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Evaluation of Practical Chromatographic Procedures for Identification of Clinical Isolates of Mycobacteria

M. LUQUIN,[1] V. AUSINA,[1]* F. LÓPEZ CALAHORRA,[2] F. BELDA,[1] M. GARCÍA BARCELÓ,[1] C. CELMA,[2] AND G. PRATS[1]

Departamento de Microbiología, Hospital de la Santa Cruz y San Pablo, Facultad de Medicina de la Universidad Autónoma de Barcelona,[1] and Departamento de Química Orgánica, Facultad de Químicas, Universidad de Barcelona,[2] Barcelona, Spain

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After experimental conditions were established, 366 strains of mycobacteria belonging to 23 different species were studied for fatty acids, secondary alcohols, and mycolic acid cleavage products by capillary gas-liquid chromatography. Additionally, the mycolic acid pattern was studied by thin-layer chromatography. Capillary gas-liquid chromatography allowed direct identification of the following *Mycobacterium* spp.: *M. kansasii*, *M. marinum*, *M. szulgai*, *M. xenopi*, *M. malmoense*, and *M. gordonae*. The patterns of mycolic acid methyl esters recorded for the test strains of *M. chelonae* and *M. agri* may be of value in the identification of these species. Moreover, the combined use of the two chromatographic techniques provided precise identification of the *M. tuberculosis* complex, *M. simiae*, *M. fallax*, *M. triviale*, and *M. chelonae*-like organisms. A minimal set of biochemical tests is usually required to obtain identification to the species level when chromatographic procedures alone are not sufficient. Under the reported experimental conditions, thin-layer chromatography and capillary gas-liquid chromatography are rapid and very useful techniques for the identification of mycobacteria.

Identification of mycobacteria in the clinical laboratory still remains a fastidious, difficult, and time-consuming procedure. The morphological, cultural, and biochemical tests used for mycobacterial identification require specialized knowledge and well-trained laboratory technicians (20, 22). To simplify or even replace those procedures that have been used for many years, some laboratories are working toward the development of quicker, newer techniques for the identification of mycobacteria. Analysis of lipid composition by various chromatographic methods is recognized as a useful tool for differentiating mycobacterial species (1–13, 15–19, 21, 24).

Some reports have shown the value of the determination of patterns of mycolic acids by thin-layer chromatography (TLC) as the first step in the identification of mycobacteria in clinical laboratories (2, 3, 17–19, 24). Analysis of the cellular fatty acids by gas-liquid chromatography (GLC) appears to be a particularly suitable method for the identification of mycobacteria, because it is capable of distinguishing between closely related species (7, 21, 24). GLC has also been used to detect secondary alcohols stemming from wax-ester mycolates (7, 9) and the long-chain fatty acid cleavage products ($C_{22:0}$, $C_{24:0}$, and $C_{26:0}$) that are formed when mycolic acid methyl esters from mycobacteria are heat cleaved in the injection port of the gas chromatograph (1, 4, 7, 9).

TLC and GLC are rapid, simple techniques and require only small amounts of cells (10 mg [wet weight]). GLC is used as a routine method for the identification of mycobacteria to the species level, although this has been done only in relatively few laboratories so far. Standardized procedures must be developed before the method can be expected to become more widely used (9). Differences in instrumentation and variations in analytical conditions could significantly affect the chromatographic profiles of these microorganisms. The relative amounts of mycolic acid cleavage products (MACP) and secondary alcohols detected by GLC varied considerably from one laboratory to another. (7, 9). In order to obtain reproducible results for these compounds, which are very useful markers in mycobacterial identification, the method is dependent on several factors such as the hydrolysis methods used, reaction time periods, injector port temperature, and instrumentation (4, 7, 9, 10, 21).

The purpose of our study was twofold: to evaluate the utility of TLC and capillary gas-liquid chromatography (CGC) for the routine identification of mycobacteria and to evaluate the possibility of standardizing a practical gas chromatographic method for obtaining interlaboratory reproducibility.

MATERIALS AND METHODS

Bacterial strains. The sources of the 366 strains selected for this study are given in Table 1. These organisms were obtained from the collection of the Department of Tuberculosis, Hospital de la Santa Cruz and San Pablo, Barcelona, Spain.

Media and growth conditions. For isolation of lipid components, all strains were cultivated on plates of Middlebrook 7H10 agar and incubated at 35 to 37°C in 5% CO_2. Incubation temperature for *M. marinum* and *M. fallax* strains was 30°C. The incubation period ranged from 7 to 10 days for rapidly growing species and from 25 to 32 days for slowly growing species.

All strains were identified by their rate of growth, colonial morphology, pigmentation, and biochemical properties (20). All slowly growing species were tested for niacin, urease, semiquantitative and heat-stable catalases, nitrate and tellurite reduction, and Tween 80 hydrolysis. Rapidly growing mycobacteria were tested for 3-day arylsulfatase, tolerance to 5% NaCl on Löwenstein-Jensen medium, growth on

* Corresponding author.

TABLE 1. Strains examined in this study

Group	Organism	Reference[a]	No. of strains	
			Clinical	Other sources
Slowly growing mycobacteria	*M. tuberculosis*	ATCC 27294^T	47	—[b]
	M. bovis	ATCC 19201^T	11	6
	M. kansasii	ATCC 12478^T	19	2
	M. marinum	ATCC 927^T	1	2
	M. szulgai	NCTC 10831^T	—	4
	M. scrofulaceum	ATCC 19981^T	13	—
	M. gordonae	ATCC 14470^T	26	2
	M. xenopi	NCTC 10042^T	27	4
	M. simiae	ATCC 25270^T	2	5
	M. terrae	ATCC 15755^T	10	2
	M. triviale	ATCC 23292^T	8	3
	M. avium-M. intracellulare	ATCC 29548^T	21	5
	M. malmoense	ATCC 29575^T	—	1
Rapidly growing mycobacteria	*M. fortuitum*	ATCC 6841^T	36	2
	"*M. peregrinum*"	—	4	15
	M. chelonae subsp. *chelonae*	NCTC 946^T	8	8
	M. chelonae subsp. *abscessus*	ATCC 19977^T	5	4
	M. chelonae-like	—	3	15
	M. smegmatis	ATCC 19470^T	3	4
	M. porcinum	ATCC 37776^T	—	—
	M. chitae	ATCC 19727^T	—	—
	M. agri	ATCC 27406^T	—	—
	M. fallax	CIP 8139^T	—	13
	M. flavescens	ATCC 14974^T	1	2

[a] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures, London, England; CIP, Collection Nationale de Cultures de Microorganismes, Paris, France.
[b] —, None.

MacConkey agar, nitrate reduction, iron uptake, growth on sodium citrate, and use of mannitol and inositol as a sole source of carbon in the presence of amonniacal nitrogen. All of the tests listed above were conducted by standard methods (20, 25, 26).

Preparation of samples. A spadeful of bacteria (10 mg [wet weight]) was scraped from the surfaces of Middlebrook 7H10 agar plates. The mycobacterial lipids were extracted and derivatized to methyl esters by a modification of the method of Minnikin et al. (18). The cells were mixed with 1 ml of a reagent composed of 30 ml of methanol, 15 ml of toluene, and 1 ml of concentrated H_2SO_4 in a screw-cap test tube (14 by 120 mm) fitted with a Teflon-lined cap. The mixture was heated in a covered bath at 80°C for 16 h (overnight). After being cooled at room temperature, the samples were extracted twice with 2 ml of *n*-hexane. The hexane extracts were combined, transferred to another test tube, and mixed with an equal volume of 0.3 M phosphate buffer (42.57 g of Na_2HPO_4 and 12.0 g of NaOH per liter of distilled water [pH 11 to 12]) (4). The hexane upper layer was then removed, placed in a clean tube, and evaporated to dryness in a water bath at 40°C under a stream of nitrogen. The residue was dissolved in *n*-hexane.

TLC of mycolic acid methyl esters. The methyl ester extracts (2 μl) were spotted onto aluminum high-performance silica gel (0.2 mm thick) sheets (10 by 10 cm; HPTLC Alufolien Kiesegel 60 F_{254}; Merck). One-dimensional analysis was performed by using two different elution systems: *n*-hexane–ether (85:15 [vol/vol]) solvent A (18) and dichlorometane (solvent B) (8). The presence of separated components was revealed by spraying with 10% (wt/vol) molybdophosphoric acid in ethanol followed by charring.

CGC of MACP, cellular fatty acids, and alcohols. The fatty acid methyl esters, MACP, and 2-alcohols were analyzed on a fused-silica capillary column (15 m by 0.25 mm [inner diameter]) with cross-linked methyl silicone (SPB-1; Supelco, Inc., Bellefonte, Pa.) as the stationary phase; the column was inserted in a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector. The column was programmed to go from 175 to 300°C at 8°C /min and was maintained at 300°C for 15 min. The injector and detector temperatures were 275 and 315°C, respectively. The carrier gas was helium with a flow rate of approximately 1 ml/min; the sample size was 1 μl, with a split ratio of approximately 1:50. The chromatograms were integrated by using a Hewlett-Packard 3390A electronic integrator.

The lipid extracts obtained with the type strains of *M. tuberculosis* (ATCC 27294), *M. xenopi* (NCTC 10042), *M. kansasii* (ATCC 12478), and *M. fortuitum* (ATCC 6841) were analyzed at different injector temperatures of the gas chromatograph (250, 275, 300, and 350°C). One part of the sample was transferred to a lyophilization vial and evaporated just to dryness with nitrogen. The vial was evacuated, sealed, placed in a muffle furnace, and heated at 350°C for 3 h. After heating, the vial was cooled at room temperature and opened. The vial was then rinsed with hexane. The hexane washes were concentrated to 0.2 ml and analyzed by CGC under the same conditions as those for the unheated samples.

Identities of gas chromatographic peaks. The peaks were identified by comparing retention times with authentic methyl esters and alcohol standards (Supelco, Inc., and Sigma Chemical Co., St. Louis, Mo.) The identities of the alcohols were confirmed by trifluoroacetylation (10). The identities of all compounds were also confirmed by mass spectrometry. A Hewlett-Packard model 5988A mass spec-

TABLE 2. Specific and characteristic lipids found in some mycobacteria

Microorganism	CGC[a]			Pattern of mycolic acids on TLC[b]
	Fatty acids	Alcohols	MACP	
M. tuberculosis complex	—	—	$C_{26:0}$	I, III, IV
M. kansasii	2-M $C_{14:0}$ 2,4-DM $C_{14:0}$	—	$C_{24:0}$	I, III, IV
M. marinum	10-M $C_{16:0}$ 2,4-DM $C_{16:0}$	—	$C_{24:0}$	I, III, IV
M. szulgai	2-M $C_{14:0}$ 10-M $C_{16:0}$ 2,4-DM $C_{20:0}$ 2,4,6-TM $C_{22:0}$	—	$C_{24:0}$	I, III, IV
M. simiae	—	—	$C_{26:0}$	I, II, IV
M. gordonae	2-M $C_{14:0}$	—	$C_{22:0}$, $C_{24:0}$	I, III, IV
M. scrofulaceum	—	2-OH $C_{18:0}$ 2-OH $C_{20:0}$	$C_{24:0}$	I, IV, VI
M. xenopi	—	2-OH $C_{20:0}$ 2-OH $C_{22:0}$	$C_{26:0}$	I, (IV), VI
M. avium-M. intracellulare	—	2-OH $C_{18:0}$ 2-OH $C_{20:0}$	$C_{24:0}$	I, IV, VI
M. terrae	—	2-OH $C_{18:0}$ 2-OH $C_{20:0}$	$C_{24:0}$	I, (IV), VI
M. triviale	—	—	$C_{24:0}$	I
M. malmoense	2-M $C_{20:0}$ 2,4,6-TM $C_{24:0}$	—	$C_{24:0}$	I, II, IV
M. fortuitum	—	—	$C_{24:0}$	I, (II), V
M. chelonae	—	—	$C_{24:0}$	I, II
M. smegmatis	—	—	$C_{24:0}$	I, II, V
M. fallax	—	—	$C_{22:0}$, $C_{24:0}$	I
M. agri	—	—	$C_{24:0}$	I, II, III
M. chelonae-like	—	2-OH $C_{18:0}$ 2-OH $C_{20:0}$	$C_{22:0}$, $C_{24:0}$	I, (IV), VI

[a] Numbers to the left of the colon indicate the number of carbon atoms; numbers to the right of the colon indicate the number of double bonds. 2-M, 2,4-DM, and 2,4,6-TM indicate methyl groups at the 2, 2 and 4, and 2, 4, and 6 positions, respectively. 2-OH indicates secondary alcohol. —, Not detected.

[b] I, α-Mycolates; II, α′-mycolates; III, methoxy-mycolates; IV, keto-mycolates; V, epoxy-mycolates; VI, wax-ester mycolates (ω-carboxymycolates and secondary alcohols). Parentheses indicate that only trace amounts were detected.

trometer equipped for both electron impact ionization and chemical ionization was used. The mass spectrometer was interfaced to a Hewlett-Packard 5890A gas chromatograph.

Numerical taxonomy analysis. Numerical classification was carried out as follows. The results of lipid analysis were recorded as + or −, and the matching coefficient (*M* value) was calculated by the following equation $M\ (\%) = (ns \times 100)$ $(ns + nd)$, where ns is the number of characters that gives the same results (++ or −−) between two strains, and nd is the number of characters that gives different results (+ or −). The program used is included in the packet of programs clustan 2 (Computing Laboratory, University of St. Andrews, Fife, Scotland). The results of the numerical classification are shown as a dendrogram, which was prepared by the single-linkage method.

RESULTS

TLC of mycolic acid methyl esters. The patterns of mycolic acid methyl esters of each species studied are shown in Table 2. The results of mycolic acid compositions obtained in this study are in general agreement with previously reported data (1, 13, 18, 19, 24). As was expected, the mycolic acid patterns were complex, with several spots whose R_f values varied as a result of their different structures. As described Daffé et al. (2) and Minnikin et al. (18, 19), acid methanolysis cause the breakdown of epoxy-mycolates, and characteristic pairs of polar mycolates were observed in TLC plates. Because accurate differentiation between α′-mycolates and methoxy-mycolates is necessary, we used two developing systems, as recommended by Daffé et al. (1); α′-mycolates and methoxy-mycolates showed similar R_f values in solvent A and both migrated just below the α-mycolates. However, in dichloromethane (solvent B), methoxy-mycolates migrated below the α′-mycolates and their R_f values were similar to those of keto-mycolates (Fig. 1).

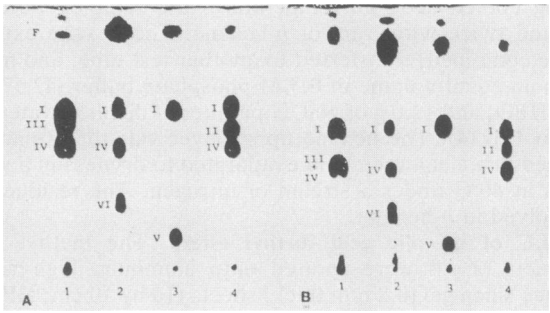

FIG. 1. Thin-layer chromatograms of methyl mycolates from *M. tuberculosis* ATCC 272294^T (lanes 1), *M. intracellulare* ATCC 29548^T (lanes 2), *M. fortuitum* ATCC 6841^T (lanes 3), and *M. simiae* ATCC 25270^T (lanes 4). The analysis was performed with two different elution systems: *n*-hexane–ether (85:15 [vol/vol]) for two runs (A) and dichloromethane (B). See footnote *b* of Table 2 for an explanation of mycolate designations I to VI.

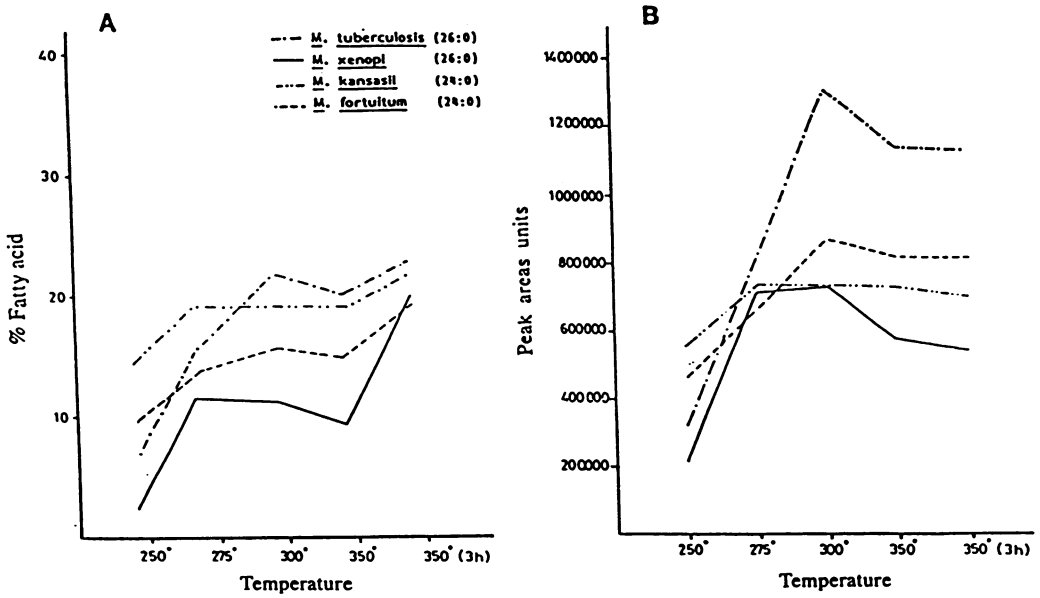

FIG. 2. Variations of values corresponding to relative percentages (A) and peak areas (B) of methyl esters of tetracosanoic ($C_{24:0}$) and hexacosanoic ($C_{26:0}$) acids, depending on injector temperature.

CGC. The $C_{22:0}$, $C_{24:0}$, and $C_{26:0}$ fatty acids are formed as methyl esters in situ upon pyrolysis of the complex branched-chain mycolic acid structures in the heated injector of the gas chromatograph. We therefore decided to study the influence of injector temperature in obtaining these compounds. Results are shown in Fig. 2. The relative quantity of pyrolysis esters increased significantly when the samples were analyzed at an injector temperature above 250°C, and the maximum yield was reached at between 275 and 300°C. No significant increases were recorded on increasing injector temperature or heating time of the sample. From these results, the injector temperature of 275°C was chosen for chromatographic analysis.

The average percentage of constituent fatty acids, alcohols, and MACP found in strains of the species examined are listed in Table 3.

The major cellular fatty acids in all species studied were hexadecanoic ($C_{16:0}$), octadecenoic ($C_{18:1}$), and octadecanoic ($C_{18:0}$) acids. Tuberculostearic acid (TBSA, 10-methyloctadecanoic acid) was detected in all species tested except *M. gordonae*. Trace to small percentages of tetradecanoic ($C_{14:0}$), pentadecanoic ($C_{15:0}$), hexadecenoic ($C_{16:1}$), and heptadecanoic ($C_{17:0}$) acids were detected in some species, although these compounds were not useful in discriminating between mycobacterial species. The secondary alcohols, mono- and polymethyl branched-chain fatty acids, and MACP presented a discontinuous and characteristic distribution for the mycobacterial species tested (Table 2 and Fig. 3).

***M. tuberculosis* complex.** The strains of *M. tuberculosis* and *M. bovis* presented by TLC a pattern of α-, methoxy-, and keto-mycolates. Characteristic fatty acids or secondary alcohols were not observed by CGC. Hexacosanoic acid ($C_{26:0}$) was the primary MACP of all the strains tested.

Slowly growing photochromogenic mycobacteria. Patterns composed of α-, methoxy-, and keto-mycolates were found in *M. kansasii*, *M. marinum*, and *M. szulgai*. These mycobacterial species contained the following characteristic methyl branched-chain fatty acids: 2,4-dimethyltetradecanoic (2,4-DM-$C_{14:0}$), 2,4-dimethylhexadecanoic (2,4-DM-$C_{16:0}$), 2,4-dimethyleicosanoic (2,4-DM-$C_{20:0}$), or 2,4,6-trimethyldocosanoic (2,4,6-TM-$C_{22:0}$) Tables 2 and 3 and Fig. 3). *M. simiae* had a very characteristic pattern of α-, α′-, and keto-mycolates, and the primary MACP was $C_{26:0}$.

Slowly growing scotochromogenic mycobacteria. *M. gordonae* was the only species of this group with a pattern of α-, methoxy-, and keto-mycolates. Furthermore, *M. gordonae* was the only mycobacterial species tested in which TBSA was not detected and contained characteristic 2-methyltetradecanoic acid (2-M-$C_{14:0}$). *M. kansasii* and *M. szulgai* showed only trace amounts of 2-M-$C_{14:0}$, which was present in *M. gordonae* in amounts ranging from 3 to 5% (Table 2).

The strains of *M. scrofulaceum* showed a TLC pattern of α-, keto-, and wax-ester mycolates; two secondary alcohols, 2-octadecanol (2-OH-$C_{18:0}$), and 2-eicosanol (2-OH-$C_{20:0}$) were detected in gas chromatograms of this species. *M. xenopi* strains contained α-, keto-, and wax-ester mycolates and showed a gas chromatographic profile with two peaks corresponding to secondary alcohols, 2-OH-$C_{20:0}$ and 2-docosanol (2-OH-$C_{22:0}$). The last alcohol was not detected in any other mycobacterial species. The primary MACP of *M. xenopi* was $C_{26:0}$.

Slowly growing nonchromogenic mycobacteria. The strains of *M. avium-M. intracellulare* and *M. terrae* showed a TLC pattern of α-, keto-, and wax-ester mycolates. Additionally, the strains of *M. terrae* contained small amounts of keto-mycolates. All strains of these two species contained 2-OH-$C_{18:0}$ and 2-OH-$C_{20:0}$, and the primary MACP was $C_{24:0}$. *M. triviale* strains presented a characteristic pattern of only α-mycolates, which is very useful in differentiating this species from all other slowly growing nonchromogenic mycobacteria. TLC analysis of *M. malmoense* showed α-, α′-, and keto-mycolates. Moreover, the two strains studied contained characteristic methyl branched-chain fatty acids, 2-methyleicosanoic acid (2-M-$C_{20:0}$), and 2,4,6-trimethyltetracosanoic acid (2,4,6-TM-$C_{24:0}$). These findings suggest that lipid analysis is a very useful approach in the species identification of *M. malmoense* (23).

TABLE 3. Fatty acids, alcohols, and MACP found in mycobacteria

Species	No. of strains	Total % ± SD of the following compounds[a]:											
		$C_{14:0}$	2-M-$C_{14:0}$	2,4-DM-$C_{14:0}$	$C_{15:0}$	$C_{16:1}$	$C_{16:0}$	10-M-$C_{16:0}$	2,4-DM-$C_{16:0}$	2-OH-$C_{18:0}$	$C_{17:0}$	$C_{18:1}$	$C_{18:0}$
M. tuberculosis	48	1.3 ± 0.8	ND	ND	0.5 ± 0.5	4.7 ± 1.1	28.2 ± 2.5	ND	ND	ND	1.7 ± 0.3	16.8 ± 3.5	8.4 ± 2.0
M. bovis	18	1.0 ± 0.5	ND	ND	1.0 ± 0.5	4.4 ± 1.1	26.3 ± 2.1	ND	ND	ND	2.5 ± 1.0	13.5 ± 1.5	8.2 ± 1.9
M. kansasii	22	2.0 ± 0.4	0.6 ± 0.2	3.4 ± 0.9	0.5 ± 0.6	7.2 ± 1.6	25.6 ± 1.5	ND	ND	ND	1.4 ± 0.9	20.4 ± 2.5	6.9 ± 2.2
M. marinum	4	1.8 ± 0.2	ND	ND	ND	7.2 ± 1.3	21.1 ± 1.6	0.5 ± 0.2	3.4 ± 0.8	ND	ND	29.2 ± 2.3	8.1 ± 1.4
M. simiae	8	5.3 ± 0.3	ND	ND	0.6 ± 0.2	4.1 ± 0.6	32.0 ± 0.9	ND	ND	ND	ND	18.5 ± 2.8	7.2 ± 2.9
M. szulgai	5	2.9 ± 0.4	1.5 ± 0.7	ND	ND	7.5 ± 0.9	34.1 ± 1.5	1.3 ± 0.3	ND	ND	0.7 ± 0.1	20.1 ± 1.9	6.8 ± 1.0
M. scrofulaceum	14	3.2 ± 0.5	ND	ND	0.5 ± 0.4	8.3 ± 2.3	23.9 ± 1.4	ND	ND	2.5 ± 1.6	ND	17.5 ± 2.4	4.4 ± 1.3
M. gordonae	29	6.7 ± 1.2	3.3 ± 1.2	ND	ND	10.0 ± 3.2	28.9 ± 2.1	ND	ND	ND	0.4 ± 0.7	28.1 ± 4.2	5.2 ± 2.2
M. xenopi	32	0.9 ± 0.4	ND	ND	ND	2.6 ± 1.6	27.7 ± 2.8	ND	ND	ND	0.5 ± 0.2	6.4 ± 3.3	4.6 ± 1.7
M. terrae	13	4.0 ± 1.2	ND	ND	0.4 ± 0.6	5.7 ± 1.8	21.6 ± 3.2	ND	ND	1.3 ± 0.9	ND	18.5 ± 2.8	7.2 ± 2.9
M. triviale	12	ND	ND	ND	1.3 ± 0.0	8.5 ± 1.3	25.8 ± 0.2	ND	ND	ND	1.6 ± 0.3	19.6 ± 3.0	4.3 ± 0.9
M. avium-M. intracellulare	27	3.6 ± 0.8	ND	ND	0.7 ± 0.4	8.0 ± 0.9	27.8 ± 1.2	ND	ND	ND	0.1 ± 0.1	14.7 ± 4.4	4.3 ± 2.0
M. malmoense	2	1.6 ± 0.4	ND	ND	1.6 ± 0.1	8.9 ± 0.9	19.8 ± 1.2	ND	ND	ND	0.6 ± 0.2	12.2 ± 1.9	2.6 ± 0.4
M. flavescens	4	2.6 ± 0.3	ND	ND	0.9 ± 0.2	10.5 ± 2.3	26.4 ± 2.1	ND	ND	2.2 ± 0.9	ND	14.6 ± 3.1	4.1 ± 0.2
M. fortuitum	39	4.1 ± 0.2	ND	ND	ND	6.6 ± 0.7	29.7 ± 0.7	ND	ND	ND	ND	19.0 ± 1.7	6.2 ± 0.0
"*M. peregrinum*"	19	4.7 ± 0.3	ND	ND	ND	6.4 ± 1.2	29.6 ± 0.6	ND	ND	ND	ND	15.9 ± 3.6	6.9 ± 1.2
M. chelonae subsp. *chelonae*	12	5.9 ± 0.3	ND	ND	ND	8.6 ± 1.4	27.2 ± 2.0	ND	ND	ND	ND	25.6 ± 1.2	3.5 ± 0.5
M. chelonae subsp. *abscessus*	5	9.0 ± 0.6	ND	ND	ND	2.6 ± 0.4	29.9 ± 1.9	ND	ND	ND	ND	22.1 ± 1.9	6.7 ± 0.9
M. smegmatis	8	3.6 ± 2.2	ND	ND	0.4 ± 0.4	10.1 ± 0.9	25.5 ± 1.8	ND	ND	ND	0.4 ± 0.3	20.1 ± 4.2	3.5 ± 0.9
M. porcinum	1	4.1	ND	ND	ND	5.5	27.6	ND	ND	ND	ND	17.9	6.3
M. chitae	1	4.3	ND	ND	ND	11.3	32.5	ND	ND	ND	ND	15.7	9.8
M. agri	1	4.7	ND	ND	ND	7.8	28.2	ND	ND	ND	ND	7.6	6.1
M. fallax	14	5.6 ± 1.2	ND	ND	1.1 ± 0.3	6.4 ± 1.3	31.9 ± 3.5	ND	ND	ND	1.8 ± 0.6	20.6 ± 2.7	9.8 ± 1.3
M. chelonae-like	18	5.2 ± 1.8	ND	ND	ND	9.6 ± 2.2	30.4 ± 2.9	ND	ND	3.8 ± 1.6	ND	22.2 ± 1.8	9.3 ± 2.7

[a] Values are mean ± standard deviation percentages of total acids. The number to the left of the colon indicates the number of carbon atoms; the number to the right of the colon indicates the number of double bonds. 2-M, 2,4-DM, and 2,4,6-TM indicate methyl groups at the 2, 2 and 4 and 2, 4, and 6 positions, respectively; 2-OH indicates secondary alcohol; TBSA, tuberculostearic acid (10-methyloctadecanoic acid), Un, unidentified compound; ND, not detected.

Continued on following page

TABLE 3—*Continued*

Total % ± SD of the following compounds[a]:													
TBSA	2-OH-$C_{20:0}$	$C_{20:0}$	2-M-$C_{20:0}$	2,4-DM-$C_{20:0}$	2-OH-$C_{22:0}$	Un	$C_{22:1}$	$C_{22:0}$	2,4,6-TM-$C_{22:0}$	$C_{24:1}$	$C_{24:0}$	2,4,6-TM-$C_{24:0}$	$C_{26:0}$
13.5 ± 3.1	ND	1.3 ± 0.4	ND	ND	ND	ND	ND	1.0 ± 1.1	ND	ND	3.9 ± 1.2	ND	15.9 ± 2.1
18.9 ± 1.9	ND	1.7 ± 0.6	ND	ND	ND	ND	ND	0.4 ± 0.3	ND	ND	4.1 ± 1.0	ND	12.7 ± 2.9
9.3 ± 2.7	ND	0.9 ± 0.3	ND	ND	ND	ND	ND	2.4 ± 0.6	ND	ND	14.9 ± 3.2	ND	ND
7.8 ± 2.2	ND	0.6 ± 0.1	ND	ND	ND	ND	ND	1.0 ± 0.5	ND	ND	11.2 ± 1.8	ND	1.3 ± 0.7
9.9 ± 3.6	7.5 ± 0.8	1.9 ± 2.2	ND	ND	ND	ND	ND	3.9 ± 0.7	ND	ND	3.8 ± 0.6	ND	11.9 ± 3.0
10.1 ± 0.7	ND	1.7 ± 0.9	ND	ND	ND	ND	ND	3.4 ± 1.3	3.3 ± 0.2	ND	10.7 ± 1.4	ND	ND
12.1 ± 2.0	5.6 ± 0.8	0.7 ± 0.5	ND	ND	ND	ND	ND	0.9 ± 0.7	ND	ND	9.8 ± 0.6	ND	ND
ND	ND	1.2 ± 0.2	ND	ND	ND	ND	ND	4.2 ± 0.8	ND	ND	4.4 ± 0.8	ND	ND
30.4 ± 2.6	1.7 ± 0.5	0.2 ± 0.3	ND	ND	8.9 ± 3.0	1.7 ± 0.7	ND	ND	ND	ND	0.3 ± 0.4	ND	11.1 ± 2.6
9.9 ± 3.6	7.5 ± 0.8	1.9 ± 2.2	ND	ND	ND	ND	ND	1.2 ± 1.0	ND	ND	12.3 ± 2.1	ND	ND
14.7 ± 1.0	ND	0.9 ± 0.2	ND	ND	ND	ND	ND	0.6 ± 0.5	ND	ND	14.4 ± 0.3	ND	ND
14.2 ± 2.2	4.3 ± 2.2	1.0 ± 0.6	ND	ND	ND	ND	ND	1.3 ± 0.9	ND	ND	7.2 ± 3.8	ND	ND
20.6 ± 3.5	ND	0.8 ± 0.0	3.1 ± 1.0	ND	ND	ND	ND	ND	ND	ND	3.0 ± 0.2	4.3 ± 1.4	14.9 ± 2.8
12.4 ± 0.8	6.6 ± 0.1	1.3 ± 0.6	ND	ND	ND	ND	ND	2.9 ± 1.1	ND	ND	10.8 ± 0.9	ND	ND
8.6 ± 0.3	ND	1.9 ± 0.2	ND	ND	ND	ND	ND	3.2 ± 0.2	ND	2.1 ± 0.1	17.4 ± 1.7	ND	ND
13.8 ± 5.8	ND	1.5 ± 0.4	ND	ND	ND	ND	0.9 ± 0.3	1.9 ± 0.8	ND	5.1 ± 1.6	9.7 ± 2.0	ND	ND
9.5 ± 1.9	ND	2.1 ± 0.8	ND	ND	ND	ND	0.6 ± 0.6	2.9 ± 0.5	ND	2.6 ± 1.3	8.3 ± 1.2	ND	ND
5.6 ± 1.8	ND	3.2 ± 0.4	ND	ND	ND	ND	ND	4.6 ± 1.2	ND	ND	14.8 ± 1.6	ND	ND
13.1 ± 3.4	ND	0.8 ± 0.6	ND	ND	ND	ND	ND	1.7 ± 0.8	ND	2.1 ± 0.6	13.9 ± 1.4	ND	ND
9.7	ND	2.0	ND	ND	ND	ND	ND	3.8	ND	1.1	21.1	ND	ND
11.4	ND	2.1	ND	ND	ND	ND	ND	2.5	ND	0.8	8.8	ND	ND
18.2	ND	3.0	ND	ND	ND	ND	0.7	3.0	ND	3.8	9.5	ND	ND
2.4 ± 1.4	ND	0.4 ± 0.5	ND	ND	ND	ND	0.8 ± 0.9	8.8 ± 2.3	ND	ND	3.7 ± 1.7	ND	ND
0.5 ± 0.3	2.1 ± 0.2	2.7 ± 0.7	ND	ND	ND	ND	1.2 ± 0.8	6.5 ± 1.7	ND	1.0 ± 0.6	5.4 ± 1.4	ND	ND

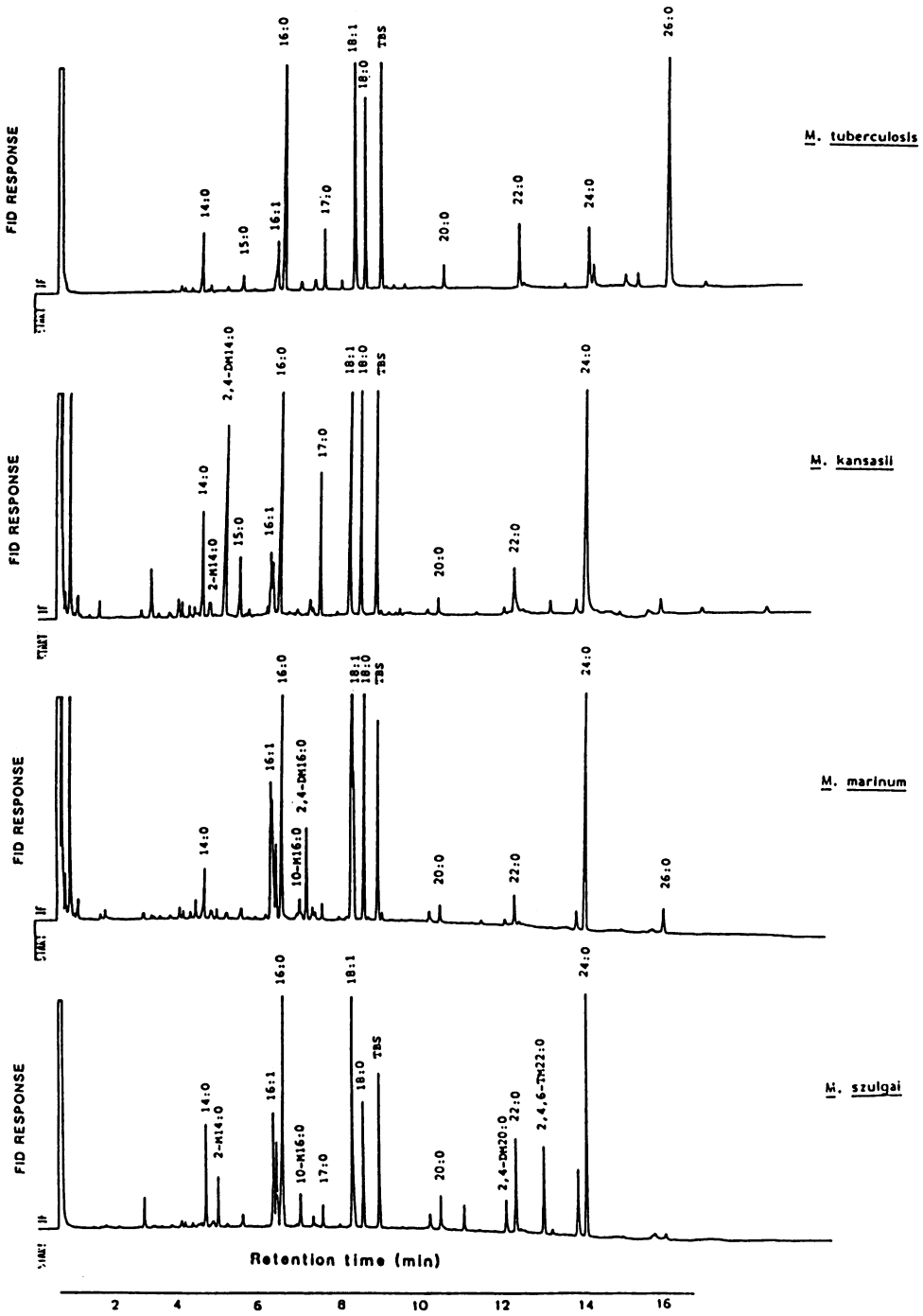

FIG. 3. Gas chromatograms of fatty acid methyl esters and MACP of some mycobacterial species. See text and footnote *a* of Table 2 for an explanation of acid designations. FID, Flame ionization detector; TBS, TBSA.

Rapidly growing mycobacteria. *M. flavescens*, like *M. avium-M. intracellulare* and *M. scrofulaceum*, showed a TLC pattern of α-, keto-, and wax-ester mycolates, containing 2-OH-$C_{18:0}$ and 2-OH-$C_{20:0}$, and the primary MACP was $C_{24:0}$. Patterns of α-, α′-, and epoxy-mycolates were characteristic of strains of *M. fortuitum*, "*M. peregrinum*," *M. smegmatis*, *M. porcinum*, and *M. chitae*. The presence of epoxy-mycolates was confirmed by degradation on acid methanolysis to characteristic polar mycolates (17). The mycolic acid compositions appeared to be sufficient for the definitive identification of *M. chelonae* and *M. agri* because these species present typical patterns that have not been found in other mycobacteria studied previously. As described previously (14), all strains of *M. fallax* exclusively formed α-mycolates; to date, the only mycobacterial species known to produce α-mycolates are *M. triviale*, a nonpathogenic, slowly growing, nonchromogenic mycobacterial species, and *M. fallax*. In this study, these eight rapidly growing

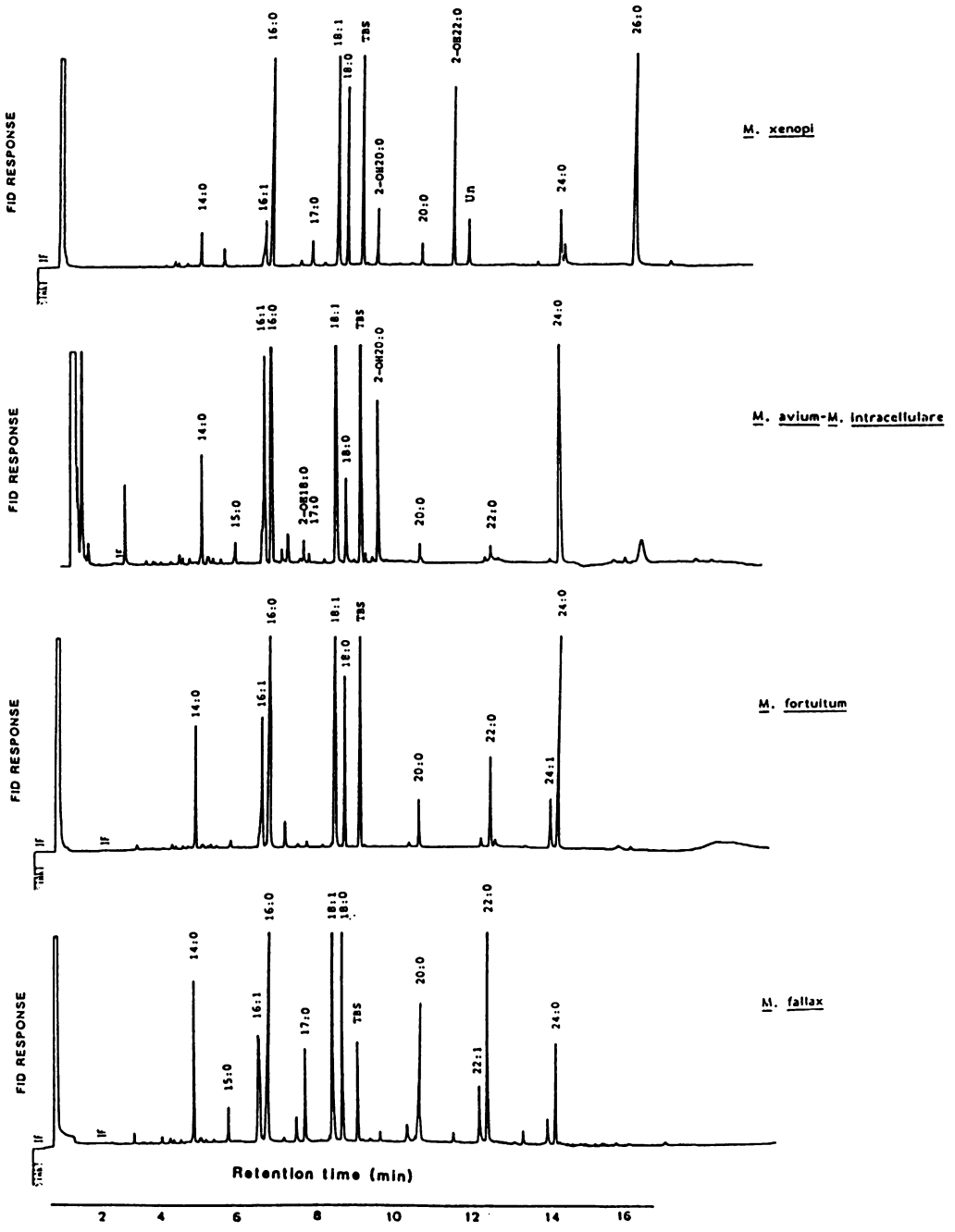

FIG. 3—*Continued*

nonchromogenic mycobacterial species did not contain any characteristic compound in gas chromatographic analysis. The *M. chelonae*-like strains could also be differentiated from all other rapidly growing nonchromogenic mycobacterial species because they contained α-, keto-, and wax-ester mycolates and two secondary alcohols, 2-OH-$C_{18:0}$ and 2-OH-$C_{20:0}$. The former was always present in relatively greater amounts.

Numerical analysis. The results of our numerical analysis according to the lipid content are shown as a dendrogram in Fig. 4. Four clusters were observed. The first cluster was composed of strains belonging to the species *M. tuberculosis*, *M. simiae*, *M. malmoense*, and *M. xenopi*; the level of similarity was 81%. All strains from rapidly growing nonchromogenic mycobacterial species, including *M. chelonae*-like strains, formed a second large cluster; the level of similarity was 79%. The third cluster was made up of *M. kansasii*, *M. marinum*, *M. szulgai*, and *M. gordonae*; the level of similarity was 88%. Finally, strains of *M. scrofulaceum*, *M. avium-M. intracellulare*, *M. terrae*, and *M. flavescens* were grouped into a fourth cluster; the level of similarity was 88%.

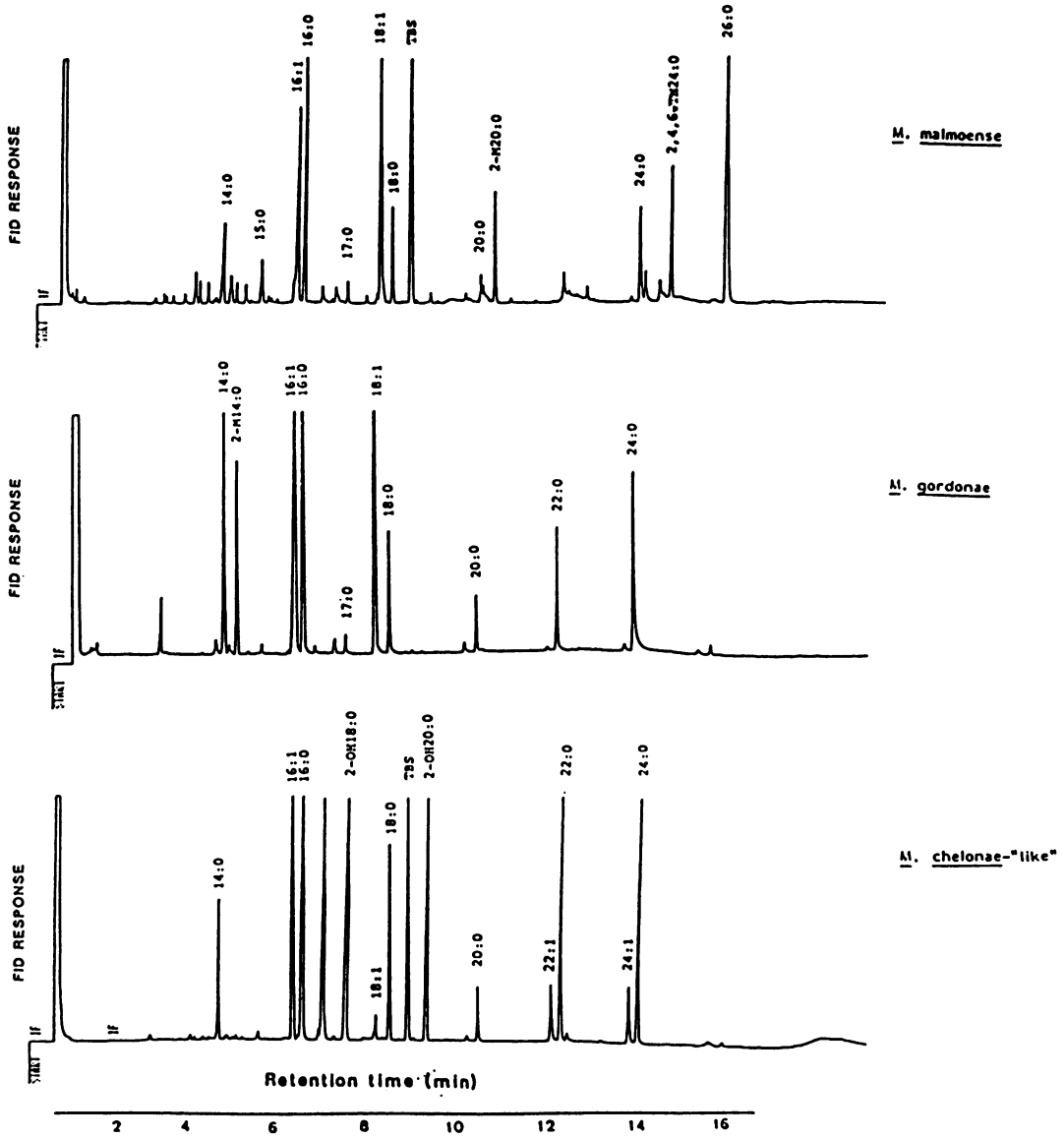

FIG. 3—*Continued*

DISCUSSION

Lipid analysis has provided useful in the classification and identification of mycobacteria (1, 3, 16, 17). Differences within some structural types of lipids provide a tool to distinguish members of the genus *Mycobacterium* from related taxa and to differentiate species within this genus (14). Several reports (4, 6, 7, 9, 10, 15, 24) have emphasized the potential value of gas chromatographic analysis as a diagnostic key to identification of mycobacteria to the species level. Moreover, the mycolic acid pattern from whole acid or alkaline methanolysates can be used as a criterion to separate *Mycobacterium* species from allied bacteria and to characterize mycobacterial species (1, 3, 18, 19). Despite the clear advantages of lipid analysis, chromatographic techniques for identification of mycobacterial species have been used in very few clinical laboratories. We have used combined TLC and CGC to identify clinical isolates of mycobacteria as routine identification techniques for several years. This was possible because of the standardization and adaptation of these chromatographic techniques to the needs of a clinical laboratory. This adaptation is fundamentally based on the choice of single but reliable reproducible procedures. Thus, with only 10 mg of bacterial mass, whole-acid methanolysates provide the lipid extract necessary to study the mycolic acid pattern by TLC and the fatty acids profile by CGC.

TLC has proven to be an easily standardized technique (1, 18). High-performance TLC sheets allowed a better resolution of the different types of mycolic acids than conventional silica gel plates did. In comparison with TLC, CGC requires a more careful standardization of working conditions if a reproducible profile of cellular fatty acids, secondary alcohols, and MACP is required. It is essential to choose an adequate chromatographic column. Apolar capillary columns are the most useful for separating long-chain fatty acids and alcohols.

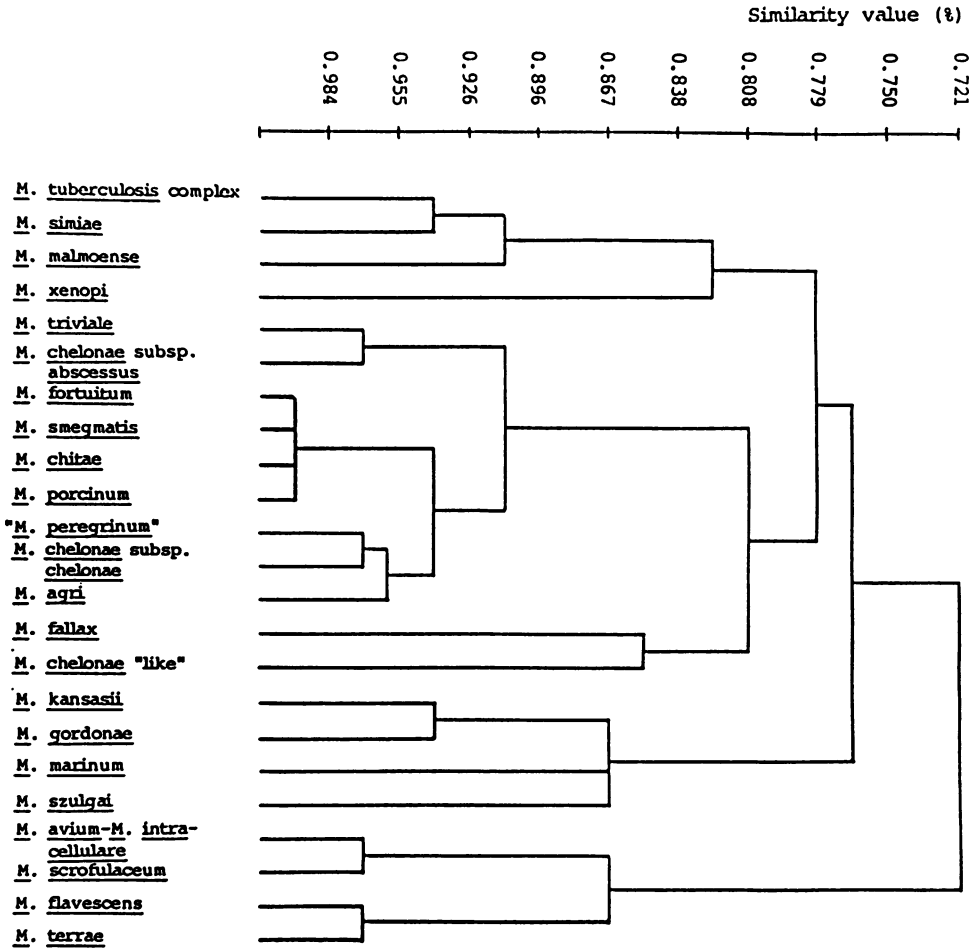

FIG. 4. Dendrogram showing the relationship of the 366 mycobacterial strains according to data obtained from lipid composition.

As seen from our results, secondary alcohols and MACP are excellent markers for the identification of mycobacterial species. The relative amounts of these compounds varied considerably from one laboratory to another (4, 10, 19). Tisdall et al. (21) did not find secondary alcohols in any of the 18 mycobacterial species they studied. Larsson (8) demonstrated that traces of nonmethylated free fatty acids in the samples injected led to absorption of alcohols in the chromatographic column. Therefore, these compounds were neither eluted nor detected. This may be avoided by a final washing of the lipid extracts in a buffered alkaline solution (8). Nevertheless, after some time, and still using a final washing with a buffered alkaline solution, we observed that the area of the chromatographic peaks corresponding to alcohols decreased and the base widened. After cleaning the column with an adequate organic solvent, we again recovered heights and resolutions of the chromatographic peaks corresponding to those of secondary alcohols. This finding demonstrates the importance of working with bonded-phase capillary columns, which allow this type of washing to be carried out.

The ability to obtain MACP, as well as alcohols, varies greatly from laboratory to laboratory (4, 7, 9). Results of the present study thus reconfirm the need to work at injector temperatures of between 275 and 300°C in order to obtain these compounds. Working at these temperatures allows us to obtain the fatty acid profile and MACP in a single chromatogram, thus eliminating the need to perform two injections of the samples, one at a low temperature and the other at a high temperature. In contrast to Lambert et al. (7), we did not observe any significant increase of these compounds when the samples were heated above 300°C, nor when the samples were heated to 350°C for several hours in a previously evacuated lyophilization vial. Although the relative percentage of MACP increased with respect to the total amount of compounds that were chromatographed, this increase was due to decreases in the amounts of other methyl esters, mainly those corresponding to unsaturated forms (Fig. 2).

In the present study, the presence of characteristic and specific chromatographic peaks was very reproducible; only slight variations in retention times and minor peak fluctuations were occasionally noted. We were able to demonstrate that analysis of mycolic acid patterns by TLC allows the mycobacteria to be classified into several groups according to their content of these compounds. This technique also facilitates direct identification of *M. chelonae* and *M. agri*. Furthermore, the CGC profile of fatty acids, secondary alcohols, and MACP allows direct identification of all those mycobacterial species containing specific lipids (*M. kansasii*, *M. marinum*, *M. szulgai*, *M. xenopi*, and *M. malmoense*). CGC is also useful for identifying *M. gordonae*, the

only mycobacterial species which does not form TBSA (5, 23). It is interesting that the combined use of the two chromatographic techniques may identify other mycobacterial species such as the *M. tuberculosis* complex, *M. simiae*, *M. fallax*, *M. triviale*, and *M. chelonae*-like organisms. For the other mycobacterial species, a minimal set of tests may be sufficient to obtain a precise identification once the chromatographic lipid profile has been determined. Routine use of TLC and CGC for the identification of clinical isolates of mycobacteria is attractive, in that these techniques eliminate several time-consuming and laborious biochemical tests.

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