2. The properties are arranged as follows. Properties used for the assignment to the *M. fortuitum-M. chelonae* complex are listed in section

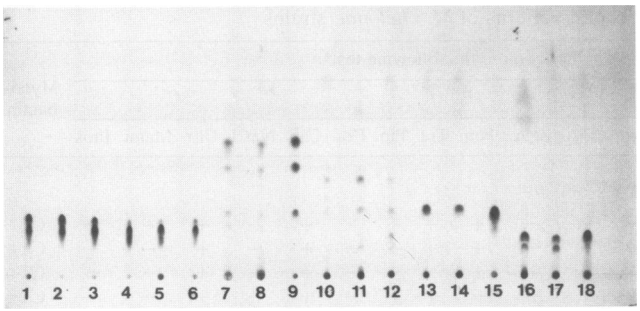

FIG. 1. Mycobactin patterns of *M. fortuitum* and *M. chelonae* strains. TLC plates were eluted by using ethanol-petroleum ether-ethyl acetate (1:4:6, vol/vol/vol). Lane numbers refer to the following strain designations (21): 1, *M. fortuitum* ATCC 6841^T; 2, *M. fortuitum* subsp. *fortuitum* 110; 3, *M. fortuitum* subsp. *fortuitum* 117; 4, *M. fortuitum* subsp. *peregrinum* 111; 5, *M. fortuitum* subsp. *peregrinum* 831804; 6, *M. fortuitum* subsp. *peregrinum* 831125; 7, *M. chelonae* subsp. *chelonae* 810071; 8, *M. chelonae* subsp. *chelonae* 801468; 9, *M. chelonae* (miscellaneous strain) 810039; 10, *M. chelonae* subsp. *chelonae* 420003; 11, *M. chelonae* subsp. *chelonae* 420006; 12, *M. chelonae* subsp. *chelonae* 840775; 13, *M. chelonae* subsp. *abscessus* 821894; 14, *M. chelonae* subsp. *abscessus* 821890; 15, *M. chelonae* (miscellaneous strain) 821895; 16, *M. chelonae* subsp. *abscessus* 420009; 17, *M. chelonae* subsp. *abscessus* 831109; 18, *M. chelonae* subsp. *abscessus* 831117. Lanes 1 to 3 correspond to pattern F, lanes 4 to 6 correspond to pattern P, lanes 7 to 9 correspond to pattern C1, lanes 10 to 12 correspond to pattern C2, lanes 13 to 15 correspond to pattern A1, and lanes 16 to 18 correspond to pattern A2. Miscellaneous refers to a strain that could not be assigned to a subspecies on the basis of its biochemical test results (21).

A, properties useful for the differentiation of *M. fortuitum* from *M. chelonae* are listed in section B, and properties useful for the recognition of subgroups within each species are listed in section C. The mycolate content for all strains in Table 1 was characteristic of *M. fortuitum*, with α- and epoxy-mycolates, whereas strains in Table 2 all shared the mycolate profile of *M. chelonae*, consisting of α- and α′-mycolates, as previously described (5, 6, 21, 26).

Mycobactin patterns. Because compounds analyzed were extracted from cell mass by using organic solvent and because they are able to bind iron, we assumed that they correspond to true mycobactins (and not to extracellular exochelins or water-soluble iron-binding proteins).

The six different types of TLC mycobactin patterns of the *M. fortuitum* (Fig. 1, lanes 1 to 6) and *M. chelonae* (Fig. 1, lanes 7 to 18) strains are shown in Fig. 1. Weekly examination of strains showing the different types of patterns determined for up to 5 or 8 weeks demonstrated the stability of mycobactin content upon aging.

Profile types of *M. fortuitum* subsp. *fortuitum* and *M. fortuitum* subsp. *peregrinum*, representing three spots, differ regarding their major products, either the top spots (the two less polar spots) (Fig. 1, lanes 1 to 3) or the spot with an intermediate migration coefficient (Fig. 1, lanes 4 to 6). These two types of patterns were encountered exclusively in the *M. fortuitum* strains and were named type F and type P, respectively. It seems relevant to us that among the biochemically classified *M. fortuitum* subsp. *fortuitum* strains, 8 of 11, or 73% (including the *M. fortuitum* type strain), present the F profile, and that with the *M. fortuitum* subsp. *peregrinum* strains 9 of 12, or 75% (including the *M. fortuitum* subsp. *peregrinum* reference strain), present the P profile (Tables 1 and 3). Of the *M. fortuitum* strains that did not fit any subspecies on the basis of biochemical test results, 78% show the type P pattern and 22% show type F (Tables 1 and 3).

Among the *M. chelonae* strains, four different patterns have been observed, referred to as C1 (Fig. 1, lanes 7 to 9), C2 (Fig. 1, lanes 10 to 12), A1 (Fig. 1, lanes 13 to 15), and A2 (Fig. 1, lanes 16 to 18). Type C1 presents as many as seven spots, with three major spots. Type C2 also has three major spots, but they do not migrate as high as those in type C1. Types A1 and A2 have simpler patterns. The type A1 profile is characterized by a single major spot (Fig. 1, lanes 13 and 14) associated in some strains with a somewhat more polar minor spot (Fig. 1, lane 15). The A2 profile (Fig. 1, lanes 16 to 18) is characterized by two spots. In some strains with the A2 profile, the more polar spot is present in a lesser amount (Fig. 1, lane 18). Of the 14 strains identified as *M. chelonae* subsp. *chelonae* (Tables 2 and 3), type C1 has been found in 4, i.e., 28% (including the *M. chelonae* subsp. *chelonae* type strain), and type C2 has been found in 9, i.e., 65% (including the reference strain, "*M. borstelense*"). Types C1 and C2 thus represent 93% of the *M. chelonae* subsp. *chelonae* strains. The remaining strain showed an A2 profile. In the *M. chelonae* subsp. *abscessus* group (Tables 2 and 3), 36% of the 14 strains are of the A1 type (including the reference type, "*M. runyonii*"), 50% are of the A2 type (including the reference type, *M. chelonae* subsp. *abscessus*), and the remaining 14% (two strains) present the C2 profile. Thus, the C and A patterns are shared by 93% of the *M. chelonae*

TABLE 3. Percent distribution of *M. fortuitum* and *M. chelonae* strains on the basis of biochemical identification and mycobactin patterns

Biochemical identification (no. of strains)	% Showing mycobactin pattern:									
	F	P	C1	C2	A1	A2	C1/C2	A1/A2	F/P	C1/C2/A1/A2
M. fortuitum subsp. *fortuitum* (11)	73[a]	27							100	
M. fortuitum subsp. *peregrinum* (12)	25	75[b]							100	
M. fortuitum miscellaneous[c] (9)	78	22							100	
M. chelonae subsp. *chelonae* (14)			28	65[d]		7	93	7		100
M. chelonae subsp. *abscessus* (14)				14	36	50[e]	14	86		100
M. chelonae miscellaneous (5)			20	60	20		80	20		100

[a] Including the *M. fortuitum* subsp. *fortuitum* type strain.
[b] Including the *M. fortuitum* subsp. *peregrinum* reference strain.
[c] Miscellaneous refers to a strain that could not be assigned to a subspecies on the basis of its biochemical test results (21).
[d] Including *M. chelonae* subsp. *chelonae* and the "*M. borstelense*" reference strain, which belongs to the genomic group of *M. chelonae* subsp. *chelonae* (22).
[e] Including the *M. chelonae* subsp. *chelonae* type strain and the "*M. runyonii*" reference strain, which belongs to the genomic group of *M. chelonae* subsp. *abscessus* (22).

subsp. *chelonae* strains and 86% of the *M. chelonae* subsp. *abscessus* strains. In the group of *M. chelonae* strains that cannot be assigned to a subspecies on the basis of biochemical features, A1, C1, and C2 patterns were observed (Tables 2 and 3).

In both *M. fortuitum* and *M. chelonae* groups, disagreement observed between biochemical identification and mycobactin analysis could not be related to any peculiar strain source; e.g., of the three strains biochemically identified as *M. fortuitum* subsp. *peregrinum* that provide the mycobactin F profile (Table 1), one consisted of an incidental human isolate from sputum, one was the etiologic agent of an abscess, and the third one was an environmental isolate.

For comparative purposes, mycobactin content was also examined in four strains, reference type strains of other rapidly growing mycobacteria that have a mycolic acid composition similar to that of *M. fortuitum* (*M. chitae*, *M. porcinum*, *M. senegalense*, *M. smegmatis*) (5, 6, 22). Mycobactin patterns of *M. chitae* and *M. smegmatis* were found to be distinct from those of *M. fortuitum*. However, *M. senegalense* and *M. porcinum* provided patterns similar to those of *M. fortuitum* subsp. *fortuitum* and *M. fortuitum* subsp. *peregrinum*, respectively (data not shown).

DISCUSSION

The mycobactins are lipid-soluble, iron-chelating compounds produced by mycobacteria under conditions of iron limitation. Two reviews were devoted to these compounds by Snow and Ratledge, dealing with structural elucidation, biological properties, and functions in iron uptake (29, 34). In 1970, Snow and White suggested the role of mycobactins as chemotaxonomic markers for identification of mycobacteria to the species level as different mycobactin structures were determined on the basis of the species examined (35). Mycobactin analysis of diverse species of mycobacteria and related genera has been performed mainly by Ratledge and collaborators (2, 3, 11–14, 30). However, the method has not been widely applied for identification purposes, owing to constraining conditions of cell cultivation compatible with mycobactin production. Iron concentration available in culture medium is critical for mycobactin production. In the original method, all glassware was cleaned with 2% KOH–ethanol followed by 8 M HNO_3 (10). A synthetic liquid medium composed of asparagine-glycerol-monopotassic phosphate was then used. Metal ions present in this medium were removed by addition of alumina. The medium was further supplemented with divalent ions, including zinc, magnesium, manganese, and limited iron to allow cell growth. This tedious procedure was inadequate for a large survey of mycobactin distribution in mycobacteria. However, the same medium, solidified with agar, was found to be adequate for mycobactin production even without any pretreatment for the removal of iron (10). Nevertheless, large volumes of this basal medium (500 ml) were required to get a consistent amount of cells. A well-controlled iron concentration was achieved only in this minimal medium, as experiments using richer media, such as Löwenstein-Jensen medium or glucose-yeast extract medium, failed to show mycobactin accumulation. Moreover, some strains cultivated in the minimal medium, especially *M. chelonae* strains, failed to produce detectable mycobactins (10). When grown on glycerol-asparagine-agar medium, *M. chelonae* yielded trace amounts from large cell quantities, such as 7.5 g of dry cells (11).

In the procedure presented here, a rich liquid medium was developed to allow satisfactory growth of mycobacterial cells. Iron limitation was achieved by the addition of EDDA, a potent iron chelator, which is not metabolized by mycobacteria. The concentration of 100 μg/ml was found to be the most adequate concentration to ensure both cell growth and mycobactin accumulation. Under such conditions, mycobactin extracted from as little as 5 ml of mycobacterial culture, cultivated for 2 weeks, can be analyzed by TLC. It is interesting that the method described here allows determination of the mycobactin contents of all strains, especially *M. chelonae*, with the same standard procedure applied to all mycobacteria.

The procedure developed in this study for mycobactin detection can be integrated into the classical scheme of mycobacterial identification because it requires only the inoculation of one tube containing 5 ml of the appropriate medium. This tube can be inoculated at the same time as Löwenstein-Jensen slants containing different inhibitors, such as thiophene-2-carboxylic acid hydrazide, thiacetazone, *p*-nitrobenzoic acid, hydroxylamine chloride, etc., usually included in the routine identification of mycobacteria. The determination of mycobactin content will not introduce any delay because the analysis can be achieved in one day after 2 weeks of cell cultivation. Mycobactin results may thus be available before the results of some biochemical tests, e.g., the Tween hydrolysis test, which is performed on a subculture (minimum of 5 days of incubation) and may require up to 14 days for the last score. TLC does not represent the introduction of an additional technique, as TLC analysis of mycolic acids or specific lipids is already part of the mycobacterial identification in several reference laboratories (7, 17, 24).

Our data confirm the results of Hall and Ratledge, who distinguished two spots in the *M. fortuitum* profile in some of the TLC procedures that they used (11). Moreover, with our method minor spots are shown, indicating that this latter method provides for more sensitive detection.

Extracts from the type strains of *M. fortuitum* subsp. *fortuitum* and subsp. *peregrinum* provided distinct mycobactin patterns, designated F and P, respectively. Similarly, extracts from the type strains of *M. chelonae* subsp. *chelonae* and subsp. *abscessus* provided distinct patterns, designated C2 and A2, respectively. However, two additional patterns were encountered within the *M. chelonae* strains. All strains identified as *M. fortuitum* produced only patterns F and P, and those identified as *M. chelonae* produced only patterns C1, C2, A1, and A2. This confirms that differentiation between *M. fortuitum* and *M. chelonae* on the basis of mycobactin analysis is relevant.

M. fortuitum and *M. chelonae* mycobactin patterns are characteristic of each species. However, similar patterns were found for *M. fortuitum* subsp. *fortuitum* and *M. senegalense*, as well as for *M. fortuitum* subsp. *peregrinum* and *M. porcinum*, confirming the previous demonstration of the equivalence of mycobactins from *M. senegalense* and *M. fortuitum* subsp. *fortuitum* (13) as well as DNA relatedness results (1). Among rapidly growing mycobacteria, intermediate percentages of homology, in the range of 47 to 57%, were determined between strains of *M. senegalense*, *M. fortuitum* subsp. *fortuitum*, and *M. fortuitum* subsp. *peregrinum*, whereas for other distinct species homology did not exceed 30% (1). Therefore, it appears that elucidation of the taxonomic status of *M. senegalense* and *M. porcinum* as species distinct from *M. fortuitum* deserves further genomic analyses.

The contribution of mycobactin analysis to the character-

ization of the *M. fortuitum* and *M. chelonae* subspecies appears to be valuable. The type strains of both species produced different patterns. Comparison of the data from a previous DNA-DNA relatedness study performed within *M. chelonae* with data on some strains included in the present study showed that strains belonging to the genomic group of *M. chelonae* subsp. *chelonae* shared the C2 profile of the type strain (except for one strain that produces an A2 profile). Similarly, strains of the genomic group of *M. chelonae* subsp. *abscessus* showed the A2 profile of the type strain; one strain of this genomic group presented the A1 profile. Neglecting the differences between C1 and C2 on one hand and A1 and A2 on the other hand, all *M. chelonae* strains showed either a C or an A pattern. Similarly, all of the *M. fortuitum* strains presented either an F or a P profile. As summarized in Table 3, results of the two methods, mycobactin analysis and biochemical profiles, are the same in more than 75% of the strains. Discrepancies may be attributed to the low reliability of the tests on which the biochemical differentiation relies. It has been shown that biochemical subspecies differentiation leaves about 30% of both *M. fortuitum* and *M. chelonae* strains out of any subspecies (21). According to our data, mycobactin analysis represents a valuable taxonomic marker because 100% of the strains of both species can be characterized on the basis of mycobactin patterns.

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