A triacyltrehalose containing 2-methyl-branched unsaturated fatty acyl groups isolated from *Mycobacterium fortuitum*

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A variety of glycolipids were found in a collection of strains of *Mycobacterium fortuitum* and two patterns were established by thin-layer chromatography. A compound present in all the strains studied was identified as 2,3,4-triacyltrehalose and its overall structure determined by infrared spectroscopy, nuclear magnetic resonance spectroscopy and gas-liquid chromatography-mass spectrometry. Fatty acyl groups present in this molecule were identified as tetradecanoyl, hexadecanoyl, hexadecanoyl, octadecanoyl, octadecanoyl and a variety of 2-methyl-branched unsaturated (α -methyl, α,β -unsaturated) acyl groups. The 2-methyl-branched compounds ranged from 17 to 21 carbon atoms, the most abundant being 2-methyloctadecen-2-oyl. Trehalose was the only sugar detected in the glycolipid. This substance immunoreacted with IgG and IgM present in sera from tuberculosis patients, as demonstrated by an enzyme-linked immunosorbent assay.

Introduction

Representatives of the genus *Mycobacterium* contain a great variety of glycolipids (Asselineau & Asselineau, 1978; Brennan, 1989; Minnikin, 1982), mostly located in the cell envelope (McNeil & Brennan, 1991; Minnikin, 1991). Their discontinuous distribution in the genus has potential taxonomic value; moreover, many of them are antigenic and constitute, in some cases (e.g. glycopeptidolipids), the molecular basis on which serovars are defined within a given species (Denner *et al.*, 1992). On the other hand, some of them have been implicated in the pathogenesis of infections caused by mycobacteria (Draper, 1989; Rastogi & David, 1988).

Mycobacterium fortuitum, a species mainly involved in skin and soft tissue infections (Good, 1985), currently includes two biovars, *fortuitum* and *peregrinum*; however, some differences were reported in their lipid profiles (Tsang *et al.*, 1984) and recent genetic studies have demonstrated their separate taxonomic status (Kusonoki

& Ezaki, 1992). *M. fortuitum* contains alkali-labile glycolipids, tentatively assigned to the lipo-oligosaccharide type (Tsang *et al.*, 1984), whose structure remains to be elucidated. In the course of a re-examination of the lipid composition of *M. fortuitum* we have found a new trehalose-containing glycolipid differing from those previously described (Daffé *et al.*, 1988, 1989; Lemassu *et al.*, 1991; Minnikin *et al.*, 1985; Ridell *et al.*, 1992). This paper gives data on its structure and immunoreactivity.

Methods

Strains and culture conditions. Twelve strains of *Mycobacterium fortuitum* were analysed: M-50 (ATCC 6841^T), M-59, M-61, M-63, M-64, M-70, M-80, M-327, M-313, M-326 (NCTC 2291), M-430 and M-431 (NCTC 8697). The origin of some of these strains can be found elsewhere (Valero-Guillén & Martín-Luengo, 1983). M-59, M-61, M-63, M-64, M-70 and M-80 are clinical isolates from this laboratory and were identified by lipid and biochemical analyses (Valero-Guillén & Martín-Luengo, 1983). For comparative purposes, *M. peregrinum* M-328, M-472 (ATCC 23041), M-470 (Valero-Guillén & Martín-Luengo, 1983) and M-71 (clinical isolate, this laboratory), and *M. chelonae* TMC 1542 were also included. For recovery of lipids, the strains were grown in Sauton agar at 35 °C for 4 d.

Extraction and analysis of lipids. Wet cells (routinely 100–200 mg) were extracted three times with chloroform/methanol (2:1, v/v) overnight at room temperature. The extracted lipids were evaporated to dryness (N₂, 37 °C), redissolved in 0.5 ml chloroform/methanol

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Abbreviations: ¹H-¹H COSY, homonuclear, ¹H-¹H, chemical shift correlated spectroscopy; ¹H-¹³C HETCOR, heteronuclear, ¹H-¹³C, chemical shift correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer.

Fig. 1. TLC of acetone-soluble lipids from *M. fortuitum* (lane 1, M-80; 2, M-64; 3, M-326; 4, M-50; 5, M-63; 6, M-313; 7, M-61; 10, M-430; 11, M-431 [NCTC 8697]), *M. chelonae* TMC 1542 (lane 8) and *M. peregrinum* M-470 (lane 9). Lane 12 contains a mixture of purified glycolipids A and B. The solvent was chloroform/methanol/water (60:12:1, by vol.). Plates were sprayed with orcinol/H₂SO₄. The positions of glycolipids A and B are indicated.

(2:1, v/v) and precipitated in cold methanol (−20 °C, overnight, three times). The lipids insoluble in methanol were recovered by centrifugation (1500 g, 4 °C, 15 min), and the fraction soluble in this solvent was precipitated with cold acetone (as above). The acetone-soluble extract was evaporated to dryness, redissolved in 0.5 ml chloroform and examined by analytical TLC on aluminium-backed silica gel 60 plates (Merck), employing chloroform/methanol (9:1, v/v) or chloroform/methanol/water (60:12:1, by vol.) as solvent. Plates were sprayed with molybdophosphoric acid (5%, w/v, in ethanol) to detect lipids, and with orcinol (0.2%, w/v, in 40%, v/v, H₂SO₄) or anthrone (1%, w/v, in H₂SO₄) for sugars; plates were heated for 10–15 min at 120 °C. To distinguish between alkali-labile and alkali-stable glycolipids the extracts were subjected to mild alkaline hydrolysis (Brennan *et al.*, 1978).

Structural analysis of the major glycolipid of *M. fortuitum*. Glycolipid A (see Fig. 1) of *M. fortuitum* NCTC 8697 was selected for structural analysis. The glycolipid was purified by silica gel column chromatography. The column was successively eluted with methanol (0, 2, 5, 7, 10 and 15%) in chloroform; the glycolipid was recovered in the 5–7% fractions. Purity was assessed by high-performance TLC (Merck 13727) with chloroform/methanol (2:1, v/v) as solvent. IR spectroscopy was performed in a Nicolet MX-1 apparatus using a NaCl cell. A UV spectrum was recorded in methanol with an Hitachi 220 spectrophotometer. NMR (¹H, ¹H–¹H COSY, ¹³C, DEPT and ¹H–¹³C HETCOR) spectra were obtained with a Varian Unity 300 spectrometer with the sample (20 mg ml^{−1}) in deuteriochloroform. The presence of trehalose was confirmed by the analysis of sugars obtained by the treatment of 2 mg glycolipid with 15% (w/v) KOH in methanol (90 °C, 4 h). The reaction mixture was partitioned between hexane and water and the aqueous fraction was desalted with Amberlite MB3 and evaporated to dryness. The sugars were converted to trimethylsilyl ethers (Daffé *et al.*, 1988) and analysed by GLC, employing a capillary column (DB1, 15 m × 0.3 mm) and a separation temperature from 60 to 100 °C, at 10 °C min^{−1}, then to 180 °C at 6 °C min^{−1}, and finally to 290 °C at 20 °C min^{−1}. The sugars extracted were also hydrolysed in 2 M-CF₃COOH at 120 °C for 1 h; partial hydrolysis was also achieved in 2 M-CF₃COOH at 95 °C for 1 h (Daffé *et al.*, 1991b). The structures of the fatty acids released by saponification were established by GLC and GLC–MS of methyl esters (Valero-Guillén *et al.*, 1987).

Analysis of glycolipid B. The compound designated B (see Fig. 1) was purified by column silica gel chromatography, as for glycolipid A, being recovered in the 10–15% methanol fractions; the presence of trehalose and the structures of the fatty acyl groups were established in a similar way.

Immunoreactivity of glycolipid A. Serological activity was assessed by ELISA (Daffé *et al.*, 1991a) on polystyrene plates (Nunc-Immuno Module MaxiSorp F16). Glycolipid A was dissolved in methanol and 50 µl (2 µg) was applied to the wells; afterwards 50 µl carbonate/bicarbonate buffer (0.05 M, pH 9.6) was added. BSA was employed as a blocking solution and also as diluent for sera (1/100). Affinity-purified peroxidase conjugated to goat anti-human IgG or IgM (Sigma) was employed, and the substrate solution was *o*-nitrophenyldiamine dihydrochloride and sodium perborate in phosphate/citrate buffer (0.05 M, pH 5), prepared from Sigma tablets. Absorbance was read at 492 nm using an automatic ELISA reader (Diagnostic Pasteur). Sixteen sera from clinically and microbiologically diagnosed patients were studied along with five negative (healthy) control sera. All of them were analysed in duplicate.

Results

Glycolipid composition of the strains studied

When acetone-soluble lipids from the different strains were separated by TLC, a variety of glycolipids were seen (Fig. 1). Overall, two patterns could be distinguished in *M. fortuitum*, but both included the compound designated glycolipid A. This compound was alkali-labile, unlike the glycolipids with similar chromatographic mobility present in *M. peregrinum* and *M. chelonae* (these were probably glycopeptidolipids, as previously demonstrated: López Marín *et al.*, 1991; Tsang *et al.*, 1984). That the substance identified as glycolipid A constituted the same molecule in all the *M. fortuitum* strains studied was suggested by the finding that trehalose was the only sugar present, and that the fatty acid profiles were similar (Table 1). Chromatographic behaviour of fatty acyl groups (as methyl esters) from glycolipid A and their characteristic fragments obtained by mass spectrometry are given in Table 2.

The glycolipid designated B in Fig. 1 was characterized as an acyl trehalose, yielding fatty acids identified as hexadecenoic (the major one), hexadecanoic, octadecenoic, octadecanoic and 2-methyloctadecen-2-oic.

Table 1. Fatty acid composition (molar %) of glycolipid A purified from different strains of *M. fortuitum*

Fatty acid*	Strain											
	M-50	M-59	M-61	M-63	M-70	M-80	M-327	M-313	M-326	M-430	M-64	M-431
14:0	2.3	6.2	9.9	5.3	3.3	6.3	4.1	5.1	2.5	2.6	2.4	3.9
16:1†	3.2	4.3	4.5	5.6	3.5	3.2	1.8	7.1	2.3	2.6	1.8	3.6
16:0	21.4	25.7	30.3	26.9	20.7	29.6	29.1	30.0	15.0	14.0	10.2	24.2
2-Me 16:1	2.6	6.5	6.5	5.4	7.2	6.7	6.1	4.7	8.2	8.3	11.4	5.3
18:1	19.2	7.2	3.7	8.3	3.4	5.7	2.1	5.0	4.2	2.2	1.6	6.7
18:0	9.8	8.7	7.5	6.5	10.4	7.2	25.9	8.7	9.1	4.6	3.6	14.3
2-Me 17:1	1.6	3.7	1.4	2.7	1.1	4.1	1.2	2.5	7.8	2.7	2.4	3.5
2-Me 18:2†	6.4	6.3	5.8	6.6	13.9	4.4	4.3	5.6	14.8	10.4	13.2	11.7
2-Me 18:1	21.3	27.0	27.0	28.9	31.0	29.6	22.6	26.4	28.1	46.8	47.8	21.5
2-Me 20:2	8.5	2.5	1.8	2.8	4.5	1.9	2.6	2.7	6.3	2.8	3.8	4.0
2-Me 20:1	3.7	1.8	1.5	1.0	1.0	1.2	1.1	2.1	1.7	2.9	1.6	1.2

* Identified by their number of carbon atoms and double bonds. Me, methyl.

† Eluted as two peaks with identical mass spectra (see Table 2).

Table 2. Chromatographic behaviour and mass spectrum features of fatty acid methyl esters from glycolipid A

Fatty acid*	Retention time relative to 16:0†	Molecular ions and other characteristic fragments
14:0	0.766±0.005	242, 74, 87
16:1a	0.957±0.003	268, 55, 74, 236
16:1b	0.965±0.003	268, 55, 74, 236
16:0	1.000	270, 74, 87
2-Me 16:1	1.121±0.002	282, 55, 88, 101, 250
18:1	1.168±0.003	296, 55, 74, 264
18:0	1.206±0.004	298, 74, 87
2-Me 17:1	1.224±0.004	296, 55, 88, 101, 264
2-Me 18:2a	1.284±0.005	308, 55, 88, 101, 127, 277
2-Me 18:2b	1.298±0.005	308, 55, 88, 101, 127, 277
2-Me 18:1	1.326±0.006	310, 55, 88, 101, 278
2-Me 20:2	1.433±0.008	336, 55, 88, 101, 127, 304
2-Me 20:1	1.504±0.009	338, 55, 88, 101, 306

* See Table 1 for identification of the peaks.

† Mean value±SD of ten different chromatograms.

Structural analysis of glycolipid A

The IR spectrum of glycolipid A shows absorption bands at (cm⁻¹) 808 (α,α' -D-trehalose), 1130–1190 (trehalose esters), 1360 (methyl branch), 1470 (methylene), 1460 (unsaturation in position 2), 2800–2900 (methyne) and 3300 (–OH) (Daffé *et al.*, 1988). The presence of trehalose was also established by GLC analysis of the aqueous extract obtained after saponification; in the conditions described in Methods, this disaccharide eluted at 25.23±0.07 min; moreover, it decomposed into glucose after acid hydrolysis. Glucose and trehalose were both present in the reaction mixture obtained by mild acid hydrolysis.

Lipid material derived from glycolipid A, after saponification or hydrolysis, was identified as exclusively fatty acids by TLC and GLC. Fatty acids ranged from 14 to 21 carbon atoms and were identified by GLC and

GLC–MS as tetradecanoic, hexadecenoic, hexadecanoic, octadecenoic, octadecanoic, 2-methylhexadecen-2-oic, 2-methylheptadecen-2-oic, 2-methylnonadecadienoic (one double bond in position 2), 2-methyloctadecen-2-oic, 2-methyleicosadienoic (one double bond in position 2) and 2-methyleicosen-2-oic. The predominant fatty acids were hexadecanoic and 2-methyloctadecen-2-oic (Table 1). The location of a double bond in position 2 in methyl-branched acids (α -methyl, α,β -unsaturated) was deduced from the UV spectrum (absorption at 219 nm), IR spectrum (as mentioned above), chromatographic behaviour (Table 2) and also by some details provided by NMR (see below). Moreover, the complete structure of 2-methyloctadecen-2-oic acid has been previously reported (Valero-Guillén *et al.*, 1987). The mass spectra of the 2-methyl-branched unsaturated acid methyl esters showed, in general, a base peak at $m/z = 88$ (Ryhaage *et al.*, 1961), a fragment at $m/z = M^+ - 32$ (or $M^+ - 31$) (loss of methanol) and a decreased intensity of the fragment at $m/z = 74$, the base peak for the straight-chain series (Table 2).

The ¹H NMR spectrum of glycolipid A (Fig. 2) contained signals from 0.86 to 6.74 p.p.m., with a typical triplet centred at 0.87 p.p.m. (terminal –CH₃), and a broad singlet at 1.25 p.p.m. (–CH₂–). A signal at 1.71 p.p.m. was attributed to methyl branch, and those at 5.34 and 6.74 p.p.m. to –CH=CH– (*cis*-double bond) and –CH=C(CH₃) (conjugated double bond), respectively, confirming some evidence of the lipid moiety of the molecule. Anomeric and other deshielded protons were located between 4.80 and 5.80 p.p.m., with signals centred at 4.85, 4.98, 5.19, 5.49 and 5.77 p.p.m. (Fig. 2). The signal centred at 5.19 p.p.m. is assigned to H-1' of trehalose (Daffé *et al.*, 1988, 1989) and those at 4.85 and 4.98 p.p.m. to a deshielded H-2, H-2' and/or H-4, H-4'. The assignments of these and the remaining signals were

Fig. 2. ^1H NMR spectrum of glycolipid A from *M. fortuitum* M-431 (NCTC 8697) (300 MHz, CDCl_3). The labelled resonances (expanded region) correspond to several protons of trehalose (see Table 3).

Table 3. ^1H - ^1H NMR analysis of glycolipid A: chemical shifts of the protons assigned to trehalose

Protons	Chemical shift (p.p.m.)
H-1	5.49
H-2	4.85
H-3	5.77
H-4	4.98
H-5	4.06
H-6	ND
H-1'	5.19
H-2'	3.57
H-3'	4.10
H-4', H-5', H-6'	ND

ND, Not determined (included in the region 3.00–4.00 in each case).

achieved by ^1H - ^1H COSY (spectrum not shown; see Table 3): connectivities of H-1 through H-5 resonances, and of H-1' through H-3' resonances, were observed. Moreover, it was calculated that $J_{1,2} = 3.7$ Hz, $J_{2,3} =$

Fig. 3. ^{13}C NMR spectrum of glycolipid A from *M. fortuitum* M-431 (NCTC 8697). Principal signals assigned to the lipid moiety resonances and trehalose carbon resonances are illustrated.

9.9 Hz and $J_{3,4} = 9.7$ Hz. These data indicated: (1) an α, α' configuration for trehalose, and (2) the presence of acyl groups in positions 2, 3 and 4 of trehalose. The ^{13}C spectrum (Fig. 3) confirmed the presence of trehalose, since two signals were obtained in the region of anomeric carbons: 92.28 and 95.53 p.p.m., which corresponded, respectively, to C-1 and C-1', as deduced from ^1H - ^{13}C HETCOR. This technique combined with ^{13}C DEPT established the location of C-2 to C-6 and C-2' to C-6' between 60.74 and 73.62 p.p.m. Other signals given by ^{13}C NMR confirmed some details of the lipid moiety: α, β -unsaturation (145.49 p.p.m.), *cis*-double bond (129.53 p.p.m.), methyl branch in position 2 (126.26 p.p.m.) and carbonyl groups (three signals centred at about 167 p.p.m.), thus supporting the notion that glycolipid A was a 2,3,4-triacyltrehalose, with 2-methyl-branched unsaturated fatty acyl groups.

Immunoreactivity of glycolipid A

A_{492} values for the sera tested are given in Table 4. The cut-off point was established at 0.120 for IgG and at 0.123 for IgM (mean values of respective controls plus

Table 4. Reactivity of sera from tuberculosis and controls to glycolipid A

	A_{492}		Seropositivity rate	
	IgG	IgM	IgG	IgM
Tuberculosis patients ($n = 16$)	0.173 ± 0.103	0.124 ± 0.072	12 (75%)	10 (62%)
Controls ($n = 5$)	0.054 ± 0.022	0.054 ± 0.023	0	0

3 $\times$ SD). Twelve sera were considered positive for IgG and 10 for IgM. All control sera were negative.

Discussion

Acylated trehaloses, other than cord-factor (Asselineau & Asselineau, 1978), sulpholipids (Goren *et al.*, 1976) and those containing phleates (Asselineau *et al.*, 1972), have been investigated in a limited number of mycobacteria and detailed structures are only known for *Mycobacterium tuberculosis* (Daffé *et al.*, 1988, 1989; Lemassu *et al.*, 1991; Minnikin *et al.*, 1985; Ridell *et al.*, 1992) and a strain named '*M. fortuitum*' (Vilkas & Rojas, 1964). Trehalose, however, also forms part of the oligosaccharide structure of other glycolipids (lipo-oligosaccharides) characterized in *M. kansasii* (Hunter *et al.*, 1983), *M. szulgai* (Hunter *et al.*, 1988), *M. malmoense* (McNeil *et al.*, 1987), *M. tuberculosis* (Daffé *et al.*, 1991 b) and *M. smegmatis* (Kamisango *et al.*, 1985). This disaccharide is always acylated at several positions, so that acylated trehaloses could constitute a simplified version of these molecules.

In *M. tuberculosis* two acylated trehaloses have recently been described (Daffé *et al.*, 1988, 1989; Lemassu *et al.*, 1991; Minnikin *et al.*, 1985; Ridell *et al.*, 1992), differing in the number and location of acyl substituents: a polyacylated trehalose and a diacyl trehalose. The polyacylated molecule contained five fatty acyl substituents, at positions 2, 2', 3', 4 and 6', most of them being phthienoyl (mycolipenyl) groups, with minor amounts of straight-chain components (Daffé *et al.*, 1988; Minnikin *et al.*, 1985). The structures of the fatty acids from the diacyl molecule were different from the penta-acylated compound, and they occupied positions 2 and 3 of one glucose residue of trehalose (Lemassu *et al.*, 1991; Ridell *et al.*, 1992).

Two diacyltrehaloses were reported in a strain named '*M. fortuitum*': a 6,6'-diacyltrehalose (with hexadecanoyl and octadecanoyl groups) (Vilkas & Rojas, 1964) and a 4,6-diacyltrehalose (Vilkas *et al.*, 1968). Although no detailed structures for acyl groups from the second compound were given, the authors pointed out that they contained 19 carbon atoms with a molecular mass (296 Da) that, according to our results, could satisfy the structure of a methyloctadecenoyl fatty acyl group. However, these glycolipids differed in the location and nature of acyl substituents from glycolipid A described in this work. Based on preliminary ¹H NMR spectra of glycolipid B (unpublished observations), it seems that this substance may represent a mixture of the two acylated trehalose homologues previously found in the strain of '*M. fortuitum*' (Vilkas & Rojas, 1964; Vilkas *et al.*, 1968).

The triacyl nature of glycolipid A was justified by the

molar ratio of trehalose to fatty acyl groups (1/3), in part by ¹³C NMR (three signals for carbonyl groups) and also by ¹H NMR spectra (chemical shift of H-3). In the last case, the chemical shift for H-3 seemed a little high, as compared with data from related substances (Daffé *et al.*, 1988, 1989; Lemassu *et al.*, 1991) but the difference could be attributed to the effects caused by the additional substitution at position 4 of trehalose (M. Daffé, personal communication). From the ¹H NMR data, and taking into account the integrated proton signal, we deduce the presence of two 2-methyl unsaturated fatty acyl substituents (mainly 2-methyloctadecen-2-oyl) and one straight-chain fatty acyl group (mainly hexadecanoyl).

The complex composition of fatty acids from glycolipid A appeared different from that described for 2,3-diacyltrehalose of *M. tuberculosis* (Lemassu *et al.*, 1991). Although the overall structures of the fatty acyl groups from both glycolipids are mainly of the methyl-branched type, differences in the chain length and number of methyl groups must be pointed out. The di-unsaturated nature of some methyl branched fatty acids from glycolipid A was unexpected, although they have been previously described in *M. tuberculosis* (see Minnikin, 1982).

The presence of three acyl groups on one glucose residue of the trehalose indicated that, like most glycolipids from *Mycobacterium* (Brennan, 1989), glycolipid A is an amphipathic molecule. In accordance with the structural molecular models proposed for the wall of these bacteria (McNeil & Brennan, 1991; Minnikin, 1982, 1991) a location on the cell envelope of *M. fortuitum* was postulated and led us to consider the possible antigenicity of glycolipid A. Previous reports on the antigenicity of 2,3-diacyltrehalose from *M. tuberculosis* (Minnikin *et al.*, 1987; Papa *et al.*, 1989; Ridell *et al.*, 1992) indicated that such a component was one of the strongest antigens among the variety of glycolipids characterized in this species (Daffé *et al.*, 1989; Ridell *et al.*, 1992). By an ELISA method, glycolipid A proved to be immunoreactive, being recognized by both IgG and IgM; however, more extensive analysis would be required to check the possible use of this substance in serological diagnosis of mycobacteriosis and/or tuberculosis. The use of some glycolipids in this context has been recently questioned (Daffé *et al.*, 1991a) and the predictive value of antibodies in tuberculosis seems to be very low (Daniel, 1989; Ivanyi *et al.*, 1988).

In conclusion, *M. fortuitum* contains an unusual trehalose-containing glycolipid that is antigenic. The taxonomic implication of the overall structure of this compound is clear and was anticipated by others when alkali-labile glycolipids were detected in *M. fortuitum* unlike *M. peregrinum* (Tsang *et al.*, 1984), which

possesses glycopeptidolipids (López Marín *et al.*, 1991). The evaluation of these components favours the separation of these species, as recently proposed (Kusonoki & Ezaki, 1992).

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