JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1986, p. 835–839
0095-1137/86/110835-05$02.00/0

Vol. 24, No. 5

Mycolic Acid Analysis for Clinical Identification of *Mycobacterium avium* and Related Mycobacteria

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We examined the mycolic acid composition of 133 strains belonging to MAIS complex (*Mycobacterium avium-M. intracellulare-M. scrofulaceum*) and MAIS intermediate strains and the related species *M. asiaticum*, *M. malmoense*, *M. shimoidei*, and *M. simiae*. The analysis revealed that about 10% of the strains identified as *M. avium-M. intracellulare* complex by conventional cultural and biochemical tests were in fact *M. simiae* strains according to their mycolate composition. Of 25 strains previously studied by the International Working Group on Mycobacterial Taxonomy, 2 (MAIS intermediate and *M. asiaticum*) presented patterns incompatible with the clusters to which they had been assigned. *M. malmoense* and both *M. simiae* serovars shared the same pattern with α-, α′-, and ketomycolates. We describe here the method used to identify the mycolic acid profiles in detail. We found it to be highly reproducible and convenient for use in mycobacterial reference laboratories.

The mycolic acids formed by representative strains of most mycobacterial species were isolated and purified. Six molecular types of mycolic acids were thus described, corresponding to the long nonoxygenated mycolates, also referred to as α-mycolates (11, 12); the short nonoxygenated mycolates, also designated α′-mycolates (15); the methoxymycolates (9, 23); the ketomycolates (23); the epoxymycolates (7); and the dicarboxylic mycolates (10, 19). Several comprehensive reviews have been published (1, 13, 21, 26), and it has been shown that mycolate composition is a stable taxonomic characteristic (5, 8, 22). The methyl esters of mycolic acids were found to migrate on thin-layer chromatography (TLC) silica gel plates more in accordance with the nature of their characteristic functions than with other differences in their molecular structures (16). Thus, for example, the ketomycolates from *Mycobacterium tuberculosis* and *M. paratuberculosis* differ by the presence of a cyclopropane ring and by the total number of carbon atoms, but their methyl esters cochromatograph on TLC plates (2, 9, 17). Catalogs of mycolic acid compositions of mycobacterial species have since been established by using TLC methods (5, 8).

One consequence of these studies was the development of simple and reproducible methods for mycolic acid analysis (5), and since then we have applied them to the clinical identification of strains that are difficult to characterize. We present here a detailed description of the methods we used and show their usefulness in identifying organisms of the MAIS complex (*M. avium-M. intracellulare-M. scrofulaceum*), including the MAIS intermediate described by Hawkins (14), and the related species *M. asiaticum* (25), *M. malmoense* (25), *M. shimoidei* (24), and *M. simiae* (25).

MATERIALS AND METHODS

Bacterial strains. The sources of the 133 strains selected for this study are given in Table 1. The type strains *M. tuberculosis* ATCC 27294, *M. avium* ATCC 25291, *M. chelonei* NCTC 946, and *M. fortuitum* ATCC 6841 were used for the preparation of mycolic acid standards (see below).

Identification scheme. The strains were identified by conventional methods (18, 20, 27).

Extraction of mycolic acids. A spadeful of bacteria (ca. 10 mg, wet weight) was scraped from the surface of Löwenstein-Jensen slants. The cells were suspended in 2 ml of ethylene glycol monomethyl ether containing 5% (wt/vol) potassium hydroxide and 12% (vol/vol) distilled water. The tubes were stoppered with Teflon-lined screw caps, and the mixtures were saponified by heating at 110°C for 2 h. This method allows the quantitative saponification of sterically constrained esters such as dicarboxylic mycolates, mycocerosates, and phthioceranates (5, 6). The saponification must be complete because the dicarboxylic mycolates still esterified by secondary alcohols have the same chromatographic properties as the ketomycolates (5).

After saponification, the mixtures were allowed to cool and then acidified by adding 1 ml of a 20% (vol/vol) sulfuric acid solution. The fatty acids were extracted into 5 ml of ether. The ether layers were transferred to new test tubes and extensively washed with distilled water to remove the sulfuric acid and the ethylene glycol monomethyl ether. The ether layers were recovered and evaporated to dryness.

Preparation of methyl esters. Lipids were methylated with diazomethane freshly prepared from a commercially available compound, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazogen; Janssen-Chimica, Beerse, Belgium). For this purpose 2.15 g of Diazogen was dissolved in 20 ml of ether and added to a precooled solution containing 600 mg of KOH, 1 ml of distilled water, 3.5 ml of diethylene glycol monoethyl ether, and 1 ml of ether. The mixture was gently warmed to 50°C, and the diazomethane generated was trapped through a U-bottom glass tube into 20 ml of ether cooled in ice. The reaction was allowed to stand for 15 to 20 min. A 1-ml portion of the ether solution of diazomethane was poured out on each tube containing the dried lipids, and methylation was achieved within 5 min. Diazomethane was then evaporated, and 10-μg/ml lipid solutions were prepared in ether.

TLC of mycolic acid methyl esters. The lipid-ether solutions were spotted onto two K6 silica gel plates (Whatman, Inc., Clifton, N.J.). One plate, previously washed by one passage of the appropriate solvent, was developed three times by using petroleum ether-ether (90:10; vol/vol [solvent

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TABLE 1. TLC mycolic acid patterns of 133 *Mycobacterium* strains

Organism[a]	No. of strains		Mycolic acids of type strains[d]	No. of strains with expected patterns		% Frequency of discrepancies	
	IWGMT study[b]	Other sources[c]		IWGMT study	Other sources	IWGMT study	Other sources
MAIS-1	6	0	I, IV, VI	5	0	16.7	
MAI complex	0	95	I, IV, VI	0	83		12.6
M. scrofulaceum	0	5	I, IV, VI	0	5		0
M. malmoense	6	0	I, II, IV	6	0	0	
M. simiae	9	8	I, II, IV	9	8	0	0
M. asiaticum	4	0	I, III, IV	3	0	25.0	
M. shimoidei	0	0	I, II, IV, VI	0	0		

[a] The bacteria were identified according to current cultural and biochemical properties as indicated in the text.

[b] OES numbers refer to the coded numbers of the strains in the open-ended study of slowly growing mycobacteria performed by the International Working Group on Mycobacterial Taxonomy (IWGMT) (28). The strains included here were MAIS-1 (MAIS intermediate) strains OES 90002, OES 90004, OES 90018, OES 90023, OES 90034, and OES 90035; *M. malmoense* strains OES 90045, OES 90047, OES 90049, OES 90050, and OES 90051; *M. simiae* strains OES 90006, OES 90007, OES 90008, OES 90009, OES 90010, OES 90012, OES 90021, OES 90022, and OES 90025; and *M. asiaticum* strains OES 90028, OES 90041, OES 90052, and OES 90053.

[c] These strains were mainly clinical isolates submitted to our laboratory for reference purposes. All of the MAI complex strains were clinical isolates; 16 were from patients with the acquired immunodeficiency syndrome (AIDS) diagnosed in Paris. the five *M. scrofulaceum* strains were clinical isolates. All of the *M. simiae* strains had been previously studied either by Boisvert and Truffot (4) or by Baess and Magnusson (3); these strains included isolates from human (ER 4238 serotype 1 [4], HB 4393 serotype 2 [4], and MNC 413 serotype 1 [3]), monkey (MNC 838 serotype 2, MNC 946 serotype 2, MNC 947 serotype 1, and MNC 1322 serotype 2 [3]), and a leprosy-infected armadillo (78-2663 [4]). The MNC strains were kindly provided by I. Baess, Statens Seruminstitut, Copenhagen, Denmark.

[d] The type strains were *M. intracellulare* ATCC 13950, *M. scrofulaceum* ATCC 19981, *M. malmoense* ATCC 29571, *M. simiae* ATCC 24275, and *M. asiaticum* ATCC 25276.

1]), and the other plate was developed once by using dichloromethane (solvent 2). The fatty acids were revealed by spraying the plates with a 0.1% (wt/vol) solution of rhodamine B in ethanol diluted 10 times just before use in 0.25 M sodium phosphate.

Eleven strains could be tested on each set of two plates, and the mycolic acids of the reference strains served as landmarks on each plate for the proper identification of the mycolic acids extracted from the unknown strains. The mycolic acids of the reference strains were as follows: for *M.*

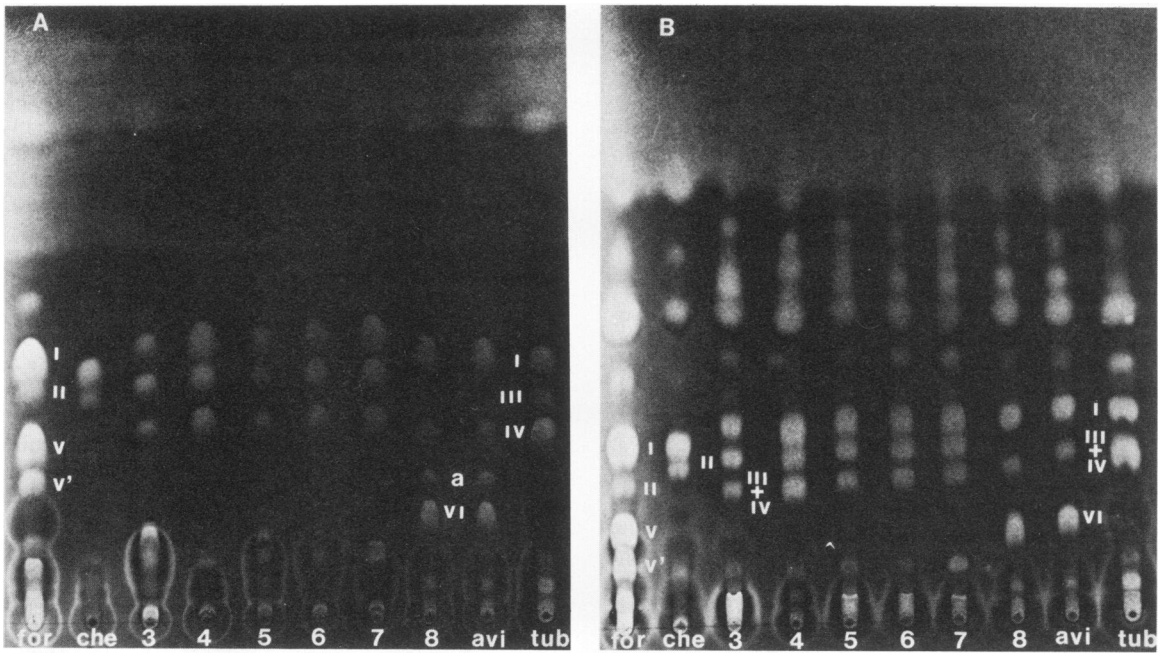

FIG. 1. TLC mycolic acid patterns of 10 mycobacterial species. The solvents used were petroleum ether-ether (90:10, vol/vol; solvent 1) in panel A and dichloromethane (solvent 2) in panel B. Plates were photographed under UV light to improve the contrast; however, this revealed quenching spots that were not visible with white light (red spots were obtained with rhodamine B), especially in the lower part of the plates between the origin and mycolate VI. The two lanes on the left and right sides of each plate correspond to type strains *M. fortuitum* ATCC 6841 (for), *M. chelonae* NCTC 946 (che), *M. avium* ATCC 25291 (avi), and *M. tuberculosis* ATCC 27294 (tub). The other lanes correspond to the following strains: 3, *M. malmoense* ATCC 29571; 4, *M. simiae* ATCC 25275 (serotype 1); 5, *M. simiae* MNC 838 (serotype 2); 6, *M. simiae* MNC 946 (serotype 2); 7, *M. simiae* 78-2663 (from armadillo); and 8, *M. asiaticum* OES 90028. The sources of these strains are given in Table 1. The mycolate designations are as previously described (5): I, α-mycolate; II, α′-mycolate; III, methoxymycolate; IV, ketomycolate; V, epoxymycolate; VI, dicarboxylic mycolate; and a, alcohol-esterifying dicarboxylic mycolate. V′ refers to a polar component sometimes found in association with epoxymycolate, probably a diol generated by hydratation of the epoxy function. It must be noted that the α- and α′-mycolates of *M. malmoense* and *M. simiae* are longer, and so migrate farther than the α- and α′-mycolates of other species.

TABLE 2. Mycolic acid compositions of strains giving discrepant data with respect to their biochemical identification

Biochemical identification	Strain[a]	Source	Mycolic acid composition	Biochemical and mycolate pattern identification
MAIS-1	OES 90018	IWGMT study	I, III, IV	*M. asiaticum*
MAI complex	CIPT 841198[b]	Blood (France)	I, II, IV	*M. simiae*
MAI complex	CIPT 841267[b]	Rectal biopsy (France)	I, II, IV, VI	*M. simiae* plus MAI complex
MAI complex	CIPT 840934[c]	Lung (Reunion island, France)	I, II, IV	*M. simiae*
MAI complex	CIPT 850782[c]	Sputum (Reunion island, France)	I, II, IV	*M. simiae*
MAI complex	CIPT 840958	Unknown (Reunion island, France)	I, II, IV	*M. simiae*
MAI complex	CIPT 850646	Unknown (Portugal)	I, II, IV	*M. simiae*
MAI complex	CIPT 850931[d]	Sputum (Reunion island, France)	I, II, IV	*M. simiae*
MAI complex	CIPT 850447	Gastric washing (France)	I, II, IV	*M. simiae*
MAI complex	CIPT 851039	Skin (Brazil)	I, II, IV	*M. simiae*
MAI complex	CIPT 850407	Urine (Reunion island, France)	I, II, IV	*M. simiae*
MAI complex	CIPT 850734	Gastric washing (France)	I, II, IV	*M. simiae*
MAI complex	CIPT 851430	Sputum (France)	I, II, IV	*M. simiae*
M. asiaticum	OES 90028	IWGMT study	I, IV, VI	MAI complex

[a] OES refers to the coded numbers of the strains of the open-ended study of slowly growing mycobacteria by the IWGMT (28). CIPT indicates strains isolated from patients and then submitted to our laboratory for reference purposes.

[b] Strains isolated from the same patient who had AIDS. Strains biochemically identified as MAI complex were isolated from the blood culture and the rectal biopsy. Single-colony isolates showed mixed cultures consisting of two different clones. One formed mycolates I, IV, and VI and was biochemically identified as MAI complex. The other one formed mycolates I, II, and IV and could be identified as *M. simiae* on the basis of positive niacin or urease tests.

[c] Strains isolated from the same patient.

[d] Strong positive niacin test after 3 months of incubation.

avium, the α-, keto-, and dicarboxylic mycolates, including the secondary alcohol released from dicarboxylic mycolate during saponification; for *M. chelonei*, the α- and α′-mycolates; for *M. fortuitum*, the α-mycolate and epoxymycolate; and for *M. tuberculosis*, the α-, methoxy-, and ketomycolates.

RESULTS

A typical thin-layer chromatogram of the mycolic acid methyl esters from 10 strains is shown in Fig. 1. Lanes 1, 2, 9, and 10 represent the mycolic acids of the reference strains. Because the chromatographic behavior of mycolic acids correlates with the nature of their main function, they were designated by roman numerals as follows: type I, long nonoxygenated mycolates (α-mycolates); type II, short nonoxygenated mycolates (α′-mycolates); type III, methoxymycolates; type IV, ketomycolates; type V, epoxymycolates; and type VI, dicarboxylic mycolates as noted by Daffé et al. (5).

Two plates were needed to identify the various mycolates (Fig. 1). Solvent 1 resolved the mixture into seven spots corresponding to the six mycolate types and to the secondary alcohol, which always esterified the dicarboxylic mycolates. The alcohol (usually eicosanol, seldom docosanol) was released upon saponification and was coextracted with the fatty acids. With both solvents the alcohol migrated in the same region as did the mycolic acids, whereas the common fatty acids migrated farther towards the front. Solvent 1 did not clearly distinguish between mycolates II and III and between mycolates IV and V. On the other hand, solvent 2 clearly separated these mycolates, but mycolate VI and the secondary alcohol were not clearly resolved and mycolates III and IV cochromatographed as a single spot. The proper interpretation of the chromatograms could therefore only be made if the plates eluted with solvents 1 and 2 were compared to each other.

The mycolic acid compositions of the 133 strains analyzed here are presented in Table 1. The mycolic acid compositions of the strains identified as *M. malmoense*, *M. scrofulaceum*, and *M. simiae*, as well as 88% of the MAI (*M. avium-M. intracellulare*) complex strains and three of the *M. asiaticum* strains, were in agreement with the compositions of the type strains of the corresponding species. In addition, five MAIS-intermediate strains described by Hawkins (14) and classified in a cluster designated MAIS-1 (28) presented the same mycolate pattern as did MAIS strains, i.e., mycolates I, IV, and VI. However, another MAIS-intermediate strain (OES 90018) that was also classified in the MAIS-1 cluster formed mycolates I, III, and IV. This strain could not be placed in any recognized serovar (28). However, the strain could be identified as *M. asiaticum* on the basis of a positive Tween hydrolysis test and a compatible mycolate pattern. Discrepant data were also found among the strains identified as MAI complex and *M. asiaticum* (Table 2). The OES 90028 strain was included in the *M. asiaticum* cluster (28), but it formed mycolates I, IV, and VI, whereas the type strain and three other *M. asiaticum* strains formed mycolates I, III, and IV. This OES 90028 strain was found to match the "*M. paraffinicum*" marker strain at a score of more than 85% (28). Finally, 12 clinical isolates identified as MAI complex strains had negative test results for photochromogenicity and for niacin and urease production but formed the mycolic acid pattern characteristic of *M. simiae* or *M. malmoense*. All of these MAI complex strains were slowly pigmented; niacin and urease tests sometimes turned positive when the cultures became old (>8 weeks).

DISCUSSION

DNA-DNA hybridization experiments established that *M. simiae* strains do not belong to MAIS or *M. asiaticum* species (3). In addition, *M. simiae* serotype 1 is not homologous to *M. simiae* serotype 2 (3). These genotypic differences were confirmed by comparative reciprocal intradermal sensitin reactions in guinea pigs (3), by enzyme-linked immunosorbent assay of glycolipid antigens (30), and, partially, by seroagglutination performed according to the Schaefer method (4). Numerical taxonomy studies generated different clusters corresponding to *M. simiae*, *M. malmoense*, MAIS-1, *M. asiaticum*, and *M. shimoidei* species (28, 29), but did

TABLE 3. Key tests used in the clinical identification of *M. avium* and related species

Organism	Key identification tests						Mycolic acid profile					
	Pigmentation		Niacin	Tween 80 hydrolysis (10 days)	Urease[a]	Thermoresistant catalase	I	II	III	IV	V	VI
	Dark	Light										
M. avium	−	−	−	−	−	+	+			+		+
M. intracellulare	−	−	−	−	−	+	+			+		+
M. scrofulaceum	+	+	−	−	+	+	+			+		+
M. malmoense	−	−	−	+	−	−	+	+		+		
M. simiae	−	+	+	−	+	+	+	+		+		
M. asiaticum	+	+	−	+	−	+	+		+	+		
M. shimoidei	−	−	−	+	−	+	+	+		+		+

[a] The urease test was performed as described in previous evaluation studies (17, 19).

not yield satisfactory key tests for the identification of strains of these species and of the MAIS complex (28, 29). The differential identification of these organisms in clinical laboratories is therefore not easy to perform either because the test characters used are variable in their results or because only a single test character is available (28, 29). Thus, the strains that do not fit the test character profiles summarized in Table 3 are usually identified as MAI complex.

Our study showed that the determination of the mycolic acid composition of the mycobacterial strains may help to solve some of the diagnostic difficulties. Using this technique, we easily differentiated *M. malmoense* and both *M. simiae* serovars from the MAIS complex (including MAIS intermediate), *M. asiaticum*, and *M. shimoidei*. TLC mycolate patterns represent a useful criterion for identifying the *Mycobacterium* species mentioned above because the mycolate composition is a stable feature shared by all strains of the same species (5, 8, 22). Strains erroneously assigned to MAIS-1 or *M. asiaticum* clusters could also be detected by this method. In addition, the mycolic acid composition was particularly useful for identifying *M. simiae* and *M. malmoense* since we found that 10% of the strains with physiological and biochemical properties characteristic of the MAI complex had a mycolate pattern specific for *M. malmoense* or *M. simiae*. It should be pointed out that the *M. malmoense* and *M. simiae* patterns are not shared by other species (5, 8). However, this analysis has to be completed by using other characteristics since the mycolate patterns were not sufficient to differentiate either *M. malmoense* from *M. simiae* or between the various *M. simiae* serovars.

The technique described here is easy to perform, can be carried out in a few hours, and is very reliable and reproducible. Mycolate profile determination is a useful method that complements the usual identification schemes and so helps to prevent the misidentification of strains related to the MAIS complex, and it is especially convenient for the detection of *M. simiae* strains which may show uncharacteristic features in other biochemical tests.

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