The Mycolic Acids of *Mycobacterium chelonei*

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(Received 13 May 1981)

Two-dimensional thin-layer chromatography of whole-organism acid methanolysates of *Mycobacterium chelonei* gives a characteristic pattern of two non-polar mycolic acid methyl esters which allows the organism to be distinguished from all other mycobacteria including *Mycobacterium fortuitum*. The mycolic acids from *Mycobacterium chelonei* were composed of approximately equal amounts of a diunsaturated α-mycolate and a lower molecular weight α'-mycolate, though minor amounts of a different α-mycolate were also detected. These mycolic acids are the first examples of natural mixtures from mycobacteria lacking major amounts of acids having oxygen functions in addition to the 3-hydroxy acid unit.

**INTRODUCTION**

High molecular weight 3-hydroxy, 2-alkyl-branched fatty acids, the mycolic acids, are characteristic components of the cell envelopes of all strains of *Mycobacterium* examined to date (Minnikin & Goodfellow, 1980). Mycobacterial mycolic acids are homologous compounds having between 60 and 90 carbons, and they usually occur as complex mixtures of so-called α-mycolates and acids having oxygen functions (\(-\text{CO}=\text{O}, \text{-CH-OCH}_3, \text{-COOH}\)) in addition to the 3-hydroxy acid unit. The carbon skeletons of both oxygenated and α-mycolic acids may contain either double bonds or cyclopropane rings and methyl branches. In contrast, mycolic acids from true corynebacteria, nocardiae, rhodococci and related strains have between 20 and 70 carbons, and the only additional functional groups are varying numbers of double bonds (Minnikin & Goodfellow, 1980). A recently developed procedure, involving thin-layer chromatography of acid methanolysates, has revealed the presence of three characteristic patterns of mycobacterial mycolic acid methyl esters (Minnikin et al., 1980). The mycolates of *Mycobacterium tuberculosis*, consisting of α-mycolates, ketomycolates and methoxymycolates, are an example of the first pattern. The second pattern, exemplified by *Mycobacterium avium*, comprises α-mycolates, ketomycolates and an α-carboxymycolate co-occurring with 2-eicosanol and its homologues. In the third pattern, two characteristic unidentified polar mycolates co-occur with α-mycolates in acid methanolysates of *Mycobacterium fortuitum* and *Mycobacterium smegmatis*. The present study shows that a fourth pattern is characteristic of acid methanolysates of representatives of *Mycobacterium chelonei*.

**METHODS**

*Strains and cultivation.* Cultures (Table 1) were maintained on glucose yeast agar (Gordon & Mihm, 1962) at room temperature. Strains were grown in shake flasks of modified Sauton's medium (Mordarska et al., 1972) for 5
to 7 d at 30 °C. The organisms were checked for purity at maximum growth, killed with formaldehyde (1%, v/v), harvested by centrifugation, washed with distilled water and freeze-dried.

Mycolic acid analysis. Freeze-dried bacteria (50 mg) were degraded by acid methanolysis, and petroleum ether (b.p. 60–80 °C) extracts were examined for long chain components by thin-layer chromatography (t.l.c.) as described previously (Minnikin et al., 1980). Analytical two-dimensional t.l.c. was performed using small plates (10 x 10 cm) cut from Merck 5554 silica gel 60 F254 aluminium sheets (20 x 20 cm) using a triple development with petroleum ether (b.p. 60–80 °C)/acetone (95:5, v/v) in the first direction followed by a single development in the second direction with toluene/acetone (97:3, v/v) (Minnikin et al., 1980). The positions of separated components were revealed by spraying with a 10% ethanolic solution of molybdophosphoric acid followed by heating at 120 °C for 15 min. Individual mycolic acid methyl esters were isolated from strains M350, M354, M365 and M366 by preparative t.l.c. on layers (1 mm) of Merck 7748 silica gel PF254, separated bands being detected with ultraviolet light (366 nm).

Mass spectra of mycolic acid methyl esters were taken on an AEI MS9 instrument using a direct insertion probe, an ionizing voltage of 70 eV and a temperature range of 190 to 220 °C. Nuclear magnetic resonance (n.m.r.) proton spectra (220 MHz) were recorded for deuterochloroform solutions by the Physico-Chemical Measurements Unit, Harwell.

RESULTS

Two-dimensional thin-layer chromatography of whole-organism acid methanolysates gave, in all cases, a simple pattern of three major long-chain components as exemplified by the pattern for M. chelonei M350 (Fig. 1); copies of the patterns for all other species examined have been deposited with the British Library Lending Division, Boston Spa, Yorkshire LS23 7BQ, as Supplementary Publication No. SUP 28011 (3 pages). (Copies may be obtained from the BLLD on demand; where possible, requests should be accompanied by prepaid coupons, held by many university and technical libraries and by the British Council.) The most mobile component corresponded to non-hydroxylated fatty acid methyl esters and the other two components had the chromatographic properties of mycolic acid methyl esters (Fig. 1). The relative proportions of the two mycolates appeared to be quite constant in the strains studied.

The mass spectra of both mycolic acid methyl esters from strains M350, M354, M365 and M366 all contained peaks at m/e 382 corresponding to pyrolytic release in the mass spectrometer (Etémadi, 1967a) of methyl tetracosanoate. The spectra of the more polar mycolates from these strains were all very similar and contained peaks at m/e 546 and 574

Table 1. Designation and sources of test strains

<table>
<thead>
<tr>
<th>Mycobacterium chelonei subsp. abscessus</th>
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<tr>
<td>*M353</td>
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<td>M361</td>
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<table>
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<tr>
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<td>*M349</td>
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<tr>
<td>M350</td>
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<td>M351, M352</td>
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<td>M357, M358</td>
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<td>M363</td>
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* Type strains. ATCC, American Type Culture Collection, Rockville, Md., U.S.A.; NCTC, National Collection of Type Cultures, London, U.K.
Mycolic acids of *M. chelonei*

Fig. 1. Two-dimensional t.l.c. of whole-organism acid methanolysate of *M. chelonei* M350. A triple development with petroleum ether (b.p. 60–80 °C)/acetone (95:5, v/v) was used in the first direction followed by a single development with toluene/acetone (97:3, v/v) in the second direction. Abbreviations: F, non-hydroxylated fatty acid methyl esters; A, α-mycolate; A', α'-mycolate.

(main components in italics) corresponding to monounsaturated meroaldehydes having 38 and 40 carbons produced by the pyrolytic decomposition noted above. Complementary pairs of peaks corresponding to anhydromycolates having 62 and 64 carbons were observed at *m/e* 910 and 938.

The less polar mycolates from the four strains under study gave more complex mass spectra. Peaks due to anhydromycolates were not consistently recorded but homologous series of meroaldehyde fragments were readily identified. Peaks of the predominant series were found at *m/e* 698, 726, 754, 782 and 810 corresponding to diunsaturated meroaldehydes having 49, 51, 53, 55 and 57 carbons; peaks of the minor series were found at *m/e* 684, 712, 740, 768 and 796 corresponding to diunsaturated meroaldehydes with 48, 50, 52, 54 and 56 carbons. For the latter minor series the main component in all cases was that at *m/e* 768; for the main series *m/e* 782 was the principal component for strain M365 but in the other three strains *m/e* 754 was the largest peak in this series. Taking together the data for these two series of meroaldehyde fragments and the size of the chain in the 2-position, the least polar mycolates from these strains appear to comprise a minor series having even numbers of carbon atoms (*C*72 to *C*80) and a major series with odd numbers of carbon atoms (*C*73 to *C*81).

Larger amounts of mycolic acid methyl esters were isolated by preparative t.l.c. of the methanolysate from 1300 mg of *M. chelonei* M354 to give 25 mg of the least polar mycolate and 15 mg of the more polar ester (Fig. 1). Examination of both mycolic esters by 220 MHz proton n.m.r. showed the presence of resonances characteristic of the 2-branched, 3-hydroxy methyl ester unit and two terminal methyl groups (Etémadi, 1967b; Minnikin & Polgar, 1966). The more polar mycolate contained a clear triplet (2 H) at δ 5.38 characteristic of a *cis* double bond (Frost & Gunstone, 1975) but for the least polar mycolate a similar triplet at δ 5.38 (2 H) was accompanied by a multiplet (2 H) at δ 5.32. The spectrum of the least polar mycolate also contained a doublet (3 H) at δ 0.95 corresponding to a methyl group on a carbon adjacent to a double bond (Etémadi *et al.*, 1967). Signals characteristic of cyclopropane rings (Minnikin, 1966; Longone & Miller, 1967) were not found in the n.m.r. spectra of either of these mycolic esters.

The experimental data lead to the conclusion that the least polar mycolates from *M. chelonei* (Fig. 1) are the so-called α-mycolates (Etémadi, 1967b). The principal series (*C*73 to
C_{81} appears to correspond closely to the methyl-branched mycolates (C_{71} to C_{83}) having the structure (I), characterized previously from *Mycobacterium smegmatis* (Etézadi, 1967b; Etézadi et al., 1967; Wong et al., 1979). The composition of the minor series (C_{72} to C_{80}) of \( \alpha \)-mycolates cannot be assigned with similar confidence but it is considered more likely that they are similar to the di-cis-alkenic acids (C_{74} to C_{82}) (II) rather than to cyclopropyl mycolic acids (C_{76} to C_{84}) (III), both isolated previously from *M. smegmatis* (Etézadi, 1967b; Etézadi et al., 1967). The most polar mycolates from strains of *M. chelonei* (Fig. 1) correspond very closely to the so-called monounsaturated \( \alpha' \)-mycolates also previously characterized from *M. smegmatis* (Krembel & Etézadi, 1966; Etézadi, 1967b; Wong & Gray, 1979). The mass spectrometric data summarized above indicate the structure (IV) for the \( \alpha' \)-mycolates of *M. chelonei*.

**DISCUSSION**

The mycolic acids from *M. chelonei* strains consist of approximately equal amounts of a diunsaturated \( \alpha \)-mycolate (I) and a lower molecular weight \( \alpha' \)-mycolate (IV); minor amounts of a different \( \alpha \)-mycolate (II) were also detected. Mycolic acids having the same structural types have been characterized previously from *Mycobacterium smegmatis* (Krembel & Etézadi, 1966; Etézadi et al., 1967; Wong et al., 1979; Wong & Gray, 1979). In the case of *M. smegmatis*, however, the \( \alpha' \)-mycolates are usually minor components, though oxygenated, so far uncharacterized, mycolates are also encountered (Minnikin et al., 1980). The mycolic acids of *M. chelonei* are the first examples of natural mixtures from mycobacteria lacking major amounts of acids having oxygen functions in addition to the 3-hydroxy acid unit.

*Mycobacterium chelonei* was established by Bergey et al. (1923) to accommodate Friedmann's (1903) turtle tubercle bacillus. Stanford & Beck (1969) found strains of *Mycobacterium abscessus* (Moore & Frerichs, 1953), *Mycobacterium horstelense* (Bönicke & Stottmeier, 1965) and *Mycobacterium runyonii* (Bojalil et al., 1962) to be similar to the turtle tubercle bacillus and proposed that all three taxa be reduced to synonyms of *M. chelonei*. Subsequently, Stanford et al. (1972) recognized two geographical subgroups of *M. chelonei* and these were later given subspecific rank as *M. chelonei* subsp. *chelonei* and *M.
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*chelonei* subsp. *abcessus* (Kubica et al., 1972). Strains of the two subspecies can be distinguished by a number of biochemical and physiological properties (Stanford et al., 1972; Runyon et al., 1974) but not on the basis of lipid analysis (Jenkins et al., 1971). The present finding that good representatives of the two subspecies contain the same types of mycolic acids is further evidence of the integrity of *M. chelonei*.

*Mycobacterium chelonei* and *Mycobacterium fortuitum* are rather similar and contain strains able to cause abscesses and pulmonary disease in man (Pattyn & Portaels, 1972). Strains of the two species can be distinguished by biochemical and colonial properties (Pattyn et al., 1974), bacteriocin typing (Takeya & Tokiwa, 1972), in vitro antibiotic sensitivity studies (Haas et al., 1973), polyacrylamide gel electrophoresis of cell proteins (Haas et al., 1974) and immunodiffusion studies (Kubica et al., 1972; Stanford et al., 1972; Pattyn et al., 1974) but there is still a need for a simple reliable method to distinguish between them. Thin-layer chromatography of whole-organisms methanolysates provides an easy and accurate way of distinguishing *M. chelonei* from *M. fortuitum* and all other mycobacteria.

Thanks are due to P. Kelly and S. H. Addison for mass spectra and to Judith M. Best and Carole Todd for technical assistance. D. E. M. and M. G. are indebted to the Medical Research Council (G974/522/S) and Science Research Council (GRA 88651); the S. R. C. also financed the 220 MHz proton n.m.r. spectral analysis.

REFERENCES


