Mycolic Acid Patterns of Representative Strains of *Mycobacterium fortuitum*, '*Mycobacterium peregrinum*' and *Mycobacterium smegmatis*

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Representative strains of *Mycobacterium fortuitum*, '*Mycobacterium peregrinum*' and *Mycobacterium smegmatis* were degraded by acid methanolysis and patterns of long-chain compounds were determined by two-dimensional thin-layer chromatography. The same general pattern of mycolic acid methyl esters was found in all 39 strains examined, the major components being so-called α -mycolates and characteristic pairs of polar mycolates. Analysis of alkaline methanolysates of selected strains confirmed that these polar mycolates were derived from epoxymycolic acids, as found previously.

INTRODUCTION

Mycolic acids are characteristic high molecular weight 3-hydroxy 2-alkyl branched fatty acids whose discontinuous distribution is of value in both the classification and identification of mycobacteria (Minnikin & Goodfellow, 1980). Mycobacterial mycolic acids contain between 60 and 90 carbons and different structural types include so-called α -mycolates and acids having oxygen functions ($>C=O$, $>CH-OCH_3$, $-COOH$) in addition to the 3-hydroxy acid unit (Minnikin, 1982). The carbon skeletons of both oxygenated and α -mycolic acids may contain either double bonds or cyclopropane rings and methyl branches may be present. By use of thin-layer chromatography (TLC) of methanolysates of whole organisms (Minnikin *et al.*, 1980) it has been shown that four characteristic patterns of mycobacterial mycolic acid methyl esters are commonly encountered. The mycolic acids of *Mycobacterium tuberculosis*, consisting of α -mycolates, ketomycolates and methoxymycolates, are an example of the first pattern and the second, shown by *Mycobacterium avium* for example, has ω -carboxymycolates and 2-eicosanol and homologues, together with α -mycolates and ketomycolates (Minnikin *et al.*, 1980; Minnikin & Goodfellow, 1980; Minnikin, 1982). A third pattern, found only in *Mycobacterium chelonae* and close relatives, contains only α - and α' -mycolates (Minnikin, 1982; Minnikin *et al.*, 1982*a*). The fourth main pattern discovered in *Mycobacterium fortuitum* and *Mycobacterium smegmatis* (Minnikin *et al.*, 1980) showed the presence of characteristic polar mycolates co-occurring with α -mycolates and this pattern was found to be characteristic of representatives of *Mycobacterium farcinogenes* and *Mycobacterium senegalense* (Ridell *et al.*, 1982). It has been shown that these characteristic polar mycolates are derived from epoxymycolates in these taxa (Daffé *et al.*, 1981; Minnikin *et al.*, 1982*b*). In the present paper mycolic acid patterns of representative strains of *M. fortuitum*, *M. smegmatis* and '*M. peregrinum*' have been investigated to determine their taxonomic value and to assess the distribution of epoxymycolates and their acid methanolysis products.

METHODS

Strains and cultivation. Cultures (Table 1) were maintained on glucose yeast agar (Gordon & Mihm, 1962) at room temperature and cultivated in shake flasks of modified Sauton's medium (Mordarska *et al.*, 1972) for 5 to 7 d

Table 1. *Designation and sources of test strains*

Strain no.	Designation and source*
M32	<i>Mycobacterium fortuitum</i> , Institute d'Hygiene, Lausanne, Switzerland, LA 1263
M61, M62	<i>M. fortuitum</i> , I. Baess, Statens Seruminstitut, Copenhagen, Denmark, F656, F141
M172	<i>M. fortuitum</i> , J. M. Grange, 108; J. Marks, 16575 PC; sputum
M173	<i>M. fortuitum</i> , J. M. Grange, 487; Gatti, 750; abscess
M174	<i>M. fortuitum</i> , J. M. Grange, 5; NCTC 2006; terrapin
M175	<i>M. fortuitum</i> , J. M. Grange, 630; ATCC 23021; human
M195	<i>M. fortuitum</i> , J. M. Grange, 650; ATCC 23041; abscess
M199	<i>M. fortuitum</i> , J. M. Grange, R44; Ugandan mud
M204	<i>M. fortuitum</i> , NCTC 10394; J. C. Cruz 1; cold abscess. Type strain
M226	<i>M. fortuitum</i> , J. M. Grange, 33; abscess
M227	<i>M. fortuitum</i> , J. M. Grange, 109; J. Marks, 18519 PC; sputum
M229	<i>M. fortuitum</i> , J. M. Grange, 113; J. Marks, 37379 PC; sputum
M230	<i>M. fortuitum</i> , J. M. Grange, 443; sputum
M232	<i>M. fortuitum</i> , J. M. Grange, 557; clinical isolate
M233	<i>M. fortuitum</i> , J. M. Grange, 677; abscess
M234	<i>M. fortuitum</i> , J. M. Grange, 678; zoo aquarium
M235	<i>M. fortuitum</i> , J. M. Grange, 802; gastric washings
M236	<i>M. fortuitum</i> , J. M. Grange, 806; frog
M237	<i>M. fortuitum</i> , J. M. Grange, 81; NCTC 2891; frog
M238	<i>M. fortuitum</i> , J. M. Grange, 491; urine
M6	' <i>Mycobacterium peregrinum</i> ', NCTC 10264; bronchial aspirate of child. Type strain.
M418-M423	' <i>M. peregrinum</i> ', P. A. Jenkins, <i>Mycobacterium</i> Reference Unit, University Hospital of Wales, Cardiff, Wales, UK, 7678, 7680, 14654, 14657, 14894, 14895; sputum
M424	' <i>M. peregrinum</i> ', P. A. Jenkins, 16788; peritoneal fluid
M425, M426	' <i>M. peregrinum</i> ', P. A. Jenkins, 17564; 14462; urine
M427	' <i>M. peregrinum</i> ', P. A. Jenkins, 19774; sputum
*M59	<i>Mycobacterium smegmatis</i> , I. Baess, F87
M101	<i>M. smegmatis</i> , ATCC 11468
M215	<i>M. smegmatis</i> , J. M. Grange, 1; NCTC 333
M216	<i>M. smegmatis</i> , J. M. Grange, 2; NCTC 8159. Type strain
M217	<i>M. smegmatis</i> , J. M. Grange, 77; NCTC 10265
M218	<i>M. smegmatis</i> , J. M. Grange, 138; milk
M219	<i>M. smegmatis</i> , J. M. Grange, 607; ATCC 607

* Abbreviations: NCTC, National Collection of Type Cultures, Colindale Avenue, London, UK; ATCC, American Type Culture Collection, Rockville, Maryland, USA.

at 30 °C. After checking for purity at maximum growth, organisms were killed with formaldehyde (1%, v/v), harvested by centrifugation, washed with distilled water and freeze-dried. Bacterial names that do not appear in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980), or subsequent validation lists published in the *International Journal of Systematic Bacteriology*, are written in quotation marks.

Mycolic acid analysis. Acid methanolysates of dry bacteria (50 mg) were prepared as described previously (Minnikin *et al.*, 1980). Alkaline methanolysates (West, 1975; Minnikin *et al.*, 1982*b*) of selected strains were made by treating organisms (50 mg) at 37 °C overnight with 20% methanolic tetramethylammonium hydroxide (Aldrich) (0.5 ml), toluene (0.5 ml) and methanol (1.0 ml). The supernatant was removed after centrifugation and the organisms washed with methanol/toluene (2:1, v/v) (1 ml). Dimethylformamide (3 ml) was added to the combined supernatants and approximately 3 ml of methanol and toluene were removed by evaporation under a stream of nitrogen. After adding a 10% solution of iodomethane in dimethylformamide (1 ml) and mixing for 1 h, the mixture was extracted with petroleum ether (b.p. 60–80 °C) (4 ml followed by 2 × 2 ml). The extracts were evaporated to about 1 ml in volume and the residue was then extracted with more petroleum ether (1 ml followed by 2 × 0.5 ml) to remove residual dimethylformamide. The final extract was evaporated to dryness under a stream of nitrogen.

Analytical two-dimensional TLC (Minnikin *et al.*, 1980) was performed at first on 20 × 20 cm layers (0.5 mm) of Merck 7739 silica gel 60 HF₂₅₄, but subsequently 10 × 10 cm pieces of Merck 5554 silica gel 60 F₂₅₄ aluminium sheets were used. A triple development with petroleum ether (b.p. 60–80 °C)/acetone (95:5, v/v) in the first direction was followed in the second direction by a single development with toluene/acetone (97:3, v/v). The presence of separated components was revealed by spraying with 10% ethanolic molybdophosphoric acid followed by charring at 150 °C for 15 min.

RESULTS AND DISCUSSION

Samples of all the test strains (Table 1) were subjected to acid methanolysis and selected organisms to alkaline methanolysis. The results of TLC analysis of both acid and alkaline methanolysates of representatives of *M. fortuitum*, *M. smegmatis* and '*M. peregrinum*' are shown in Fig. 1. In all three cases acid methanolysates contained the characteristic pairs of polar mycolates (Fig. 1, I, J, N, O) found previously in strains of the former two species (Minnikin *et al.*, 1980; Minnikin, 1982). These components were absent in the alkaline methanolysates (Fig. 1) being replaced by a component 'M' shown in previous studies to be an epoxymycolic acid methyl ester (Minnikin *et al.*, 1982*b*). All three representative strains have components corresponding to α -mycolates and non-hydroxylated fatty acid methyl esters in both types of methanolysates (Fig. 1). The strain of *M. smegmatis* studied has substantial amounts of an α' -mycolate and the '*M. peregrinum*' representative contained a small amount of an uncharacterized acid-stable component labelled 'K' (Fig. 1), whose presence in methanolysates of *M. fortuitum* (Minnikin *et al.*, 1980), *M. farcinogenes* and *M. senegalense* (Minnikin *et al.*, 1982*b*; Ridell *et al.*, 1982) has been previously noted.

TLC analysis of acid methanolysates of the remaining test strains (Table 1) showed that the same general pattern (Fig. 1), containing the characteristic components I, J, N, and O, was present in all cases. Copies of these patterns have been deposited with the British Library Lending Division, Boston Spa, Yorkshire LS23 7BQ, UK, as Supplementary Publication no. SUP 28015 (6 pp.). (Copies may be obtained from the BLLD on demand; wherever possible, requests should be accompanied by prepaid coupons, held by many university and technical libraries and by the British Council.)

In addition to the components F, A, I, J, N and O found in all acid methanolysates some of the minor components were discontinuously distributed. The spot labelled 'L' in the acid methanolysate of '*M. peregrinum*' M6 (Fig. 1) was detected as a minor component in *M. fortuitum* M32, M61, M172, M174, M175, M230, M232, M233, M234 and M238 but not in any of the other strains of '*M. peregrinum*'. The structure of component 'L' is unknown, but, as it is an artefact formed in an erratic manner by acid methanolysis of epoxymycolates (Minnikin *et al.*, 1982*b*), it is not a reliable chemotaxonomic character. Component 'K' is, however, consistently present in both acid and alkaline methanolysates, as shown for '*M. peregrinum*' M6 in Fig. 1, and it may have possible chemotaxonomic value. All strains of *M. fortuitum* and '*M. peregrinum*' contained small amounts of K in their methanolysates with the exceptions of *M. fortuitum* M199, M204 (Fig. 1), M227, M235 and '*M. peregrinum*' M422, M423, M425; this component was present only in one representative, M216, the type strain of *M. smegmatis*.

The other potentially valuable components in methanolysates of these mycobacteria are the lower molecular weight α' -mycolates represented by the spot A' in the methanolysates of *M. smegmatis* M59 (Fig. 1). When large amounts of α' -mycolates are present in methanolysates of, for example, *M. chelonae* (Minnikin *et al.*, 1982*a*) their presence can be readily seen on TLC but small amounts are less easily distinguished from the α -mycolates (spot A, Fig. 1). All representatives of '*M. peregrinum*' appeared to lack α' -mycolates but they were present in all representatives of *M. smegmatis* excepting M216. *Mycobacterium fortuitum* M61, M172, M195, M199, M226, M227, M229, M230, M235 and M237 did not contain clearly recognizable amounts of α' -mycolates. The detection of small amounts of α' -mycolates may require the use of quantitative chromatographic techniques such as high-performance liquid chromatography (Steck *et al.*, 1978).

The related patterns shown by the methanolysates of the representative strains of *M. fortuitum* and *M. smegmatis* confirm and extend the results of an earlier study based upon single strains of the two species (Minnikin *et al.*, 1980). *Mycobacterium fortuitum* and *M. smegmatis* form distinct and separate clusters in numerical phenetic surveys (Kubica *et al.*, 1972; Tsukamura, 1981) and have little DNA in common in homology studies (Baess & Weis-Benzon, 1978; Baess, 1982) but the lipid data raise the possibility that they might have a fairly close phylogenetic relationship. The mycolic acid data do not contradict the classification of '*M. peregrinum*' (Bojalil *et al.*, 1962) as a subjective synonym of *M. fortuitum* (Kubica *et al.*, 1972; Grange & Stanford, 1974; Pattyn *et al.*, 1974; Tsukamura, 1981), although TLC analysis of mycobacterial mycolates does not

Fig. 1. Two-dimensional TLC of whole-organism acid and alkaline methanolysates of *M. fortuitum* M204, *M. smegmatis* M59 and '*M. peregrinum*' M6. 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; K, unknown mycolate; M, epoxymycolate; I, J, L, N and O, components derived from epoxymycolates by acid methanolysis.

necessarily give data sufficiently sensitive for the circumscription of species (Minnikin & Goodfellow, 1980; Ridell *et al.*, 1982). '*Mycobacterium peregrinum*' can, however, be distinguished from *M. fortuitum* using chemical and serological criteria (Jenkins *et al.*, 1971) and it has been suggested that the taxon be revised as a separate species on the basis of results from DNA homology studies (Baess, 1982).

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