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Systematic Study of the 3-Hydroxy Fatty Acid Composition of Mycobacteria

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Twenty-seven strains belonging to 12 *Mycobacterium* species were studied for 3-hydroxy fatty acid composition. Mycobacterial cells were subjected to both mild and strong acid methanolysis, after which the liberated hydroxy fatty acids were purified and analyzed by gas chromatography-mass spectrometry as methyl ester trimethylsilyl ether derivatives. Altogether, 21 3-hydroxy fatty acids containing 14 to 28 carbon atoms were detected; 10 were straight chain, 6 were 2-methyl branched chain, and 5 were 2,4,6-trimethyl branched chain. The mycobacterial strains were classified in groups according to 3-hydroxy fatty acid patterns.

Mycobacterial cell walls have been shown to contain type-specific antigenic glycolipids, such as phenolic glycolipids, glycopeptidolipids (GPL), and trehalose-containing lipooligosaccharides (LOS) (4, 5). Long-chain 3-hydroxy fatty acids (3-OH-FAs) have been identified in several glycolipids, for example, in GPL from *Mycobacterium peregrinum* and *M. smegmatis* (7, 12), in LOS from *M. szulgai* and *M. tuberculosis* Canetti (9, 10), and in acyltrehaloses from *M. tuberculosis* Canetti and H37Rv (3, 6). Furthermore, 3-OH-FAs have been detected in other bioactive mycobacterial structures, i.e., in ornithine-amide lipids and in lipopeptides in rough mutants of *M. avium* (2, 11). The present work was undertaken to systematically study the 3-OH-FA composition of several clinically important mycobacteria.

MATERIALS AND METHODS

Chemicals. *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Fluka Chemica (Buchs, Switzerland), diethyl ether and dichloromethane were from Janssen Chemica (Beerse, Belgium), pyridine and acetylchloride were from Merck (Darmstadt, Germany), 3-hydroxytridecanoic acid (3-OH $C_{13:0}$) was from Larodan Lipids (Malmö, Sweden), and the solvents *n*-hexane and methanol were from Lab-Scan (Dublin, Ireland).

Mycobacteria. Altogether, 27 strains belonging to 12 *Mycobacterium* species were analyzed. Twenty-five of the strains were grown at 37 or 33°C (*M. marinum*) in Sauton medium, and two (*M. peregrinum* and *M. chelonae*) were grown in Dubos medium (Table 1). The cultures were autoclaved, harvested by centrifugation, washed twice with sterile distilled water, and freeze-dried.

Methanolysis and derivatization. Portions (8 mg) of dried mycobacterial cells were divided into two equal parts; one was subjected to mild methanolysis, i.e., heating in 2 M methanolic HCl (60°C, 2 h), and the other was subjected to strong methanolysis, i.e., heating in 4 M methanolic HCl (100°C, 18 h). After cooling, 50 ng of 3-OH $C_{13:0}$ methyl ester was added as an internal standard, and the reaction mixture was extracted with 1 ml of hexane-water (1.5:1, vol/vol). The hexane phase was collected, and the lower phase was subjected to a second hexane extraction. The two hexane phases were then combined, dried, and redissolved in 1 ml of hexane-dichloromethane (1:1, vol/vol). Hydroxylated and nonhydroxylated fatty acid methyl esters were separated by using a disposable silica gel column (1 ml; Bond-Elut; Analytichem, Harbour City, Calif.). Prior to use, the column was washed with 1 ml of diethyl ether and then with 1 ml of hexane-dichloromethane (1:1, vol/vol). The methyl ester preparation was applied to the column, and two 1-ml volumes of hexane-dichloromethane (1:1, vol/vol) were added; the eluate was discarded. The column was then washed with two 1-ml volumes of diethyl ether to collect the 3-OH-FA methyl esters, and the preparations were dried. Trimethylsilyl (TMS) derivatives were prepared by adding 50 μl of BSTFA and 5 μl of pyridine to the sample and then heating at 80°C for 15 min. After cooling to room temperature, excess pyridine was removed from the reaction mixture under a stream of nitrogen, after which 50 μl of hexane was added. A sample volume of 1 μl was injected into a gas chromatograph (GC) mass spectrometer (MS).

GC-MS. A VG Trio-1 S GC-MS system (Manchester, England) was used. The GC was a Hewlett-Packard model 5890 (Avondale, Pa.) equipped with a fused-silica capillary column (25 m by 0.25 mm [inner diameter]) containing cross-linked OV-1 as the stationary phase. Splitless injections were made with a Hewlett-Packard model 7673 autosampler. The carrier gas, helium, was used at an inlet pressure of 7 lb/in^2. The temperature of the column was programmed to rise from 120 to 260°C at 20°C/min; both the injector and the interface (between GC and MS) temperatures were kept at 260°C. The ionization was performed at 70 eV. The temperature of the ion source was 220°C in the electron impact (EI) mode and 200°C in the positive ion-chemical ionization (PCI) mode (with ammonia as the reagent gas).

RESULTS

Mass spectra. Straight-chain 3-OH-FAs ranging from 14 to 28 carbon atoms were identified. Their methyl ester TMS ether (Me/TMS) derivatives gave EI mass spectra showing the characteristic fragment at *m/z* 175 (cleavage between C_3 and C_4) and the molecular-specific ions *m/z* M-15 (loss of CH_3). Branched-chain 2-methyl (2-Me) and 2,4,6-trimethyl (2,4,6-tri-Me) 3-OH-FAs with 19 to 28 carbon atoms were also detected; their EI spectra (as Me/TMS derivatives) showed prominent ions at *m/z* 189 (cleavage between C_3 and C_4) and *m/z* M-15

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TABLE 1. Mycobacterial strains analyzed

Species	Strain[a]	Origin	Incubation time (days)	3-OH-FA group
M. tuberculosis	CIPT 140010001 (H37Rv)	CIPT	49	A
	Clinical isolate	Sputum, non-AIDS, Sweden	41	A
	TMC 201 (H37Ra)	TMC	40	C
M. kansasii	CIPT 140110002	CIPT	76	B
	2 clinical isolates	Bronchial aspirate, non-AIDS, Belgium	48	B
M. avium	CIPT 140310005	CIPT	65	C
	Clinical isolate	Lymph node, non-AIDS, Belgium	48	C
M. marinum	ATCC 927	ATCC	41	C
	2 clinical isolates	Skin biopsy, non-AIDS, Belgium	48	C
M. xenopi	NCTC 10042	NCTC	85	C
	Clinical isolate	Sputum, non-AIDS, Belgium	52	C
M. simiae	OES 90145	OES	28	C
	Clinical isolate	Sputum, AIDS, Belgium	41	C
	Clinical isolate	Lymph node, armadillo, Louisiana, Belgium	41	C
M. intracellulare	Clinical isolate	Sputum, AIDS, Australia	52	C
M. fortuitum	Clinical isolate	Spleen, seagull, Belgium	76	C
M. peregrinum	Clinical isolate	Sputum, non-AIDS, Belgium	52	D
	Clinical isolate[b]	Sputum, non-AIDS, Belgium	19	D
M. chelonae	Clinical isolate	Sputum, non-AIDS, Belgium	34	D
	Clinical isolate	Pleural fluid, Burundi	19	D
	Clinical isolate[b]	Pleural fluid, Burundi	19	D
M. malmoense	Clinical isolate	Sputum, U.K.	65	E
	Clinical isolate	Sputum, non-AIDS, Belgium	52	E
M. scrofulaceum	Clinical isolate	Bronchial aspirate, non-AIDS, Belgium	76	F
M. gordonae	Clinical isolate	Sputum, non-AIDS, Belgium	76	F

[a] All strains were grown in Sauton medium except as noted. CIPT, Collection Institut Pasteur Tuberculose; ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; TMC, Trudeau Mycobacterial Culture Collection; OES, open-ended study (16).
[b] Grown in Dubos medium.

(loss of CH_3). In addition, 3-OH-2-Me Me/TMS derivatives exhibited a prominent ion at *m/z* M-87 that was the result of cleavage between C_2 and C_3.

Some examples of EI spectra are shown in Fig. 1. Prominent ions were seen as follows: *m/z* 175 and *m/z* 483 for 3-OH $C_{26:0}$, *m/z* 189, *m/z* 341, and *m/z* 413 for 3-OH-2-Me $C_{20:0}$, and *m/z* 189 and *m/z* 497 for 3-OH-2,4,6-tri-Me $C_{24:0}$.

The identities of the 3-OH-FA derivatives were confirmed by PCI. The PCI spectra showed an abundant molecular-specific ion at *m/z* M+1. In addition, the ion of *m/z* M+18 was also noticed for both straight-chain and 2-Me 3-OH-FAs. Thus, the PCI spectrum of 3-OH $C_{26:0}$ showed ions of *m/z* 499 and *m/z* 516, the spectrum of 3-OH-2-Me $C_{20:0}$ showed ions of *m/z* 429 and *m/z* 446, and the spectrum of 3-OH-2,4,6-tri-Me $C_{24:0}$ showed the ion *m/z* 513 (Fig. 2).

Figure 3 shows examples of mass chromatograms obtained by monitoring the ion at *m/z* 175 for the detection of straight-chain 3-OH-FAs of *M. chelonae* and the ion at *m/z* 189 for detection of 3-OH-2-Me and 3-OH-2,4,6-tri-Me FAs of *M. gordonae* and *M. tuberculosis* H37Rv, respectively.

Distribution of 3-OH-FAs in the mycobacteria. Mycobacteria were assigned to different groups according to the 3-OH-FA patterns that they exhibited (Table 1 and 2). Group A includes only *M. tuberculosis* (H37Rv and a clinical isolate), which contains straight-chain 3-OH-FAs with 14 to 26 carbon atoms and branched-chain 3-OH-FAs with 24 to 28 carbon atoms. Likewise, group B is represented by only one species, *M. kansasii* (CIPT 140110002 and clinical isolates), in which only small amounts of straight-chain 3-OH-FAs up to 24 carbon atoms were found. Straight-chain 3-OH-FAs with 14 to 26 carbon atoms are characteristic of the mycobacteria in group C, i.e., *M. avium* (CIPT 140310005 and a clinical isolate), *M. marinum* (ATCC 927 and clinical isolates), *M. xenopi* (NCTC 10042 and a clinical isolate), *M. simiae* (OES 90145

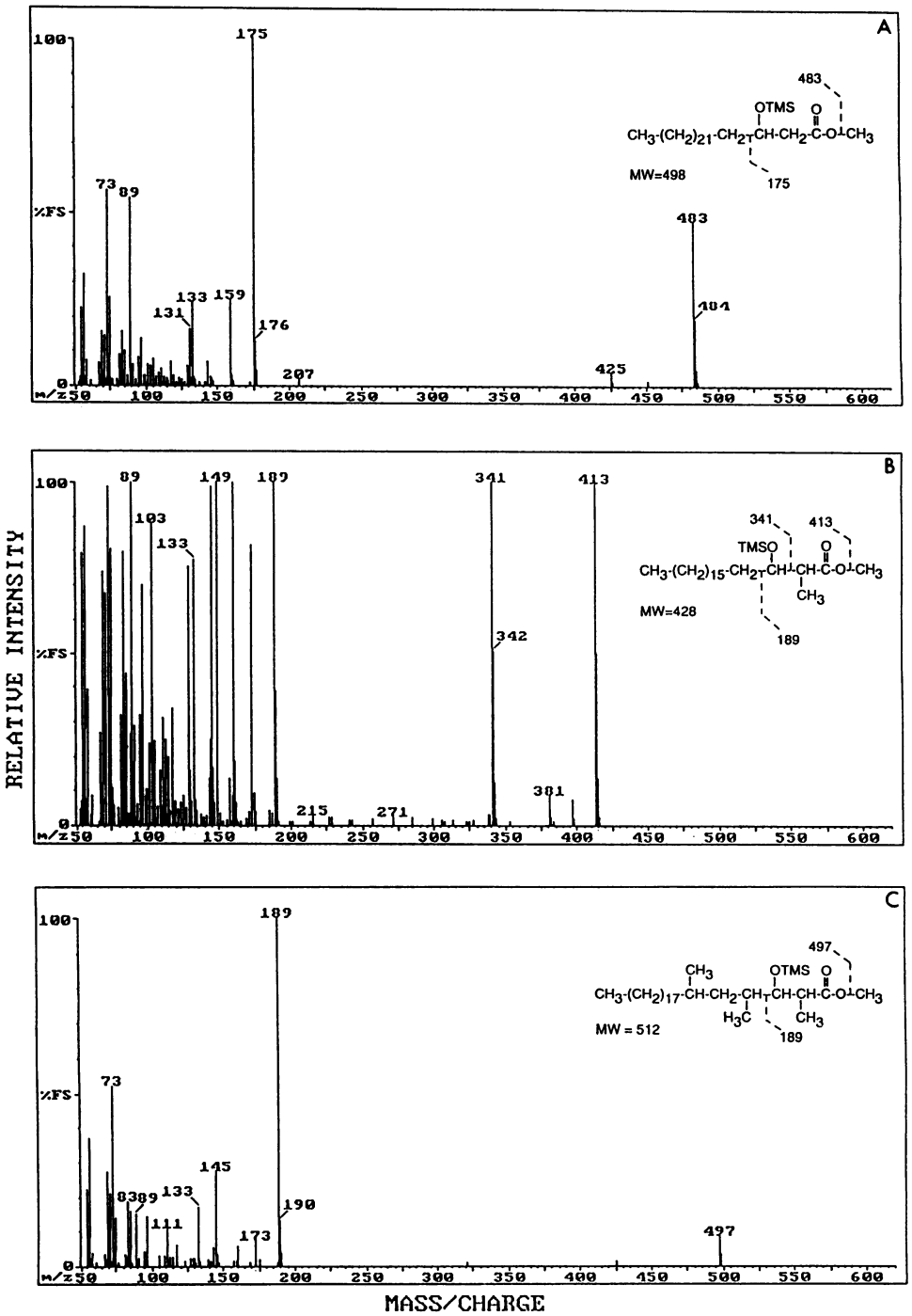

FIG. 1. EI mass spectra of the Me/TMS derivative of 3-OH $C_{26:0}$ in *M. chelonae* (A), 3-OH-2-Me $C_{20:0}$ in *M. gordonae* (B), and 3-OH-2,4,6-tri-Me $C_{24:0}$ in *M. tuberculosis* H37Rv (C).

and clinical isolates), *M. intracellulare* (clinical isolate), *M. tuberculosis* (strain H37Ra) and *M. fortuitum* (bird isolate). The species in group D, i.e., *M. peregrinum* (clinical isolates) and *M. chelonae* (clinical isolates), were found to contain straight-chain 3-OH-FAs with 14 to 28 carbon atoms; relatively large amounts of these fatty acids were long chain, i.e., had 26 to 28 carbon atoms. Group E includes only *M. malmoense* (clinical isolates), which is characterized by 3-OH-FAs with up to 26 carbon atoms and 3-OH-2-Me $C_{20:0}$. Finally, group F is comprised of *M. scrofulaceum* (clinical isolate) and *M. gordonae* (clinical isolate), which contain 3-OH-FAs with up to 26 carbon atoms and several monomethyl branched-chain 3-OH-FAs with 19 to 23 carbon atoms.

Influence of culture medium and methanolysis conditions. Use of Sauton or Dubos medium for cultivation did not influence the 3-OH-FA patterns of the *M. peregrinum* and *M.*

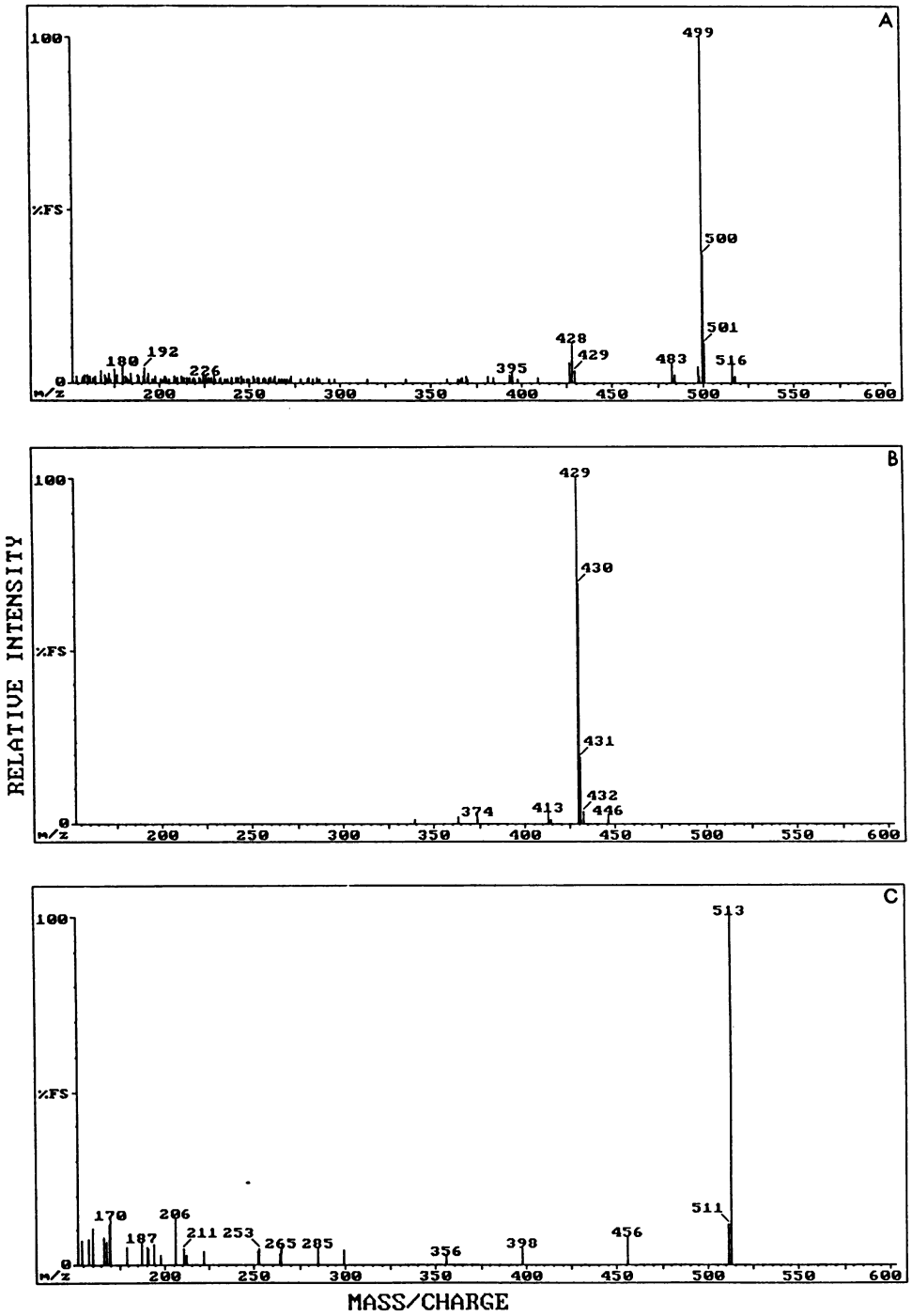

FIG. 2. PCI mass spectra of the Me/TMS derivative of 3-OH $C_{26:0}$ in *M. chelonae* (A), 3-OH-2-Me $C_{20:0}$ in *M. gordonae* (B), and 3-OH-2,4,6-tri-Me $C_{24:0}$ in *M. tuberculosis* H37Rv (C).

chelonae strains. However, with strong and mild acid methanolysis, the 3-OH-FA patterns differed markedly. Since it is known that the stronger methanolysis releases both ester- and amide-linked fatty acids and the milder methanolysis releases only ester-linked acids (13), we conclude that both of these types of linkages exist in mycobacteria. For example, in group F mycobacteria, similar amounts of 3-OH-2-Me FAs with 19 to 23 carbon atoms were detected after strong and mild methanolysis, indicating that they are mostly ester linked. However, in mycobacteria belonging to group D, the long-chain 3-OH-FAs (i.e., those with 26 to 28 carbon atoms) appear to be mainly amide linked because much larger amounts of these acids were found after stronger methanolysis (Table 2). As examples, mass chromatograms showing straight-chain 3-OH-FAs in *M. peregrinum* after both types of methanolysis are shown (Fig. 4).

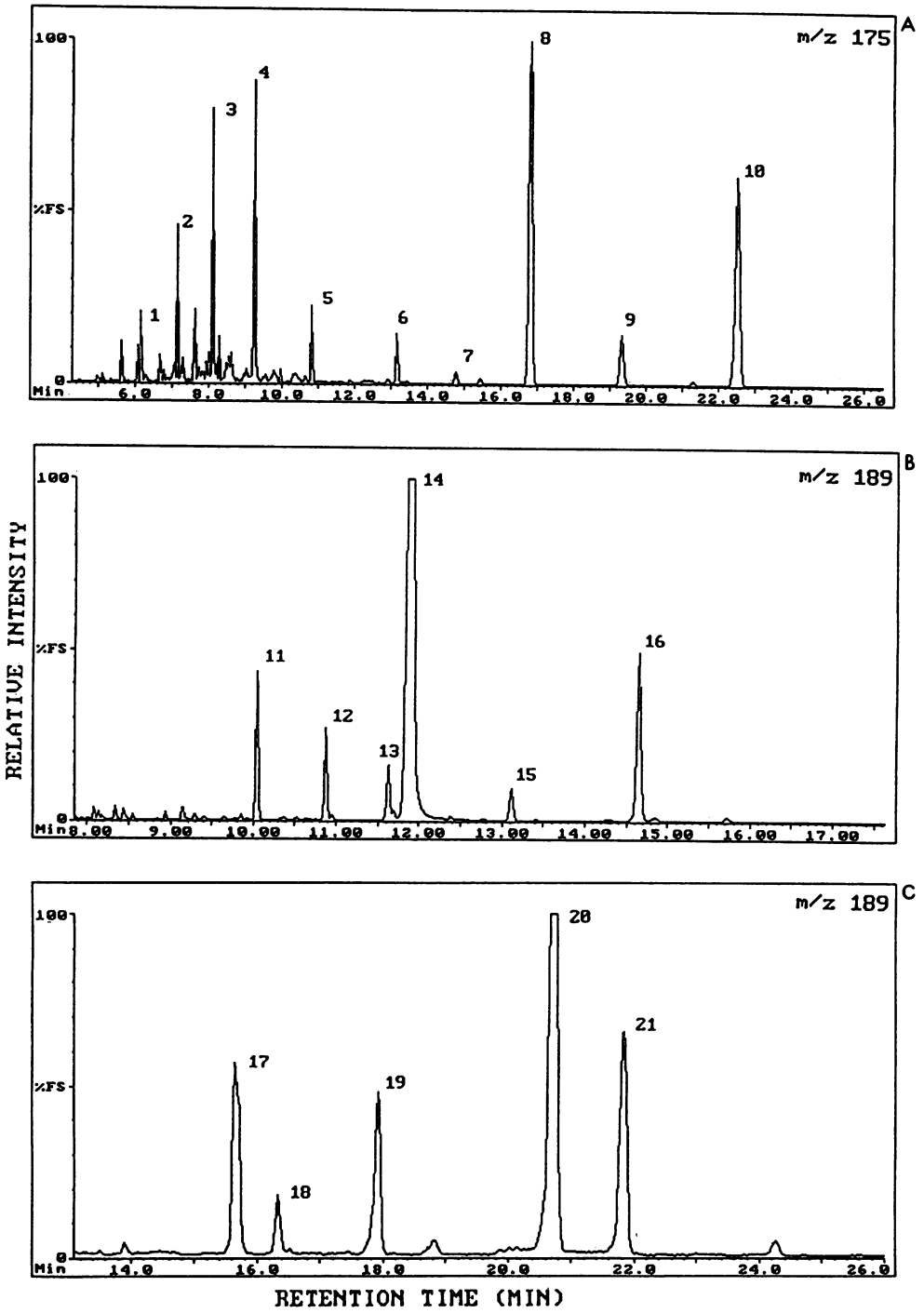

FIG. 3. EI mass chromatograms of Me/TMS-derivatized straight-chain 3-OH-FAs in *M. chelonae* (A), monomethyl branched-chain 3-OH-FAs in *M. gordonae* (B), and trimethyl branched-chain 3-OH-FAs in *M. tuberculosis* H37Rv (C). Peak identities: 1, 3-OH $C_{14:0}$; 2, 3-OH $C_{16:0}$; 3, 3-OH $C_{18:0}$; 4, 3-OH $C_{20:0}$; 5, 3-OH $C_{22:0}$; 6, 3-OH $C_{24:0}$; 7, 3-OH $C_{25:0}$; 8, 3-OH $C_{26:0}$; 9, 3-OH $C_{27:0}$; 10, 3-OH $C_{28:0}$; 11, 3-OH-2-Me $C_{18:0}$; 12, 3-OH-2-Me $C_{19:0}$; 13, 3-OH-2-Me $C_{20:1}$; 14, 3-OH-2-Me $C_{20:0}$; 15, 3-OH-2-Me $C_{21:0}$; 16, 3-OH-2-Me $C_{22:0}$; 17, 3-OH-2,4,6-tri-Me $C_{21:0}$; 18, 3-OH-2,4,6-tri-Me $C_{22:0}$; 19, 3-OH-2,4,6-tri-Me $C_{23:0}$; 20, 3-OH-2,4,6-tri-Me $C_{24:0}$; 21, 3-OH-2,4,6-tri-Me $C_{25:0}$.

DISCUSSION

It has previously been demonstrated that several 3-OH-FAs in mycobacteria are constituents of glycolipids and other bioactive moieties. Lanéelle et al. identified 3-OH $C_{18:0}$, 3-OH $C_{20:0}$, and 3-OH $C_{22:0}$ amide linked to the α-amino group of L-ornithine in slow-growing mycobacteria (11), and López-Marín et al. found 3-OH $C_{26:0}$ and 3-OH $C_{28:0}$ amide linked to D-phenylalanine in the GPL of *M. peregrinum* (12). Daffé et al. detected monounsaturated 3-OH-FAs with 28 and 30 carbon atoms in the C-mycoside of *M. smegmatis* (8). Recently, Belisle

TABLE 2. Distribution of 3-OH-FAs in mycobacteria in groups A to F[a]

Fatty acid[b]	Wt % × 1,000[c]					
	Group A	Group B	Group C	Group D	Group E	Group F
3-OH $C_{14:0}$	0.1–0.5	0.7–1	0.1–4.5	0.3–1	0.6–0.8	0.8–1
	tr–0.1	0.1–0.2	0.2–4.5	0.2–1	0.5–0.6	0.5–0.7
3-OH $C_{16:0}$	0.5–1.5	1–4	0.7–12	0.3–3	0.4–1	1–10
	0.5–1	0.6–2	0.3–10	0.3–2	0.6–1	0.6–1
3-OH $C_{18:0}$	1.5–5	0.1–0.7	2–23	0.4–20	1.5–2	0.7–4
	0.4–0.7	0.1–0.4	0.1–12	0.1–2	0.8–1	0.5–3
3-OH $C_{20:0}$	1–4	tr–0.2	1.5–20	0.2–16	1–2	1–4
	0.3–0.6	tr–0.1	0.1–5.5	0.1–3	0.8–1	1–3
3-OH $C_{22:0}$	0.4–1	tr–0.2	0.6–5	0.1–2.5	0.8–1	0.1–0.4
	0.2–0.3	tr–0.1	0.2–3	0.1–2	0.7–1	0.1–0.3
3-OH $C_{24:0}$	0.1–0.4	0–0.3	0.3–2.5	1–2	0.5–0.8	0.1–0.3
	0.1–0.3	0–0.1	0.1–2	0.1–1	0.4–0.6	0.1–0.2
3-OH $C_{25:0}$	—	—	—	0.2–0.7	—	—
				tr–0.6		
3-OH $C_{26:0}$	0.1–0.3	—	0.2–2.5	50–86	0.7–1	0.1–0.2
	tr–0.2	—	0.1–1	4–7	0.3–0.4	tr–0.1
3-OH $C_{27:0}$	—	—	—	5–13	—	—
				0.3–0.5		
3-OH $C_{28:0}$	—	—	—	60–112	—	—
				2–7		
3-OH-2-Me $C_{18:0}$	—	—	—	—		7–134
						6–72
3-OH-2-Me $C_{19:0}$	—	—	—	—	—	5–6.5
						4–5.5
3-OH-2-Me $C_{20:1}$	—	—	—	—	—	0–4
						0–2
3-OH-2-Me $C_{20:0}$	—	—	—	—	1–1.5	60–120
					0.5–0.6	47–110
3-OH-2-Me $C_{21:0}$	—	—	—	—	—	0–3
						0–2.5
3-OH-2-Me $C_{22:0}$	—	—	—	—	—	0–15
						0–11
3-OH-2,4,6-tri-Me $C_{21:0}$	0.6–0.9	—	—	—	—	—
	0.4–0.6					
3-OH-2,4,6-tri-Me $C_{22:0}$	0.2–0.7	—	—	—	—	—
	0.1–0.3					
3-OH-2,4,6-tri-Me $C_{23:0}$	0.2–0.8	—	—	—	—	—
	0.1–0.4					
3-OH-2,4,6-tri-Me $C_{24:0}$	4–7	—	—	—	—	—
	2–4					
3-OH-2,4,6-tri-Me $C_{25:0}$	0.2–0.7	—	—	—	—	—
	0.1–0.4					

[a] See Table 1 for definition of groups.

[b] 3-OH, hydroxyl group in position 3; 3-OH-2-Me, hydroxyl group in position 3 and methyl group in position 2; 3-OH-2,4,6-tri-Me, hydroxyl group in position 3 and methyl groups in positions 2, 4, and 6. Number before colon, number of carbon atoms in chain; number after colon, number of double bonds.

[c] Amounts are given as ranges among strains. Values on the top line are results of strong hydrolysis, and those on the bottom line are results of mild hydrolysis. tr, less than 0.1; —,not detected.

et al. reported the presence of both mono- and diunsaturated 3-OH $C_{32:0}$ in lipopeptides from rough mutants of *M. avium*; one of the lipopeptides also contained minor amounts of monounsaturated 3-OH-FAs with 33 to 36 carbon atoms (2). Branched-chain 3-OH-FAs have also been found. Thus, 3-OH-2-Me $C_{14:0}$ has been detected in LOS antigens from *M. szulgai* (10) and 3-OH-2-Me $C_{20:0}$ in LOS of *M. tuberculosis* Canetti (9); these acids were found to be ester linked to the terminal glycosyl residue. The latter fatty acid is also present in *M. gordonae* (7). In another study, 3-OH-2,4,6-tri-Me $C_{24:0}$ was found in acylated trehaloses from *M. tuberculosis* Canetti, and homologous acids containing 24 to 28 carbon atoms were identified in *M. tuberculosis* H37Rv (3, 16). Mycobacterial 3-OH-2-Me FAs may act as precursors of multimethyl-branched fatty acids such as phthienoic, phthioceranic, and mycocerosic acids (1).

Altogether, 21 different mycobacterial 3-OH-FAs with 14 to 28 carbon atoms, including those found in earlier studies (see above), were identified in this study. Many of them, for example, straight-chain 3-OH-FAs with 14, 16, 24, and 25 carbon atoms, as well as several methyl branched-chain 3-OH-FAs (in *M. gordonae*, *M. malmoense*, and *M. scrofulaceum*), have not been described previously. It should be noted that unlike other workers, we used whole mycobacterial cells for analysis. Moreover, we noticed the existence of both ester and amide linkages; for example, 3-OH $C_{26:0}$, 3-OH $C_{27:0}$, and 3-OH $C_{28:0}$ in mycobacteria belonging to group D appear to be mostly amide linked, whereas 3-OH-2-Me $C_{18:0}$, 3-OH-2-Me $C_{19:0}$, 3-OH-2-Me $C_{20:1}$, 3-OH-2-Me $C_{20:0}$, 3-OH-2-Me $C_{21:0}$, and 3-OH-2-Me $C_{22:0}$ in group F mycobacteria are mainly ester linked.

It is noteworthy that several 3-OH-2,4,6-tri-Me FAs are

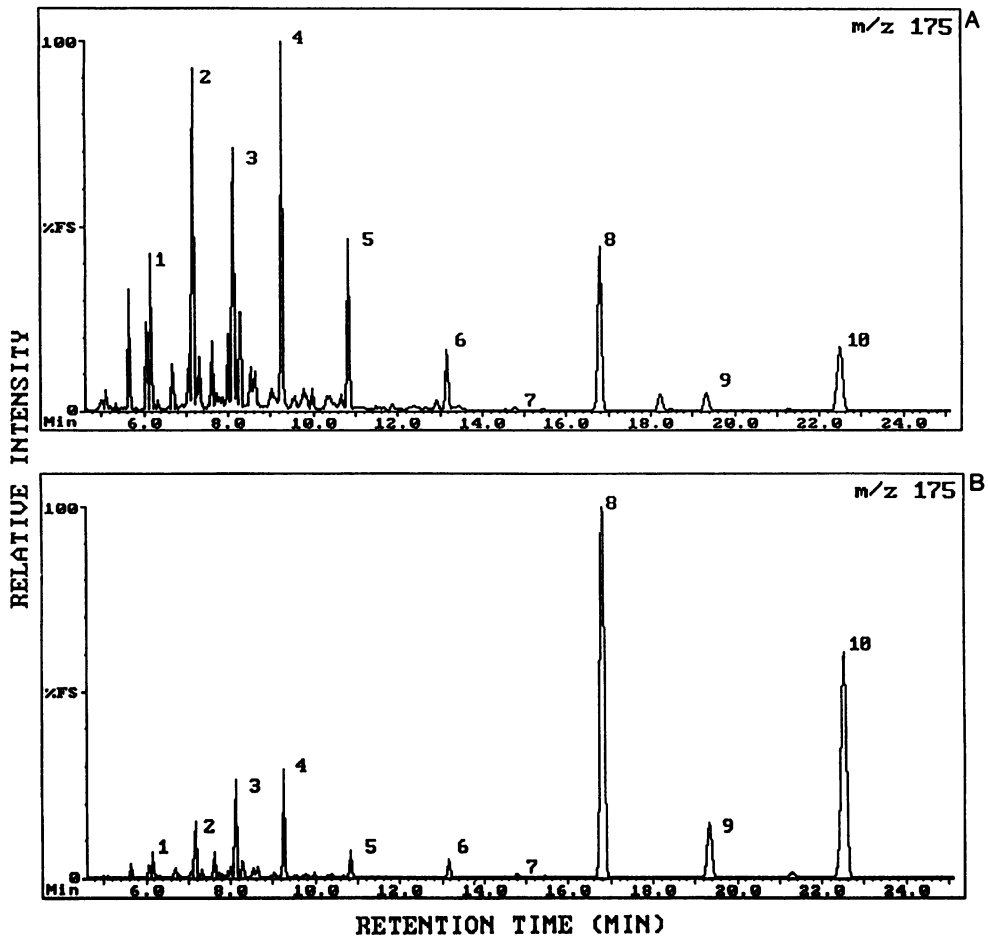

FIG. 4. Mass chromatogram of the Me/TMS derivatives of straight-chain 3-OH-FAs in *M. peregrinum* after mild hydrolysis (A) and strong hydrolysis (B). Peak identities: 1, 3-OH $C_{14:0}$; 2, 3-OH $C_{16:0}$; 3, 3-OH $C_{18:0}$; 4, 3-OH $C_{20:0}$; 5, 3-OH $C_{22:0}$; 6, 3-OH $C_{24:0}$; 7, 3-OH $C_{25:0}$; 8, 3-OH $C_{26:0}$; 9, 3-OH $C_{27:0}$; 10, 3-OH $C_{28:0}$.

present in *M. tuberculosis* (H37Rv and a clinical isolate) but not in the avirulent H37Ra strain. It has previously been reported that these acids are present in both *M. tuberculosis* H37Rv and clinical isolates as constituents of acyltrehaloses (3). Also interesting are the different 3-OH-FA patterns of *M. peregrinum* (containing 3-OH $C_{26:0}$, 3-OH $C_{27:0}$, and 3-OH $C_{28:0}$) and *M. fortuitum* (devoid of these acids). Our results are in agreement with earlier reports that amide-linked 3-OH $C_{26:0}$ and 3-OH $C_{28:0}$ are constituents of GPL in *M. peregrinum* (12) and that *M. fortuitum* is devoid of GPL (15).

Complex hydroxy fatty acid compositions have previously been reported for a few intracellularly growing gram-negative bacteria, including *Legionella* spp. (14), *Brucella abortus* (13), and *Coxiella burnetii* (17), in which these acids are part of the lipopolysaccharide. Further studies are required to elucidate the locations of the mycobacterial 3-OH-FAs described in the present report and to evaluate the plausibility of using these fatty acids as markers for the detection and quantification of bioactive mycobacterial lipids. Possible relationships between virulence and 3-OH-FA composition also deserve further attention.

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