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Fatty Acids of *Mycobacterium kansasii*

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The cellular fatty acids of 35 strains of *Mycobacterium kansasii* isolated from clinical material were analyzed to establish properties by which we could identify and characterize these acid-fast microorganisms. The fatty acids were extracted from cells grown in liquid synthetic media, and they were analyzed as methyl esters by gas-liquid chromatography. The fatty acid profiles of all strains were similar. They differed from fatty acid profiles of other mycobacteria by their content of a saturated fatty acid with a methyl group at C2.

Mycobacterium kansasii is a member of Runyon's group I (photochromogenic) mycobacteria. The distribution of this organism in nature is unknown (10, 11). *M. kansasii* is considered to be the etiological agent in certain diseases of man and it has been isolated from animals (9). In man, the disease usually occurs in the lungs and is similar to tuberculosis in symptoms, pathology, and course (11). In most instances, the pulmonary lesions are histopathologically similar to lesions induced by *M. tuberculosis* (6). At the Mayo Clinic, *M. kansasii* has been isolated from lesions of the lung, knee, testicle, elbow, and hand as well as from gastric washings and sputum.

In attempting to differentiate mycobacteria by chemical analysis, we observed that *M. kansasii* produced a unique assortment of fatty acids in comparison to *M. avium*, *M. marinum*, *M. tuberculosis*, and certain skotochromogens. During growth under standardized conditions, the fatty acids produced by *M. kansasii* contained a saturated branched-chain fatty acid as more than 1% of the mixture. Mycobacteria other than *M. kansasii* produced no more than traces of this acid. Our survey included 29 strains of *M. kansasii* from 29 patients and 6 strains which had been isolated and typed in other laboratories.

MATERIALS AND METHODS

Microorganisms. The sources of the strains of *M. kansasii* used in this study are listed in Table 1. The organisms were identified by tests for photochromogenicity (11), by nitrate reduction (14), and by serotyping (12); all exhibited nitrate reduction and all but two were photochromogenic.

Cultural procedures for fatty acid studies. To evaluate the effect of carbon source and nitrogen source with regard to the types and amounts of fatty acids synthesized, we examined different media (Table 2). The isolates were subcultured on Lowenstein-Jensen medium for 10 days, and one loopful (2-mm loop) of growth was then transferred to 300 ml of medium in 1,000-ml Erlenmeyer flasks. Cultures were incubated for 28 days at 37 C in an atmosphere containing 5% carbon dioxide. Uninoculated flasks of medium were incubated under similar conditions for control specimens. Stained smears and subcultures were made of the growth to detect contamination.

The fatty acid composition of bacteria has been shown to differ in relation to stage of growth (1, 7, 8). Therefore, the influence of duration of incubation on cellular fatty acid composition was studied. Three different strains of *M. kansasii* were grown on Proskauer and Beck liquid medium for 14, 28, and 60 days. In addition, the influence of continuous agitation on growth was assessed. Four different strains of *M. kansasii* were incubated simultaneously without agitation and with agitation by rotary mixer.

Preparation of specimens for gas-liquid chromatography (GLC). For saponification of cells and extraction of fatty acids, the cells were harvested by centrifugation for 20 min at 3,000 rev/min in a model U International centrifuge (no. 240 rotor). The packed cells were suspended in a mixture of 4 ml of 33% potassium hydroxide and 25 ml of methanol and transferred to 250-ml Erlenmeyer flasks. The mixtures were heated for 1 hr in flowing steam, and then 20 ml of distilled water was added. After thorough mixing (shaking by hand for 1 min), the mixtures were heated for an additional 30 min in flowing steam and then another 25 ml of water was added. After cooling to room temperature, the nonsaponifiable material was extracted with three 50-ml portions of hexane and discarded. The residue was acidified to *p*H 2 with 15% sulfuric acid; the fatty acids were then extracted with two 50-ml portions of distilled ligroin. The solvent was vaporized in a rotary

[1] On assignment from the United States Department of Agriculture.

evaporator, and the residue was dissolved in 5 ml of anhydrous ether–methanol mixture (9:1).

For esterification, the fatty acids were treated with diazomethane by the method of Schlenk and Gellerman (13). Diazomethane was bubbled through the solution of fatty acids until a yellow color formed. The solvent was vaporized under a gentle stream of nitrogen, and the methyl esters were dissolved in 0.5 ml of hexane. The methylated fatty acids were analyzed immediately or stored at −20 C.

For catalytic hydrogenation, a portion of the methylated fatty acid extract was hydrogenated in the presence of reduced platinum oxide at room temperature under 2 atm of pressure. Known standards containing unsaturated fatty acids were included in this experiment to establish the efficacy of the procedure (Fig. 1). This test procedure was used to facilitate the identification of saturated and unsaturated fatty acids in the unknown specimens (Fig. 2).

To determine whether the mixtures of fatty acids from mycobacteria included hydroxy acids, small portions of the methylated mixtures were exposed to acetyl bromide in hexane. The excess acetyl bromide was destroyed with water and bicarbonate, and the methyl esters were recovered by extraction with hexane. The methyl esters recovered were compared with the original mixtures of methyl esters by GLC.

GLC analysis of methyl esters. The methyl esters were analyzed by use of a gas chromatograph equipped with a thermal conductivity detector and a hydrogen flame ionization detector (F & M model 500-1609). The instrument was modified to allow injection of the specimen directly into the chromatographic column. The specimens were fractionated in 6-ft (1.8-m) columns packed with diethylene glycol succinate (15%) on siliconized Chromosorb G-AW

TABLE 1. *Sources of strains of Mycobacterium kansasii*

Source	No.
Pulmonary lesion	13[a]
Sputum	12
Gastric washings	4
Knee	2
Elbow	1
Hand	1
Testicle	1
Larynx	1

[a] Two were supplied by E. Runyon (Salt Lake City, Utah), two by W. B. Schaefer (Denver, Colo.), and two by Ann Pollak (2).

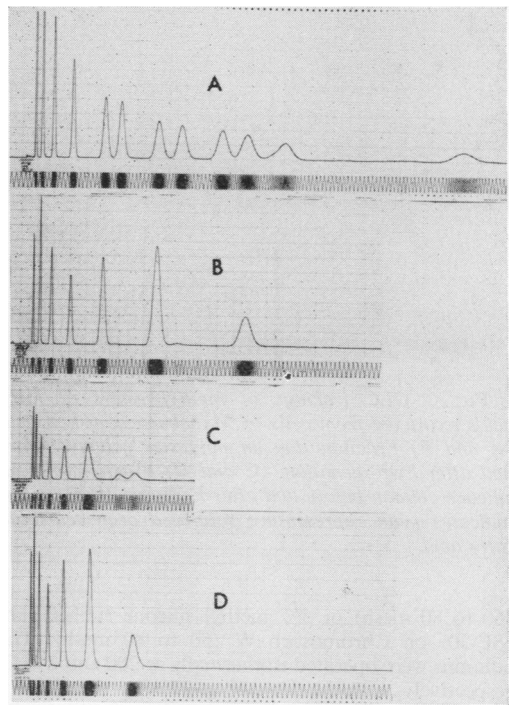

FIG. 1. *GLC tracings on polyester and silicone columns of known standard mixtures containing saturated and unsaturated methyl esters. (A) Standards; polyester column. (B) After hydrogenation of standards; polyester column. (C) Standards; silicone column. (D) After hydrogenation of standards; silicone column. Note conversion of unsaturated fatty acids to their saturated analogues.*

TABLE 2. *Media used for fatty acid studies*

Media	Carbon source[a]	Nitrogen source
1. Proskauer and Beck medium (Difco)	Glycerol, 2%; carbon dioxide	Asparagine, 5 g/1,000 ml
2. Basal medium (4) plus glycerol (5 ml)	Glycerol, 0.5%; carbon dioxide	Ammonium sulfate, 5 g/1,000 ml
3. Basal medium plus[b] biotin, 35 mg; sodium citrate, 0.5 g; potassium carbonate, 2.0 g; glycerol, 5 ml	Glycerol, 0.5%; carbon dioxide	Ammonium sulfate, 5 g/1,000 ml
4. Basal medium (same as 3) plus[b] glucose, 1.0 g	Glycerol, 0.5%; carbon dioxide; glucose	Ammonium sulfate, 5 g/1,000 ml

[a] Cultures were grown in an atmosphere of about 5% CO_2.
[b] Amounts per 1,000 ml.

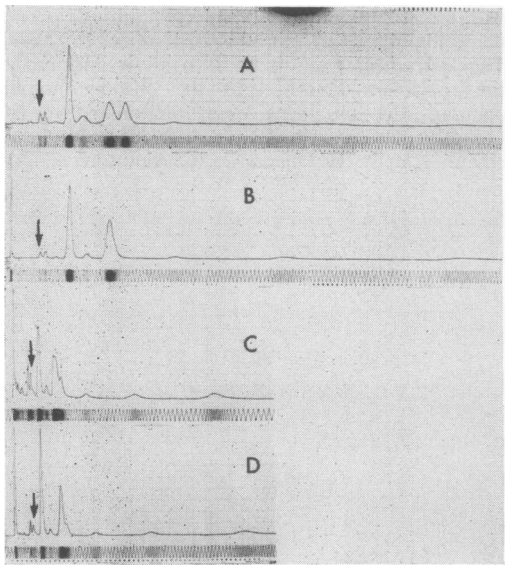

FIG. 2. *GLC tracings of methyl esters of fatty acids extracted from cells of Mycobacterium kansasii. (A and B) Fractionation on polyester column before and after hydrogenation. (C and D) Fractionation on silicone column before and after hydrogenation. Arrow indicates peak representing saturated branched-chain fatty acid.*

(60 to 80 mesh) or 5% methyl silicone rubber gum (SE-30) on Chromosorb W (60 to 80 mesh). The columns were operated isothermally at 200 and 250 C, respectively, with the detector at 275 C.

For further characterization and to facilitate the isolation of components for analysis by other techniques, the methyl esters also were chromatographed in a column packed with Apiezon L (20%) on Chromosorb W for the purpose of comparing retention times; the column [⅜ inch in diameter and 6 ft in length (0.48 cm by 1.8 m)] was operated isothermally at 250 C. The detector block was maintained at 300 C. The rate of flow of carrier gas was 50 ml/min, and the filament current of the katharometer was set at 100 ma.

For the purpose of isolation of components, chromatography was accomplished at 200 C, the detector block was maintained at 210 C, the filament current was 100 ma, and the carrier gas flow rate was 50 ml/min. The instrument was fitted with a stainless-steel exit port machined to a standard taper. Standard taper glass tubes with capillary tips were used to convey the effluent to collection tubes (16 by 150 mm) where the material was dispersed into acetone. The acetone was vaporized with the aid of a gentle stream of nitrogen at room temperature, and the residue was purified by rechromatography on the Apiezon column under the same conditions. Mass spectra of isolated methyl esters were measured with an Hitashi RMU6D single-focusing instrument at 70 ev and approximately 10^{-7} torr. (These analyses were kindly conducted by Ralph T. Holman, Hormel Institute, University of Minnesota, Austin, Minn.)

RESULTS

Identification of fatty acids. Chromatography of the mycobacterial fatty acid methyl esters before and after hydrogenation established the identity of the normal saturated acids and the normal monoenoic acids (Table 3). Neither normal saturated acids nor normal monoenoic acids having 21 and 23 carbon atoms were available in pure form for use as standards. Identification of the C_{21} and C_{23} saturated acids was based on plots of the logarithm of the retention time versus the number of carbon atoms. No evidence of the presence of C_{21} and C_{23} monoenoic acids was found.

The unique characteristic of the cellular fatty acids of *M. kansasii* was the occurrence of a saturated branched-chain fatty acid (BCFA) to the extent of more than 1% of the total fatty acids. No more than trace amounts of this BCFA were found in any other of the mycobacteria included in our survey (38 strains of *M. avium*, 20 of so-called Battey bacilli, 10 of *M. marinum*, 13 of skotochromogens, and 1 of *M. tuberculosis*). The identity of this distinctive component of *M. kansasii* was suggested by its mobility in the three chromatographic systems, its resistance to hydrogenation, and its resistance to acylation with acetyl bromide. A specimen of this BCFA isolated by gas chromatography was identified by mass spectrography as a saturated branched-chain fatty acid with a methyl group at C2.

A major component of the fatty acids of all mycobacteria included in this survey remains unidentified. In Table 3, this component is listed as compound B. Chromatographic mobility and resistance to hydrogenation and to acetylation suggest that this component is tuberculostearic acid (10-methylstearic acid). The mass spectrum of a partially purified specimen further suggests that this substance is tuberculostearic acid.

Chromatograms of standard mixtures of purified fatty acid methyl esters provided the basis for quantitative analysis of the cellular fatty acids. Relative amounts of the components in the fatty acid mixtures from *M. kansasii* were determined by integration of the strip-chart tracings of the chromatograms. The relative amounts of the cellular fatty acids are listed in Table 3. Variations in amounts were greatest for the 14:0, 15:0, and 16:1 acids; variations were relatively small for the other fatty acids. Variations among subcultures of a single strain

TABLE 3. *Fatty acids of Mycobacterium kansasii*

Carbon no.	Retention time (min) of methyl esters of fatty acids[a]			Reduced by hydrogen	Per cent of total	
	DEGS	SE-30	Ap-L		Mean	SD
8:0	1.7	0.8	1.5	No	Trace	
10:0	2.9	2.2	3.0	No	Trace	
11:0	3.7	3.0	4.2	No	Trace	
12:0	5.0	3.8	6.0	No	Trace	
14:0	8.4	6.7	11.6	No	4.1	1.3
BCFA[b]	7.2	7.9	14.1	No	3.8	0.7
15:0	10.7	8.8	16.3	No	1.3	0.5
16:0	14.5	11.0	23.8	No	35.0	3.5
16:1	17.2		21.8	Yes	3.4	1.5
17:0	19.1	14.8	32.8	No	4.7	1.4
17:B		12.8	28.0	No	Trace	
18:0	24.8	19.4	46.6	No	10.4	2.3
18:1	28.8	18.5	42.0	Yes	17.3	3.8
19:0	32.4	25.2	65.0	No	Trace	
B[c]		21.6	54.0	No	8.4	1.7
20:0	42.4	32.7	90.3	No	2.0	0.6
20:1	49.6	31.0	84.4	Yes	Trace	
21:0	56.0	42.4	140.0	No	Trace	
22:0	72.0	54.4	182.0	No	2.3	0.7
22:1	84.4	51.4	163.2	Yes	1.6	0.4
23:0	96.4	70.2	260.0	No	Trace	
24:0	126.0	90.0	364.0	No	2.8	0.6
24:1	144.4	84.6	328.0	Yes	1.6	0.3

[a] On three GLC columns: DEGS, diethylene glycol succinate coated on siliconized Chromosorb G-AW; SE-30, methyl silicone rubber gum coated on Chromosorb W; Ap-L, Apiezon L.

[b] Tentatively identified by mass spectrographic analysis as a saturated branched-chain fatty acid with a methyl group at C2.

[c] Tentative identification of this compound is 10-methylstearic acid.

were negligible (at least two subcultures of each strain were studied).

Effect of composition of culture medium. Glycerol and carbon dioxide served satisfactorily as carbon sources for the growth of *M. kansasii*. Inclusion of citrate, glucose, or additional bicarbonate (carbonate) in the medium induced no measurable change in the synthesis of fatty acids. Addition of biotin to the medium was also without effect. Either ammonium sulfate or asparagine served satisfactorily as a source of nitrogen.

Effect of duration of incubation. Each of three strains was examined after 14, 28, and 60 days of incubation in Proskauer and Beck medium. The length of incubation time did not affect the composition of the cellular fatty acids. A plot of logarithm of population density (determined by light transmission) against time demonstrated that the logarithmic phase of growth lasted about 15 days and followed a lag phase of 2 days.

Effect of continuous agitation. *M. kansasii* grew well with or without continuous agitation (which kept the cells suspended in the culture medium). The assortment and relative amounts of fatty acids produced were not affected by agitation.

DISCUSSION

The mycobacteria included in this study were isolated originally from resected lesions, from gastric washings, and from sputum. They included recent isolates and strains which had been cultured in the laboratory for several years. All of the strains which we designated as *M. kansasii* were shown by serological testing to be related.

The control of lipogenesis in *M. kansasii* does not appear to be affected by any adaptation of the organism to changes in the environment. Although our 35 isolates of *M. kansasii* came from a variety of sources, the fatty acid profiles of the cellular lipids were alike. Two strains maintained by subculture every 3 months for 15 years on Lowenstein-Jensen medium produced fatty acids similar to those of recent isolates.

Chromogenicity and the control of lipogenesis apparently represent separate genetic traits in *M. kansasii*. The independence of these traits

was exemplified by two strains which were nonchromogenic mutants but which had profiles of cellular fatty acids indistinguishable from the profiles of the photochromogenic organisms.

Cattaneo and associates (3, 5) reported the composition of cellular fatty acids of *M. kansasii* after a limited study of mycobacteria. They reported that a tridecenoic acid was a distinguishing component of *M. kansasii*, but we failed to recognize more than traces of a tridecenoic acid in our lipid extracts. In consideration of the small degree of variation of the fatty acid profile among our 35 strains of *M. kansasii*, we believe that the organisms examined by Cattaneo and associates (3, 5) must have had fatty acid profiles which were similar to those of our organisms. We do suspect that the tridecenoic acid which they reported was what we have identified as BCFA.

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