Establishment of 2-Docosanol as a Cellular Marker Compound in the Identification of *Mycobacterium xenopi*

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Received 4 April 1989/Accepted 27 June 1989

Sixteen strains of *Mycobacterium xenopi* were studied by gas chromatography and thin-layer chromatography. Data on the cellular fatty acids, fatty alcohols, mycolic acids, and glycolipids indicated that this bacterium possesses a specific lipid composition. 2-Docosanol, detected in all studied strains, was found to constitute a useful chemical marker in the identification of *M. xenopi*.

Mycobacterium xenopi, first described by Schwabacher in 1959 as a cause of skin granulomas in the South African clawed toad, *Xenopus laevis*, has been isolated from both clinical and environmental specimens (7). The pathogen was recently isolated from a patient with acquired immunodeficiency syndrome (2). *M. xenopi* ranges with *Mycobacterium avium-Mycobacterium intracellulare*, forming a slowly growing, Tween 80-negative subgroup of mycobacteria (10). Because of its extremely dysgonic growth, the demonstration of *M. xenopi* by culture growth and biochemical assays is time-consuming. The present work was undertaken to study the possibility of using lipid analysis (fatty acids, fatty alcohols, mycolic acids, and glycolipids) in the laboratory identification of *M. xenopi*.

A total of 16 strains of *M. xenopi* (10 strains from sputum, 4 from tap water samples, 1 from necrotic material from a patient with acquired immunodeficiency syndrome, and the type strain NCTC 10042) were studied. The species identity of each strain was verified by standard biochemical tests (10, 14). Before the lipid analyses, the mycobacteria were cultivated in Sula liquid medium (6) at 39°C for 5 to 6 weeks. After collection, the bacteria were washed twice in saline, autoclaved, and freeze-dried.

For analysis of the fatty acids, alcohols, and mycolic acids, 2-mg samples of the dried mycobacterial cells were heated in 1 ml of 1-*N*-methanolic hydrogen chloride (prepared by adding 0.9 ml of acetyl chloride dropwise to 10 ml of methanol) at 80°C for 1 h, extracted with 2 ml of hexane-water (2:1, vol/vol), evaporated under a stream of nitrogen, and made up to 50 μ l with hexane (8). One-microliter portions of the extracts were introduced by splitless injection onto a fused-silica capillary column (25 m by 0.25 mm) attached to a flame ionization detector, using SE-30 (cross-linked) as the stationary phase. The column temperature was programmed at a rate of 6 degrees/min from 140 to 280°C. The injector temperature was set at 320°C to facilitate pyrolysis of the mycolates (9). The identities of the fatty acids and alcohols were confirmed by gas chromatography and mass spectrometry. The methyl mycolates were separated by two-dimensional thin-layer chromatography, using silica gel plates (10 by 10 cm; E. Merck AG) and dichloromethane and petroleum ether-acetone as solvents (12); the components were identified by cochromatography

of known mycolates. The mycolate patterns were confirmed by analyzing the mycobacteria after saponification followed by esterification of the mycolic acids with iodomethane (5).

For the glycolipid analyses, the mycobacteria (approximately 5 mg) were extracted overnight in 4 ml of chloroform-methanol (2:1, vol/vol). The total-lipid fractions were applied to silica gel plates (10 by 10 cm; Merck) and separated by using a variety of combinations of chloroform-methanol-water (9:1:0, 60:12:1, and 60:35:5, vol/vol/vol) as the solvent. The plates were revealed with anthrone (0.2%, wt/vol, in sulfuric acid) and, separately, with molybdophosphoric acid (5%, wt/vol, in ethanol). The glycolipids were identified as blue spots in response to the anthrone treatment and characterized as glycolipids *sensu stricto* because of their sensitivity to mild saponification (3).

All strains contained saturated and monounsaturated fatty acids 14 to 26 carbons in length, with notably high concentrations of tuberculostearate (accounting for a mean of 30.4% of the total content of fatty acids and alcohols) and hexacosanoate (7.5%). In addition, 2-eicosanol (0.9%) and 2-docosanol (4.5%) were found (Fig. 1). The mass spectrum of the latter compound showed a base peak at m/z 45 ($\text{CH}_3\text{CH}=\text{OH}^+$) and a significant fragment at m/z 308 ($\text{M}-18$)⁺. These data are consistent with the structure of a secondary alcohol with 22 carbon atoms (1, 11). The mycolic acid composition consisted of alpha-, keto-, and dicarboxymycolates (Fig. 2). Seven spots were identified as glycolipids (including the cord factor) *sensu stricto*; all were labile to mild saponification. The best resolution was obtained when chloroform-methanol (9:1, vol/vol) was used as the solvent (Fig. 3).

Abundant amounts of hexacosanoate (originating from pyrolysis of methyl mycolates in the heated gas chromatographic injector) have previously been reported for the *Mycobacterium tuberculosis* complex (4), *Mycobacterium mageritense* (13), and *Mycobacterium simiae* (4, 11). 2-Octadecanol and 2-eicosanol are present in certain other slow-growing *Mycobacterium* species, including the *M. avium* complex, *Mycobacterium nonchromogenicum*, and *Mycobacterium terrae*, being ester linked to dicarboxymycolates, forming "wax-ester" mycolates (5). 2-Docosanol, on the other hand, appears to be unique to *M. xenopi*. Thus, of several studied reference strains of each of the presently recognized mycobacterial species (6) and hundreds of strains isolated from clinical and environmental specimens, we

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FIG. 1. Representative gas chromatogram of a strain of *M. xenopi*. Numbers before and after colons indicate carbon chain lengths and double bonds, respectively. 2-20-OH, 2-Eicosanol; 2-22-OH, 2-docosanol; TPA, tuberculopalmitate; TSA, tuberculostearate.

have, over a 10-year period, detected 2-docosanol exclusively in strains belonging to *M. xenopi*. The mycolic acid composition of *M. xenopi* is the same as that of the *M. avium* complex, for example (4, 5); however, the former mycobacteria contain glycolipids, whereas several characteristic glycopeptidolipids are found in the latter (3).

Lipid analysis provides a useful way of characterizing clinical isolates of mycobacteria. A single analysis involving detection of 2-docosanol appears to be sufficient for the definite identification of *M. xenopi*. The required gas chromatographic procedure is uncomplicated and can be recommended for routine use.

FIG. 2. Two-dimensional thin-layer chromatography pattern representing the methyl mycolates of *M. xenopi*. 1, First direction (dichloromethane, once); 2, second direction (petroleum ether-acetone, 95:5, vol/vol, three times); A, C, D, and E, alpha-, keto-, and dicarboxymycolates and secondary alcohols (2-eicosanol and 2-docosanol), respectively; F, fatty acid methyl esters; ?, artifacts.

FIG. 3. Thin-layer chromatography of glycolipids of *M. xenopi*. The plate was revealed with anthrone and then heated to 120°C for 2 to 5 min. Spot 5 corresponds to cord factor; spots 1 to 4, 6, and 7 are glycolipids sensu stricto. (A) Crude extract; (B) after mild saponification.

This work was supported by a grant from the Swedish Heart and Lung Foundation (to L. Larsson), by grant 84/819 from CAICYT (to F. Martín-Luengo and P. Valero-Guillén), and by grant T9/181/5 from the World Health Organization (to M. Kubín).

LITERATURE CITED

1. Alvin, C., L. Larsson, M. Magnusson, P.-A. Mårdh, G. Odham, and G. Westerdahl. 1983. Determination of fatty acids and carbohydrate monomers in microorganisms by means of glass capillary gas chromatography: analysis of *Mycobacterium gordonae* and *Mycobacterium scrofulaceum*. *J. Gen. Microbiol.* **129**:401–405.
2. Armstrong, D. A., J. W. Gold, J. Dryjanski, E. Whimbey, B. Polsky, C. Hawkins, A. E. Brown, E. Bernhard, and T. E. Kiehn. 1985. Treatment of infections in patients with the acquired immunodeficiency syndrome. *Ann. Intern. Med.* **103**:738–743.
3. Brennan, P. J., H. Meyer, G. O. Aspinall, and J. E. Nam Shin. 1981. Structures of the glycopeptidolipid antigens from serovars in the *Mycobacterium avium*/*Mycobacterium intracellulare*/*Mycobacterium scrofulaceum* serocomplex. *Eur. J. Biochem.* **115**:7–15.
4. Daffé, M., M. A. Lanéeelle, C. Asselineau, V. Lévy-Frebault, and H. L. David. 1983. Intérêt taxonomique des acides gras de mycobactéries: proposition d'une méthode d'analyse. *Ann. Microbiol. (Paris)* **134B**:241–256.
5. Dobson, G., D. E. Minnikin, S. M. Minnikin, J. H. Partlett, M. Goodfellow, M. Ridell, and M. Magnusson. 1985. Systematic analysis of complex mycobacterial lipids, p. 237–265. In M. Goodfellow and D. E. Minnikin (ed.), *Chemical methods in bacterial systematics*. Academic Press, Inc. (London), Ltd., London.
6. Jenkins, P. A., S. R. Pattyn, and F. Portaels. 1982. Diagnostic bacteriology, p. 441–470. In C. Ratledge and J. Stanford (ed.), *The biology of the mycobacteria*, vol. 1. Academic Press, Inc. (London), Ltd., London.
7. Kubín, M. 1984. Distribution and ecology of mycobacteria in non-living reservoirs: opportunists and pathogens, p. 1313–1338. In G. P. Kubica and L. G. Wayne (ed.), *The mycobacteria—a source book*, part B. Marcel Dekker, Inc., New York.
8. Larsson, L. 1983. Acidic methanolysis v. alkaline saponification in gas chromatographic characterization of mycobacteria: differentiation between *Mycobacterium avium-intracellulare* and *Mycobacterium gastri*. *Acta Pathol. Microbiol. Immunol. Scand. Sect. B* **91**:235–239.
9. Larsson, L., E. Jantzen, and J. Johnsson. 1985. Gas chromatographic fatty acid profiles for characterization of mycobacteria: an interlaboratory methodological evaluation. *Eur. J. Clin. Microbiol.* **4**:483–487.
10. Tsukamura, M. 1983. Numerical classification of 289 strains of slowly growing mycobacteria. Proposal of *Mycobacterium tuberculosis* series, *Mycobacterium avium* series, and *Mycobacterium nonchromogenicum* series. *Microbiol. Immunol.* **27**:315–334.
11. Valero-Guillén, P. L., and F. Martín-Luengo. 1986. 1-Tetradecanol, a new alcohol found in the cell wall of some rapidly growing chromogenic mycobacteria. *FEMS Microbiol. Lett.* **35**:59–63.
12. Valero-Guillén, P., F. Martín-Luengo, J. Jiménez, and L. Larsson. 1986. A thin-layer chromatographic method for separating methyl esters of mycobacterial mycolic acids. *Acta Pathol. Microbiol. Immunol. Scand. Sect. B* **94**:373–376.
13. Valero-Guillén, P., F. Martín-Luengo, L. Larsson, J. Jiménez, I. Juhlin, and F. Portaels. 1988. Fatty and mycolic acids of *Mycobacterium malmoense*. *J. Clin. Microbiol.* **26**:153–154.
14. Wayne, L. G., H. C. Engbaek, H. W. B. Engel, S. Froman, W. Gross, J. Hawkins, W. Kämpfer, A. G. Karlson, H. H. Kleeberg, I. Krasnow, G. P. Kubica, C. McDurmont, E. E. Nel, S. R. Pattyn, K. H. Schröder, S. Showalter, I. Tarnok, M. Tsukamura, B. Vergmann, and E. Wolinsky. 1974. Highly reproducible techniques for use in systematic bacteriology in the genus *Mycobacterium*: tests for pigment, urease, resistance to sodium chloride, hydrolysis of Tween 80, and β -galactosidase. *Int. J. Syst. Bacteriol.* **24**:412–419.