Capillary Gas Chromatographic Analysis of Mycolic Acid Cleavage Products, Cellular Fatty Acids, and Alcohols of *Mycobacterium xenopi*

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The fatty acids, alcohols, and mycolic acids of 26 strains of *Mycobacterium xenopi* were studied by capillary gas chromatography and thin-layer chromatography. All strains contained α -, keto-, and ω -carboxymycolates. The primary mycolic acid cleavage product was hexacosanoic acid. The fatty acid patterns and, especially, the presence of 2-docosanol are characteristic markers of *M. xenopi*.

Mycobacterium xenopi is a slow-growing scotochromogenic mycobacterium frequently isolated from human secretions without associated disease (20, 21). Several reports of infection caused by *M. xenopi* have appeared in the literature. Most cases have been of pulmonary disease (3, 16, 20, 21). *M. xenopi* has also been implicated in waterborne nosocomial outbreaks of pulmonary disease (3). During the past several years, disseminated infections produced by *M. xenopi* have been associated with acquired immune deficiency syndrome (5, 10). Because of its potential response to the traditional antituberculous drugs (3), it is important to distinguish this organism from the more drug-resistant *Mycobacterium avium*-*M. intracellulare* complex. Differentiation of *M. xenopi* from other mycobacterial species is usually performed by using a combination of several biochemical tests; however, this is a tedious task and differentiation between species is often unclear (19). Analyses of cellular fatty acid methyl esters and alcohols by gas-liquid chromatography and mycolic acid patterns by thin-layer chromatography have been used to identify clinical isolates of mycobacteria more rapidly than with conventional testing (1, 4, 6-9, 11, 12, 14, 15, 17, 18). In this paper, the characteristic lipid profile of *M. xenopi* determined by capillary gas-liquid chromatography and thin-layer chromatography is described.

Twenty-five strains of *M. xenopi* and the type strain of *M. xenopi* (NCTC 10042) were studied. These organisms were obtained from the collection of the Department of Tuberculosis, Hospital de la Sta. Cruz y San Pablo, Barcelona, Spain. For isolation of lipid components, all strains were cultivated on plates of Middlebrook 7H10 agar and incubated at 37°C in 5% CO₂ for 21 days. A spade-ful of bacteria (5 mg, wet weight) was scraped from the surfaces of Middlebrook 7H10 agar plates. The mycobacterial lipids were extracted and the fatty acids were derivatized to methyl esters by a modification of the method of Minnikin et al. (14). The cells were mixed with 2 ml of a reagent composed of 30 ml of methanol, 15 ml of toluene, and 1 ml of concentrated H₂SO₄ in a screw-cap test tube (16 by 125 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 Na₂HPO₄ and 12.0 g of NaOH per liter of distilled water, pH 11 to 12) (10).

The hexane upper layer was then removed, placed in a clean tube, and evaporated to dryness in a water bath at 50°C under a stream of nitrogen. The residue was dissolved in 0.1 to 0.4 ml of *n*-hexane, and 1 μ l was analyzed by capillary gas-liquid chromatography.

The fatty acid methyl esters, mycolic acid cleavage products (MACP), and 2-alcohols were analyzed on a fused-silica capillary column (15 m by 0.25 mm [inside 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 at 175 to 300°C at 8°C/min and maintained at 300°C for 14 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 μ l, with a split ratio of approximately 100:1. The chromatograms were integrated by using a Hewlett-Packard 3390A electronic integrator. The peaks were identified by comparing retention times with authentic methyl ester and alcohol standards (Supelco, Inc., and Sigma Chemical Co., St. Louis, Mo.). The identities of the alcohols were confirmed by trimethylsilylation. This was performed through a chemical reaction with a mixture of trimethylchlorosilane and hexamethyldisilazane in dry pyridine (1:2:4, vol/vol/vol) (7). The identities of all compounds were also confirmed by mass spectrometry. A Hewlett-Packard model 5988A mass spectrometer equipped for both electron impact ionization and chemical ionization was used. The mass spectrometer was interfaced to a Hewlett-Packard 5890A gas chromatograph. Mycolic acid methyl esters obtained after acid methanolysis were analyzed by one- and two-dimensional thin-layer chromatography by the procedure described by Minnikin et al. (14, 15).

A typical gas chromatogram of *M. xenopi* is shown in Fig. 1. The average percentages of constituent fatty acids, alcohols, and MACP (C_{20:0} to C_{26:0}) are listed in Table 1. The major cellular fatty acids in *M. xenopi* were hexadecanoic acid (C_{16:0}) and tuberculoheptadecanoic acid (10-methyloctadecanoic acid). Each of the 26 *M. xenopi* strains tested con-

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FIG. 1. Gas chromatogram of fatty acid methyl esters, alcohols, and MACP of *M. xenopi*. TBS, Tuberculostearic acid; UN, unidentified compound.

tained 2 to 5% concentrations of hexadecenoic acid ($C_{16:1}$) and octadecanoic acid ($C_{18:0}$). Cultures of *M. xenopi* also contained very small amounts (<2%) of tetradecanoic acid ($C_{14:0}$), heptadecanoic acid ($C_{17:0}$), and an unidentified compound which eluted before docosanoic acid ($C_{22:0}$). The primary MACP of *M. xenopi* was hexacosanoic acid ($C_{26:0}$). Two peaks corresponding to secondary alcohols were detected in *M. xenopi* strains. Trace amounts to 2% of 2-eicosanol (2-OH $C_{20:0}$) eluted at the retention time of 19-cyclopropane, as well as 8 to 12% of 2-docosanol (2-OH $C_{22:0}$). The identity of this last alcohol was confirmed by trimethylsilylation and mass spectrum analysis (2, 13). The significant peaks of the mass spectrum illustrated in Fig. 2 show the following fragmentation pattern: m/z 325 ($M - 1$, 0.2%), m/z 311 ($M - 15$, 2%), m/z 308 ($M - 18$, 7.3%), m/z 280 ($M - 46$, 2.4%). The last peak is the consequence of the

loss of the 1-methyl group and the subsequent rearrangement type F from the resulting cation, with a breakdown C_2 to C_3 characteristic of 2-alcohol and corresponding to a neutral olefin ($M = 280$). The alkene which results from the primary dehydration of the molecular ion (peak at m/z 308) is the origin of the typical 14 to 1 series (m/z 41, 55, 69, 83, 97, 111, 125, 139 . . .) that are clearly observed as local maximums (2, 13). The base peak is m/z 45, corresponding to the ion $(CH_3CHOH)^+$, also characteristic of a 2-alcohol (Fig. 3). These concordant data led us to conclude that the structure of the problematic substance, as suggested Daffé et al. (4), is the aforementioned 2-docosanol. As previously described (15), all strains of *M. xenopi* studied show α -mycolates, ketomycolates, and ω -carboxymycolates by thin-layer chromatography analysis. We recently examined by gas chromatography 237 other mycobacterial strains comprising 21 species of the family *Mycobacteriaceae* (51 *Mycobacterium*

TABLE 1. Fatty acids, alcohols, and MACP found in *M. xenopi*

Compound ^a	% of total acids (SD)	
	Type strain	Others (n = 25)
$C_{14:0}$	0.7	0.9 (0.4)
$C_{16:1}$	2.9	2.6 (1.6)
$C_{16:0}$	26.9	27.7 (1.8)
$C_{17:0}$	0.6	0.5 (0.2)
$C_{18:1}$	6.2	6.4 (3.3)
$C_{18:0}$	4.2	4.6 (1.3)
TBSA ^b	29.7	30.4 (2.3)
2-OH $C_{20:0}$	1.5	1.7 (0.5)
$C_{20:0}$	0.2	0.2 (0.3)
2-OH $C_{22:0}$	8.3	8.9 (3.0)
Un ^c	1.6	1.7 (0.5)
$C_{24:0}$	0.5	0.3 (0.4)
$C_{26:0}$	7.4	7.1 (1.6)

^a The number to the left indicates the number of carbon atoms; the number to the right indicates the number of double bonds.

^b TBSA, Tuberculostearic acid (10-methyloctadecanoic acid).

^c Un, Unidentified compound.

FIG. 2. Mass spectrum of 2-docosanol of *M. xenopi*.

FIG. 3. Mass fragmentation pattern of 2-docosanol. See the text for an explanation.

tuberculosis, 9 *M. bovis*, 4 *M. africanum*, 32 *M. avium-M. intracellulare*, 9 *M. terrae*, 6 *M. triviale*, 4 *M. gastri*, 3 *M. malmoense*, 21 *M. kansasii*, 4 *M. marinum*, 3 *M. szulgai*, 2 *M. simiae*, 25 *M. gordonae*, 7 *M. scrofulaceum*, 2 *M. flavescens*, 21 *M. fortuitum*, 18 *M. chelonae*, 5 *M. smegmatis*, 8 *M. fallax*, and 2 *M. chitae* strains and 1 *M. porcinum* strain). With the exception of *M. xenopi*, 2-docosanol was not detected in any mycobacterial species (V. Ausina and M. Luquin, *Enferm. Infect. Microbiol. Clin.*, in press). Secondary alcohols are derived from wax ester mycolates and constitute useful markers in mycobacterial differentiation (1, 4, 7, 10, 11). The secondary alcohols 2-octadecanol and 2-eicosanol were previously found in a variety of mycobacterial species (1, 4, 10, 11), but 2-docosanol has been detected only in *M. xenopi*. The detection of alcohols by using capillary columns and the obtainment of characteristic thermal MACP can add to the differential diagnostic capability of the gas chromatography method used (10, 11).

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