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Analysis of Mycolic Acid Cleavage Products and Cellular Fatty Acids of *Mycobacterium* Species by Capillary Gas Chromatography

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After growth and experimental conditions were established, the mycolic acid cleavage products, constituent fatty acids, and alcohols of representative strains of *Mycobacterium tuberculosis*, *M. smegmatis*, *M. fortuitum* complex, *M. kansasii*, *M. gordonae*, and *M. avium* complex were determined by capillary gas chromatography. Reproducible cleavage of mycolic acid methyl esters to tetracosanoic (24:0) or hexacosanoic (26:0) acid methyl esters was achieved by heating the sample in a high-temperature muffle furnace. The major constituent fatty acids in all species were hexadecanoic (16:0) and octadecenoic (18:1 ω9-c, oleic) acids. With the exception of *M. gordonae*, 10-methyloctadecanoic acid was found in all species; moreover, *M. gordonae* was the only species tested which contained 2-methyltetradecanoic acid. *M. kansasii* was characterized by the presence of 2,4-dimethyltetradecanoic acid, *M. avium* complex by 2-eicosanol, and *M. tuberculosis* by 26:0 mycolic acid cleavage product. The mycolic acid cleavage product in the other five species tested was 24:0. Although a limited number of strains and species were tested, preliminary results indicate that this gas chromatographic method can be used to characterize mycobacterial cultures by their mycolic acid cleavage products and constituent fatty acid and alcohol content.

Detection of *Mycobacterium* species in clinical materials is often difficult because of special treatment procedures and the long incubation period required for growth of many species. Subsequent cultural and biochemical tests which are necessary for identification can require several weeks or months to complete (18, 22). It is also important to differentiate the rapidly growing species of *Mycobacterium* from acid-fast species in the related genera of *Nocardia*, *Rhodococcus*, or *Corynebacterium* (11, 12, 15, 18).

More rapid detection of growth and performance of tests for identification are needed so that the etiologic agent of a mycobacteriosis can be identified and appropriate antimicrobic therapy can be started as soon as possible. These methods include detection of growth by radiometric procedures (2, 16, 18) with selective inhibition tests (10), serologic tests (4, 17, 18), enzymatic tests (13, 22), and analysis of lipid components by various chromatographic methods (1–3, 6–8, 12, 14–16, 18–22). Some reports have shown that thin-layer chromatography of the lipid components can be used to characterize and identify isolates of mycobacteria (2, 5, 7, 12, 15, 18, 22). However, the method is not practical for routine analysis of cultures in clinical or reference laboratories. The procedure requires moderately large numbers of cells (approximately 50 mg), several developing solvents and visualization sprays, and skilled techniques for developing and interpreting the thin-layer chromatography patterns (7, 15, 18).

Analysis of the cellular fatty acids (as methyl esters) by gas-liquid chromatography (GLC) has been used to identify clinical isolates of mycobacteria more rapidly than with conventional testing (14, 16, 20, 21). GLC has also been used to detect long-chain fatty acid cleavage products (C20:0 to C26:0) formed when mycolic acid methyl esters from mycobacteria were pyrolyzed (3, 12) or heat cleaved in the injection port of the gas chromatograph (6). With the latter technique (6), mycolic acid cleavage products (MACP) and constituent fatty acids have been detected in small amounts of cells (5 mg, dry weight); however, the method requires careful attention by the operator, and results may not be reproducible on an interlaboratory basis because of differences in instrumentation. For these reasons, a method for cleaving mycolic acid methyl esters which is independent of the injector port temperature of the gas chromatograph was developed. Long-chain fatty acid methyl ester cleavage products and constituent fatty acids and alcohols (if present) of representative *Mycobacterium* species were determined by capillary GLC after growth in 5 ml of Middlebrook 7H9 broth. The profiles of some species were compared after growth on Lowenstein-Jensen medium and in different lots of Middlebrook 7H9 broth. The fatty acid and alcohol profiles of certain strains were also compared after transmethylation and after base hydrolysis (saponification) followed by methylation.

MATERIALS AND METHODS

The cultures examined in this study included *Mycobacterium smegmatis* ATCC 607, *M. fortuitum* biovar. *fortuitum* 84-527, *M. tuberculosis* $H_{37}Rv$, five strains of *M. kansasii*, six strains of *M. gordonae*, and eight strains of *M. avium* complex. These cultures were from the collection of the Mycobacteriology Reference Laboratory at the Centers for Disease Control, Atlanta, Ga. All strains except *M. tuberculosis* $H_{37}Rv$ were received after growth for 7 days in 5 ml of Middlebrook 7H9 broth (Carr Scarborough Microbiologicals, Inc., Stone Mountain, Ga.). Each culture was transferred to another tube of Middlebrook 7H9 broth and to a Lowenstein-Jensen (L-J) medium slant (BBL Microbiology Systems, Cockeysville, Md.). The second broth was incubated at 35°C for 7 days and used for a second analysis of each culture; the L-J slant was incubated at 35°C until growth was evident and refrigerated at 4°C for use as a stock culture. The *M. tuberculosis* cells were lyophilized and had been used in a previous study (6); they had been grown in Middlebrook 7H9 broth for 14 to 21 days and killed by heating in a 70°C water bath for 70 min.

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The broth cultures of the other five species were usually autoclaved for 30 min at 121°C and 15 lb/in^2. Before autoclaving, the tube cap of broth cultures in Pyrex tubes was replaced with a Teflon-lined cap. Both cultures grown in disposable glass tubes were transferred to a hexane-rinsed 16- by 125-mm screw-cap Pyrex tube fitted with a Teflon-lined cap to eliminate artifact production from the disposable tubes and caps. The autoclaved broth cultures were centrifuged for 15 min at 2,000 × *g*. The supernatant fluid was discarded, and the cells were washed with 1 ml of sterile distilled water. The water was removed with a capillary pipette to ensure that a minimal amount of supernatant fluid was left with the cells.

The mycobacterial cells were mixed with 2 or 3 ml of a transesterification reagent which consisted of 30 ml of methanol, 15 ml of toluene, and 1 ml of concentrated H_2SO_4 (MTS reagent [15]). The mixture was heated for 16 h at 80°C in a covered bath. The transmethylated samples were cooled to room temperature and extracted twice with an equal volume of hexane (2 or 3 ml). The hexane extracts containing the methyl esters were combined in a screw-cap tube (13 by 100 mm) and evaporated with nitrogen to approximately 1 ml. 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) was mixed with the sample. After 5 min, the hexane layer was removed to a clean tube and evaporated to 0.1 to 0.3 ml; 1 μl was analyzed by capillary GLC.

The remainder of the sample was transferred to a 1-ml lyophilization vial (Wheaton Scientific, Millville, N.J.) and evaporated just to dryness with nitrogen. The vial was evacuated, sealed with a natural gas-oxygen torch, placed in a muffle furnace (Sybron/Thermo-lyne 2000; Thermolyne Corp., Dubuque, Iowa), heated to 350°C, and held for 2 to 3 h. Some samples were heated for different periods of time (1 to 16 h) and to different temperatures (300 and 400°C). After heating, the vial was cooled to room temperature and opened, and both halves of the vial were rinsed with hexane. The hexane washes were combined in a 13- by 100-mm tube, concentrated to 0.1 to 0.3 ml, and analyzed by GLC under the same conditions as were used for the unheated samples.

The fatty acid methyl ester (FAME) and alcohol samples were analyzed on 50-m by 0.2-mm (inside diameter) fused-silica capillary columns with cross-linked methyl silicone (OV101; Hewlett Packard, Avondale, Pa.) as the stationary phase; the columns were installed in Hewlett Packard 5790A gas chromatographs equipped with flame ionization detectors. The instruments were coupled with either a Hewlett Packard 3390 reporting integrator or a 3388A terminal and automatic injector. For manual injection, the column was programmed at 180 to 275°C at 8°C/min and maintained at 275°C for 20 min. The injector and detector temperatures were 285°C. For samples analyzed on the automated gas chromatograph, the column was programmed at 200 to 275°C at 5°C/min and held at the final temperature for 14 min. The injector temperature was 265°C, and the detector temperature was 285°C. The carrier gas was hydrogen with a flow rate of approximately 0.7 ml/min; the sample size was 1 μl with a split ratio of approximately 50:1.

The FAME and alcohol peaks were identified by comparing retention times with authentic methyl ester and alcohol standards (Supelco, Inc., Bellefonte, Pa.; Applied Science Division, Milton Roy, State College, Pa.; NuCheck Prep, Inc., Elysian, Minn.; ICN Pharmaceuticals, Inc., Plainview, N.Y.; Larodan Fine Chemicals, Malmo, Sweden). The identities of the unsaturated acids and alcohols were confirmed by hydrogenation and trifluoroacetylation, respectively (9). The identities of most compounds were also confirmed by mass spectrometry (1, 8, 9).

In some experiments, each of several mycobacterial strains were grown in five tubes of Middlebrook 7H9 broth. After the cultures were autoclaved and the supernatant broth was removed, the cells were combined and divided into equal amounts. Some samples were heated in the MTS reagent for 1, 4, 8, and 16 h; others were mixed with 15% NaOH in 50% aqueous methanol (150 g of NaOH, 500 ml of methanol, 500 ml of distilled water) and saponified for 1, 4, 8, and 16 h before they were methylated, extracted (9), buffer washed, and analyzed as described above.

RESULTS AND DISCUSSION

When chromatograms from MTS samples which had been heated to 350°C were compared with those from unheated samples, several changes were observed. Examples of these are shown in Fig. 1, which shows chromatograms of *M. fortuitum* biovar. *fortuitum* 84-527 before and after the FAME sample was heated. All gas chromatographic conditions were the same, but the 24:0 peak in the heated sample (Fig. 1B) is much larger and sharper than that in the unheated sample (Fig. 1A). The smaller, skewed peak for 24:0 in Fig. 1A is from partial cleavage of the mycolic acid in the injector port of the gas chromatograph (6). Apparently, part of the myclolic acid methyl ester is cleaved at the β-hydroxy position (3, 5, 6, 11) to yield the methyl ester of 24:0 as the other sample components are volatilized in the injector of the gas chromatograph; this incomplete cleavage which is dependent on the injector port temperature continues for several seconds after volatilization of the other components. This probably accounts for the skewed peak shape for 24:0 in unheated samples. To demonstrate this cleavage, unheated and heated methyl ester samples of *M. fortuitum* cells were analyzed at injection port temperatures of 235, 260, and 285°C. The amount of 24-carbon MACP formed at these temperatures is shown in Table 1. The maximum amount of cleavage product was detected when the heated sample was injected at 260 and 285°C; 24:0 accounted for 29% of the total fatty acids found in these samples. When the unheated sample was injected at these same two temperatures, the relative amount of 24:0 decreased to 17 and 20%, which was equivalent to cleavage of 59 and 69% of the mycolic acid, respectively. Injecting the unheated sample at 235°C decreased the relative amount of 24:0 to 6% and represented cleavage of 21% of the mycolic acid. The peak for 24:0 in all unheated samples tailed and was broader than that in heated samples, indicating slower or incomplete volatilization. The 24:0 peak from heated samples was sharp and symmetrical, and the amount of this acid did not change significantly when the injection port temperature was decreased. The slightly lower percentage of 24:0 found in heated samples injected at 235°C is probably from incomplete volatilization of this fatty acid rather than incomplete heat cleavage of mycolic acid. Heating FAME samples of mycobacteria for longer than 3 h or at temperatures greater than 350°C did not significantly increase the amount of cleavage product detected.

We also observed that the amount of mycolic acid cleaved in unheated samples at an injection temperature of 285°C varied with the species tested. This amount ranged from 41% of 26:0 MACP in unheated samples of *M. tuberculosis* to 69% of 24:0 MACP in *M. fortuitum* biovar. *fortuitum*. This variation in the amount of mycolic acid cleaved may be from differences in the "mero" portion of the mycolic acid allowing some types of structures to be more readily cleaved

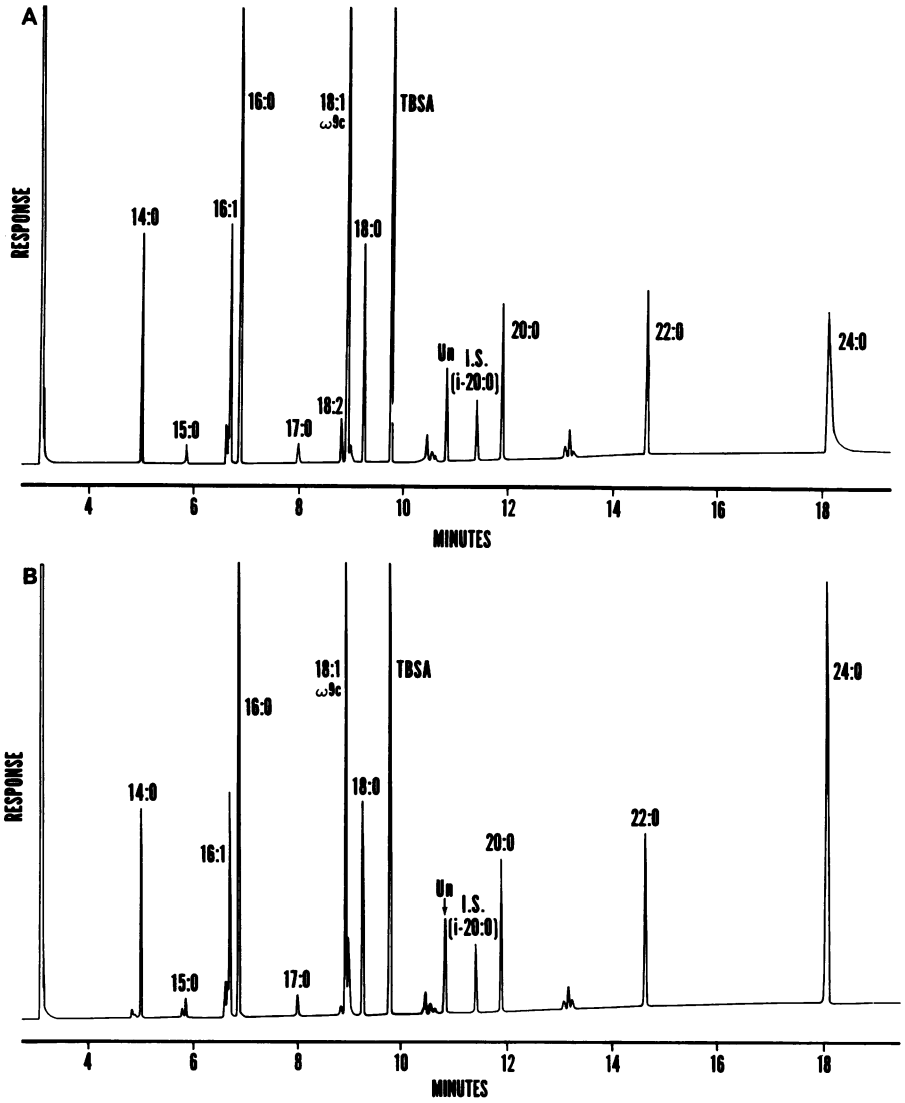

FIG. 1. Gas chromatograms of fatty acid methyl esters and MACP from transmethylated cells of *M. fortuitum* biovar. *fortuitum* before (A) and after (B) heating at 350°C. Samples were analyzed on a 50-m fused-silica capillary column. See text and Table 2, footnote *b*, for explanation of acid designations.

at the β-hydroxy position (3, 5) than others. Regardless, it is important to recognize that partial cleavage of mycolic acid methyl esters can occur in the injection port of the gas chromatograph. The uncleaved portion will eventually elute and can interfere with subsequent analyses unless adequate time is allowed or a solvent blank is injected between each analysis (6).

TABLE 1. Effect of injection port temperature on amount of tetracosanoic acid (24:0) found in methyl ester samples of *M. fortuitum* complex cells[a]

Temp of injection port (°C)	Sample temp (°C)	% of total fatty acids	% Cleaved
235	Unheated	6	21
235	350	27	93
260	Unheated	17	59
260	350	29	100
285	Unheated	20	69
285	350	29	100

[a] Values are averages of three analyses at each temperature.

The other change noted in chromatograms from unheated and heated samples was a difference in the relative amounts of the unsaturated 18-carbon acids (Fig. 1). Unheated methyl ester samples of some of the cultures contained small amounts (1 to 6%) of linoleic acid (18:2 ω6-*cis*, ω9-*cis*); however, in heated samples this acid, which eluted 0.08 min before oleic acid (18:1 ω9-*cis*), was reduced to trace (<1%) to 2% amounts or had disappeared. This was usually accompanied by the appearance of a new peak which eluted 0.05 min after oleic acid and at the retention time of two isomers of 18:1 which coelute (18:1 ω7-*cis* and 18:1 ω9-*trans*). To confirm the degradation of linoleic acid and to determine the effect of heating other unsaturated methyl esters, we diluted individual standards in hexane, placed them in lyophilization vials, and heated them to 350°C as described in Materials and Methods. The standards included methyl esters of linolenic (18:3 ω3-*cis*, ω6-*cis*, ω9-*cis*), linoleic, linoelaidic (18:2 ω6-*trans*, ω9-*trans*), oleic, elaidic (18:1 ω9-*trans*), *cis*-vaccenic

TABLE 2. Fatty acids, alcohols, and MACP found in mycobacteria

Species	% of total acids[a] of the following compounds[b]																					
	14:0	2-M 14:0	2,4-DM 14:0	15:0	16:1	16:1 ω7c	16:1	16:0	2-OH 18:0	17:0	18:2	18:1 ω9c	18:1	18:0	TBSA	2-OH 20:0	Un	20:0	22:0	Un	24:0	Un	26:0
M. tuberculosis $H_{37}Rv$	T	–	–	–	T	2	T	28	–	1	1	25	–	10	10	–	–	–	–	1	3	2	17
M. smegmatis ATCC 607	2	–	–	–	1	1	4	25	–	T	T	20	5	6	13	–	–	1	4	–	18	–	–
M. fortuitum biovar. *fortuitum* 84-527	3	–	–	–	T	T	2	25	–	T	–	16	T	7	13	–	2	3	4	–	25	–	–
M. kansasii 84-521	1	–	2	T	–	1	2	26	–	2	–	25	4	12	6	–	–	–	3	–	16	–	–
M. gordonae 84-534	3	2	–	–	–	3	3	23	–	1	1	30	4	11	–	–	–	1	3	–	15	–	–
M. avium complex 84-541	2	–	–	–	–	4	3	22	T	–	–	26	5	4	9	5	–	–	–	–	20	–	–

[a] Values are percentages of total acids and are means of at least three determinations of samples heated to 350°C; T, trace amount (<1%); –, not detected.
[b] The number to the left of the colon indicates the number of carbon atoms; the number to the right indicates the number of double bonds. 2-M and 2,4-DM indicate methyl groups at the 2 and 2 and 4 positions, respectively; ω7c and ω9c indicate the double bond position from the hydrocarbon end of the *cis* isomer; 2-OH indicates secondary alcohol; Un, unidentified.

(18:1 ω7-*cis*), palmitoleic (16:1 ω7-*cis*), and palmitelaidic (16:1 ω7-*trans*) acids. The results showed that the methyl ester of linolenic acid (18:3) was completely degraded after 1 h at 350°C, producing four small peaks which eluted between the methyl esters of heptadecanoic acid (17:0) and linoleic acid; no attempt was made to identify these peaks. The only other methyl ester affected by heating to 350°C was that of linoleic acid. Approximately 60% of this methyl ester was degraded after 1 h at 350°C, and 84% was degraded after 3 h. Two new peaks with retention times identical to those of oleic and *cis*-vaccenic (or elaidic) acid methyl esters were found. Thus, some of the relative percentages for these two unsaturated acids in heated FAME samples may be from degradation of linoleic or linolenic acids and not from constituent fatty acids.

The average percentage of constituent fatty acids and alcohols and MACP (24:0, 26:0) found in representative strains of the species examined are listed in Table 2. The major cellular fatty acids in all six species were hexadecanoic acid (16:0) and oleic acid. These two acids accounted for at least 41% of the total cellular fatty acids. Tuberculostearic acid (TBSA, 10-methyloctadecanoic) was found in all species tested except *M. gordonae*. The primary MACP in five of the six species tested was tetracosanoic acid (24:0); in *M. tuberculosis*, it was hexacosanoic acid (26:0). Although a small amount (3%) of 24:0 was also found in *M. tuberculosis*, none of the other five species contained 26:0. However, there was a slight increase in the relative concentrations of docosanoic acid (22:0) after methyl ester samples of *M. smegmatis* ATCC 607 and some strains of *M. kansasii* and *M. gordonae* were heated, indicating the presence of this cleavage product also. The relative amounts of the primary MACP (24:0 or 26:0) in heated samples were approximately twice that found in unheated samples. The relative concentrations of the other acids, except the unsaturated 18-carbon acids, usually did not change when the samples were heated.

The fatty acid profiles of *M. smegmatis* ATCC 607 and *M. fortuitum* biovar. *fortuitum* 84-527 were almost identical, except this strain of *M. fortuitum* contained a small amount (2 to 3%) of an unidentified compound which eluted before eicosanoic acid (20:0). Each of the five *M. kansasii* strains tested contained 2,4-dimethyltetradecanoic acid; this acid eluted at the retention time of 13-methyltetradecanoic acid but mass spectral studies confirmed that it was 2,4-dimethyltetradecanoic acid (8) and not 13-methyltetradecanoic acid. This compound was identified by Julák and co-workers (8) and is probably the 2-methyl branched-chain fatty acid reported previously in *M. kansasii* by Thoen and co-workers (19). None of the other species tested contained this acid, but another branched-chain fatty acid, 2-methyltetradecanoic acid, was found in all six strains of *M. gordonae* tested. The presence of 2-methyltetradecanoic acid and MACP with 24 carbon atoms and the absence of TBSA may be useful in distinguishing this common laboratory contaminant from clinically significant species of mycobacteria and species in related genera which might also lack TBSA (11, 12). All seven *M. avium* complex strains contained 2-eicosanol, and most also contained trace to 1% amounts of 2-octadecanol. These two alcohols, 2-octadecanol and 2-eicosanol, eluted at the retention times of 17 cyclopropane and 19 cyclopropane FAME, respectively. Their identity was confirmed by trifluoroacetylation or mass spectrometry (1, 9). The fatty acid profiles found for the six species tested generally agree with those reported previously (1, 6, 8, 14, 19, 21), with the exception of Tisdall and co-workers (21), who did not report any unsaturated 18-carbon acids in *M. tuberculosis*.

Each of the test strains was regrown and retested in the same lot of medium at least twice with essentially identical results. In addition, the fatty acid profiles of several mycobacterial species were compared after growth in different lots of Middlebrook 7H9 broth and on L-J slants. The profiles of a given species were quite similar, regardless of the medium; any differences were quantitative rather than qualitative ones. Table 3 shows the fatty acid composition of *M. kansasii* 84-521 after growth in two lots of Middlebrook 7H9 broth prepared at the Centers for Disease Control, two lots of commercially prepared broth, and two lots of L-J medium. Overall, the profiles were almost identical, and the acids which characterize this species (2,4-dimethyltetradecanoic acid, TBSA, and 24:0) were always present. One advantage of using growth from L-J or other solid medium is that the cells can be removed from the surface and placed directly into MTS reagent for transesterfication. However, it is almost impossible to remove cells without

TABLE 3. Fatty acid composition of *M. kansasii* 84-521 after growth in different lots of medium

Medium	% of total acids of the following compounds[a]:																	
	14:1	14:0	2,4-DM 14:0	15:0	16:1	16:1 ω7c	16:1	16:0	17:0	18:2	18:1 ω9c	18:1	18:0	TBSA	20:0	22:0	24:0	26:0
7H9, CDC lot A[b]	T	1	2	T	1	2	T	26	2	–	25	4	12	6	–	3	16	–
7H9, CDC lot B[b]	1	1	2	T	T	3	1	27	2	–	24	4	12	6	T	3	14	–
7H9, commercial lot A[c]	1	1	2	T	1	2	T	30	2	T	27	T	12	7	–	2	13	–
7H9, commercial lot B[c]	–	1	5	–	–	6	6	25	–	–	37	–	3	5	1	2	9	–
L-J, lot A[d]	–	2	2	–	–	2	3	34	T	T	26	3	7	8	T	3	10	–
L-J, lot B[d]	–	3	2	T	–	2	4	35	1	T	15	5	6	10	1	5	11	–

[a] See text and Table 2, footnotes *a* and *b*, for explanation of acid and alcohol designations and data.
[b] Middlebrook 7H9 broth prepared by the Centers for Disease Control Media Laboratory.
[c] Middlebrook 7H9 broth purchased from Carr-Scarborough Microbiologicals.
[d] Purchased from BBL.

removing some of the medium, especially if growth is sparse. Since L-J medium contains many of the same fatty acids which are found in mycobacteria, it is difficult to interpret the cellular fatty acid data when cells are contaminated with medium even when medium blanks have been included and analyzed along with the cells. To avoid these problems, we recommend that broth cultures be used for these fatty acid analyses.

The effect of autoclaving on the fatty acid profile of cells was determined after dividing broth cultures in half. One half was autoclaved and the other was not; MTS samples of both killed and live cells were prepared and analyzed as described above. When results from the two analyses were compared, the relative amounts of linoleic, oleic, and stearic (18:0) acids were usually increased 1.3 to 2.5 times in autoclaved cells; the relative amount of TBSA was usually decreased 1.2 to 1.8 times from that found in live cells. Concentrations of other acids and alcohols (if present) in the cells were not affected by autoclaving. The increased concentration of 18-carbon acids in autoclaved cells is probably from components of Middlebrook 7H9 broth such as Tween 80 and oleic acid albumin complex (23). Since autoclaving cells did not adversely affect characteristic fatty acid profiles, this step is recommended for safety reasons (18) before harvesting mycobacterial cells from broth.

The fatty acid profiles of cells which had been saponified for 1 to 16 h before methylation and extraction (9) were compared with those from transmethylated cells heated for the same amount of time. The maximum amount of mycolic acid cleavage products was detected in cells which were heated for 8 to 16 h in MTS reagent. In most samples, two to six more times mycolic acid cleavage product (24:0 or 26:0) was detected in transmethylated samples than in saponified samples, even when cells were saponified for 16 h. The relative concentrations of constituent fatty acids were the same in both cell preparations, but the amount of 2-eicosanol in *M. avium* complex was slightly increased in saponified samples. This may be due to more efficient extraction of this alcohol by the 1:1 ether-hexane used for extraction (9), rather than the hydrolysis method.

Use of a high-temperature muffle furnace to cleave mycolic acids so that long-chain fatty acid cleavage products (24:0 or 26:0) can be analyzed by GLC provides a convenient and reproducible method for the detection of the presence and length of the aliphatic chain in mycolic acids of mycobacteria (5, 6, 12). During the same gas chromatographic analysis, constituent fatty acids and alcohols can also be determined. Maximal separation of these chemical components is achieved by use of a fused-silica capillary column. The method described is a rapid way to determine whether a suspected mycobacterial isolate has a mycolic acid cleavage product characteristic of this genus or whether it belongs to another genus. Although the data reported here are from a limited number of strains and species, additional cultures are currently being tested. These preliminary results indicate that determination of MACP together with the fatty acid and alcohol content may be useful in identifying unknown cultures by comparing their profiles with those from known species. Excellent results have been obtained with 5-ml broth cultures grown to an approximate concentration of 1.5×10^8 to 4.0×10^8 cells per ml. This gas chromatographic procedure has the potential to be integrated with radiometric (2, 10, 16, 18) or other techniques for more rapid identification of mycobacteria.

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