A family of diacyltrehaloses isolated from *Mycobacterium fortuitum*

Maria A. Ariza, Francisco Martín-Luengo and Pedro L. Valero-Guillén

Author for correspondence: Pedro L. Valero-Guillén. Tel: +34 68 307100. Fax: +34 68 364150.

Departamento de Genética y Microbiología, Facultad de Medicina, Universidad de Murcia, 30100 Murcia, Spain

A trehalose-containing glycolipid was detected in several strains of *Mycobacterium fortuitum* and characterized as 2,3-di-O-acyltrehalose (DAT) by combined NMR spectroscopy, IR spectroscopy, GLC and GLC-MS. Lipid constituents of the molecule were identified as a mixture of straight-chain (14–18 carbon atoms) and methyl-branched-chain (17–21 carbon atoms) fatty acyl groups. DAT was further fractionated by reverse phase TLC into four fractions that were designated DAT-I–DAT-IV. DAT-I contained 70–75% straight-chain acyl substituents (hexadecanoyl and octadecanoyl predominating) and 25–30% 2-methyl branched substituents (mainly 2-methyl octadecadienoyl). DAT-II was composed of a mixture in which the acyl groups were almost exclusively 2-methyl branched, with 2-methyl octadecadienoyl and 2-methyl octadecen-2-oyl predominating. DAT-III, which was the major isolated fraction, consisted of compounds in which the ratio linear to branched acyl groups varied between 0.8 to 0.9, 2-methyl octadecen-2-oyl, hexadecanoyl and octadecanoyl being the most abundant. Finally, DAT-IV comprised a mixture of DAT molecules containing mostly 2-methyl octadecadienoyl, 2-methyl octadecen-2-oyl, 2-methyl eicosadienoyl and 2-methyl eicosen-2-oyl groups.

Keywords: *Mycobacterium fortuitum*, glycolipid, acyltrehalose

INTRODUCTION

Members of the genus *Mycobacterium* contain a great variety of glycolipids that can be classified into several structural types (Brennan, 1989). These molecules may play an important structural role, because most of them are located in the cell wall envelope (McNeil & Brennan, 1991; Minnikin, 1982, 1991). They also show potential taxonomic value and, in some cases, constitute the basis for the definition of serovars within a given species (Denner *et al.*, 1992). Some of these glycolipids are able to provoke non-specific membrane alterations (Fournié *et al.*, 1989; Sut *et al.*, 1990) and several physiopathological responses (Brownback & Barrow, 1988; Pabst *et al.*, 1988; Silva & Faccioli, 1988) and have been implicated in the pathogenesis of the infections produced by mycobacteria (Draper, 1989; Rastogi & David, 1988).

Apart from cord-factor (Asselineau & Asselineau, 1978), sulpholipids (Goren *et al.*, 1976) and other related trehalose-containing glycolipids (Asselineau &

Asselineau, 1978), acylated trehaloses have been described in detail only in the species *M. tuberculosis* (Daffé *et al.*, 1988; Minnikin *et al.*, 1985) and *M. fortuitum* (Gautier *et al.*, 1992; Hamid *et al.*, 1993a; Sempere *et al.*, 1993). Some structural similarities have been observed among the acyltrehaloses of the forementioned species; thus, both of them contain diacyl (Baer, 1993; Besra *et al.*, 1992a; Gautier *et al.*, 1992; Hamid *et al.*, 1993a; Lemassu *et al.*, 1991) and polyacyl (Daffé *et al.*, 1988; Gautier *et al.*, 1992; Minnikin *et al.*, 1985; Sempere *et al.*, 1993) derivatives of the disaccharide.

The diacyltrehalose (DAT) of *M. fortuitum* was only studied by Gautier *et al.* (1992) in the type strain of the species and characterized as a 2,3-di-O-acyltrehalose, with both straight-chain and 2-methyl branched fatty acyl substituents. In other studies, 2,3-di-O-acyltrehaloses were identified in 10 strains of *M. fortuitum* and their possible presence indicated in strains currently assigned to *M. senegalense* (Hamid *et al.*, 1993b). This structure appears to be related to that present in *M. tuberculosis* (Baer, 1993; Besra *et al.*, 1992a; Lemassu *et al.*, 1991). In a recent study of a collection of strains of *M. fortuitum* (Sempere *et al.*, 1993), we found two trehalose-containing glycolipids, one of which was not accurately identified (glycolipid B,

Abbreviations: DAT, 2,3-di-O-acyltrehalose; ¹H-¹H COSY, homonuclear ¹H-¹H chemical shift correlated spectroscopy; RPTLC, reverse phase thin-layer chromatography.

Fig. 1. ^1H NMR spectrum of DAT from *M. fortuitum* M-431 [300 MHz, $\text{CDCl}_3/\text{MeOD}$ (2:1, v/v)]. The labelled resonances correspond to several protons of the trehalose (see Table 1) and the lipid portion of the molecule. t, terminal; b, branched; MeOD, deuteromethanol.

see Sempere *et al.*, 1993). We have readdressed our attention to this substance and now confirm its structure as 2,3-di-*O*-acyltrehalose, similar to that of *M. fortuitum* type strain (Gautier *et al.*, 1992; Hamid *et al.*, 1993a). Our study extends this previous finding and, moreover, defines DAT as a mixture of four major compounds varying in the type and combinations of fatty acyl substituents. A more precise structural elucidation of this molecule is interesting because of its relationship to the antigenic DAT of *M. tuberculosis* (Minnikin *et al.*, 1987; Papa *et al.*, 1989; Ridell *et al.*, 1992), formerly named glycolipid SL-IV (Daffé *et al.*, 1989), for which some preliminary clinical studies have been reported (Martín-Casabona *et al.*, 1992; Ridell *et al.*, 1992).

METHODS

Strains and culture conditions. Strains M-50 (ATCC 6841^T), M-59, M-61, M-63, M-64, M-70, M-80, M-313, M-326, M-327, M-430 and M-431 (NCTC 8697) included in a previous work (Sempere *et al.*, 1993) were reanalysed in the present study, together with two new clinical isolates: M-855 and M-858. For recovery of lipids, the strains were grown in Sauton broth at 35 °C for 10 d.

Extraction and analysis of lipids. Cells were recovered by filtration and extracted overnight at 4 °C, first with chloroform/methanol (1:1, v/v), then with chloroform/methanol (2:1, v/v). The lipid extracts were combined, evaporated to dryness under N_2 (40 °C) and partitioned in chloroform/methanol/water (8:4:3, by vol.). Lipids were recovered from the lower (chloroform) phase, dried, redissolved in chloroform and analysed by TLC on aluminium-backed silica gel 60 plates (Merck), employing 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% H_2SO_4) for sugars.

Structural analysis of DAT. The diacyltrehalose, or glycolipid B (see Sempere *et al.*, 1993), was purified by column and TLC silica gel chromatography. The column was successively eluted with methanol (0, 2, 5, 7, 10, 15, 20 and 40%) in chloroform, DAT being recovered in the 10–15% methanol fractions. Final

purification was achieved by preparative TLC on plastic-backed silica gel 60 F₂₅₄ plates (Merck), with chloroform/methanol/water (60:12:1, by vol.) as solvent. DAT was detected under UV light (254 nm) and eluted from the gel with chloroform/methanol (9:1, v/v).

IR spectroscopy was performed in a Nicolet MX-1 apparatus using a NaCl cell. NMR (^1H , ^1H - ^1H COSY, and ^{13}C) spectra were obtained in a Varian Unity 300 spectrometer with the sample (15 mg ml⁻¹) dissolved in deuteriochloroform/deuteromethanol (2:1, v/v). The presence of trehalose was confirmed by GLC as previously established (Sempere *et al.*, 1993) and the structure of fatty acyl substituents determined by GLC and GLC-MS as reported previously (Valero-Guillén *et al.*, 1987).

Fractionation of DAT. Purified DATs from strains M-431, M-855 and M-858 were fractionated by reverse-phase TLC (RP-TLC) employing RP-18F₂₅₄ Splates (Merck), and chloroform/methanol/water (6:15:0.1, by vol.) as developing solvent (Besra *et al.*, 1992a). Separated components were visualized under UV light (254 nm) and eluted from the gel with chloroform/methanol (9:1, v/v). Trehalose and fatty acyl groups of the different DATs were determined as above. Fatty acid methyl esters were purified by preparative silica gel TLC, with dichloromethane as solvent, before GLC analysis, since a component of the RP-18 plates presented a similar retention time to that of 2-methyl heptadecen-2-oic methyl ester.

RESULTS

Structure of DAT

The whole glycolipid patterns of most strains studied have already been reported (Sempere *et al.*, 1993), and the current results confirm previous findings. Strains M-855 and M-858 contained two glycolipids, the more apolar corresponded to a 2,3,4-tri-*O*-acyltrehalose and the more polar to the designated glycolipid B (Sempere *et al.*, 1993), whose detailed structure is the subject of the present study.

As previously described (Sempere *et al.*, 1993), the glycolipid fraction under investigation contained tre-

Table 1. ^1H - ^1H COSY analysis of DAT of *M. fortuitum* M-431: chemical shifts of the protons assigned to trehalose

Proton	Chemical shift (p.p.m.)
H-1	5.29
H-2	4.80
H-3	5.45
H-4	3.44
H-5	4.08
H-6	3.38
H-1'	5.03
H-2'	3.76
H-3', H-4', H-5', H-6'	ND

ND, not detected (included in the region 3.4–4.0 p.p.m. in each case).

halose as the only sugar compound. The fatty acyl constituents were now precisely characterized as tetradecanoyl (14:0) (2–4%), hexadecenoyl (16:1) (3–6%), hexadecanoyl (16:0) (15–25%), 2-methyl hexadecen-2-oyl (2-Me 16:1) (3–6%), octadecenoyl (18:1) (3–7%), octadecanoyl (18:0) (8–20%), 2-methyl heptadecen-2-oyl (2-Me 17:1) (3–7%), 2-methyl octadecadienoyl (one double bond at position 2) (2-Me 18:2) (9–15%), 2-methyl octadecen-2-oyl (2-Me 18:1) (20–35%), 2-methyl eicosadienoyl (one double bond at position 2) (2-Me 20:2) (3–8%) and 2-methyl eicosen-2-oyl (2-Me 20:1) (2–5%). GLC profiles and mass spectra of these compounds were identical to those liberated from the triacyltrehalose of the same strains (Sempere *et al.*, 1993). IR spectra corroborated the presence of trehalose (absorption band at 808 cm^{-1}), and methyl branch (1360 cm^{-1}), and unsaturation at position 2 (1460 cm^{-1}) in the lipid portion of the molecule.

The ^1H NMR spectrum (Fig. 1) revealed signals between 0.8 p.p.m. and 6.7 p.p.m., with characteristic signals

centred at 0.8 p.p.m. (terminal $-\text{CH}_3$), 1.2 p.p.m. ($-\text{CH}_2-$), 1.71 p.p.m. (methyl branch), 1.93 p.p.m. ($-\text{CH}_2-\text{CH}=\text{CH}-$), 2.04 p.p.m. ($-\text{CH}_2-\text{CH}=\text{C}-\text{CH}_3$), 5.26 p.p.m. ($-\text{CH}=\text{CH}-$) and 6.65 p.p.m. ($-\text{CH}_2-\text{CH}=\text{C}-\text{CH}_3$). Anomeric protons of the trehalose resonated at 5.29 p.p.m. (H-1) and 5.03 p.p.m. (H-1'). Other ring protons of the disaccharide were assigned with the aid of ^1H - ^1H COSY NMR (Table 1): connectivities from H-1 through H-6 resonances, and from H-1' to H-2' resonances were observed. In contrast, $J_{1,2}$ was $< 4\text{ Hz}$, and $J_{2,3}$ and $J_{3,4}$ were 9.9 Hz. These data clearly proved an α,α' configuration for trehalose, and the presence of two acyl groups at position 2 and 3 (chemical shift of H-2 = 4.80 p.p.m.; chemical shift of H-3 = 5.45 p.p.m.) of the same glucosyl residue of the trehalose. These data enabled us to identify the glycolipid as 2,3-di-O-acyltrehalose.

In the ^{13}C NMR spectrum (Fig. 2) we found two signals for the anomeric carbons at 91.89 p.p.m. (C-1) and 94.80 p.p.m. (C-1'). The resonances of C-2 to C-5 and C2' to C5' were located between 69.0 p.p.m. and 75.0 p.p.m. Two resonances situated at 60.99 p.p.m. and 61.63 p.p.m. were attributed to the C-6 carbons of the sugar. The remaining signals of this spectrum confirmed the structural details of the fatty acyl carbons of the molecule, thus supporting the identification proposed above.

Fractionation of DAT

DATs from strains M-431, M-855 and M-858 were subjected to fractionation by RPTLC employing the solvent system described by Besra *et al.* (1992a). Four spots were detected and designated DAT-I ($R_F = 0.49$), DAT-II ($R_F = 0.46$), DAT-III ($R_F = 0.43$) and DAT-IV ($R_F = 0.39$) (Fig. 3). Densitometric determinations of the intensities of the spots, indicated that the major component was DAT-III (60%), followed by DAT-II (25%), DAT-IV (10%) and DAT-I (5%).

The different DATs were degraded by saponification and the liberated acids identified and quantified by GLC as

Fig. 2. ^{13}C NMR spectrum of DAT from *M. fortuitum* M-431. Principal signals assigned to the lipid moiety resonances and trehalose carbon resonances are illustrated. MeOD, deuteromethanol.

Fig. 3. RPTLC (10 × 10 cm) of DAT from *M. fortuitum* M-431. The solvent was chloroform/methanol/water (6:15:0.1, by vol.). Plates were sprayed with molybdophosphoric acid (5%, w/v, in ethanol). The positions of DAT-I–IV are indicated. O, origin; F, solvent front.

methyl esters. Table 2 shows the molar percentages of these compounds found in the four DATs of the three strains of *M. fortuitum* examined.

In DAT-I the straight-chain fatty acyl groups (70–75%) predominated over the methyl-branched ones (25–30%). In this case, and given the composition of the remaining DATs (see below), we assumed that this family was composed of two mixtures of DATs: DAT-Ia, with only straight-chain acyl substituents (a main component could be formulated as hexadecanoyl, octadecanoyl trehalose) and DAT-Ib, with two branched-chain substituents (with a probable main component formulated as di-2-methyl octadecadienoyl trehalose).

DAT-II of the strain M-855 (Table 2) was composed of mixtures of DATs in which the lipid constituents were exclusively methyl-branched fatty acyl groups. The more abundant molecular species could come from the combination of 2-Me 18:2 and 2-Me 18:1 with trehalose. The presence of linear acyl groups in DAT-II of M-431 and M-855 strains (Table 2) would derive from partial mixtures of DAT-II and DAT-III and/or DAT-I, taking into account their close TLC mobilities.

In DAT-III the fatty acyl groups ranged from 14 to 21 carbon atoms, the predominant being 16:0, 18:0 and 2-Me 18:1. The ratio straight-chain to branched-chain fatty acyl groups varied between 0.8 and 0.9.

Finally, in DAT-IV only methyl branched acyl groups were found and identified as 2-Me 18:2, 2-Me 18:1, 2-Me 20:2 and 2-Me 20:1. From the data of Table 2, a principal component comprising the combination of 2-Me 18:1 and 2-Me 20:1 with trehalose was assumed for this DAT.

DISCUSSION

From the general data presented in this work, it is now clear that the lipid formerly named 'glycolipid B' (Sempere *et al.*, 1993) is a 2,3-di-*O*-acyltrehalose, similar to

Table 2. Fatty acid composition of different DATs of *M. fortuitum*

Fatty acids were identified by the number of carbon atoms and double bonds. Me, methyl; –, not detected. The results are the mean values of two different analyses of the same lipid fraction (the SD value for the major fatty acid(s) in each DAT was < 5%).

Strain	DAT	Fatty acids (molar %)										
		14:0	16:1	16:0	2-Me 16:1	18:1	18:0	2-Me 17:1	2-Me 18:2	2-Me 18:1	2-Me 20:2	2-Me 20:1
M-431	I	8	4	29	12	11	15	5	16	—	—	—
	II	—	—	7	10	2	8	13	17	31	11	1
	III	2	2	16	2	3	25	2	5	35	5	3
	IV	—	—	—	—	—	—	—	12	48	10	30
M-855	I	5	4	24	5	10	26	—	21	—	—	—
	II	—	—	—	7	—	—	11	34	40	6	2
	III	6	1	18	—	4	16	5	10	30	5	3
	IV	—	—	—	—	—	—	—	18	41	10	31
M-858	I	5	9	21	5	16	18	4	20	—	—	—
	II	—	—	11	8	6	9	8	30	19	7	1
	III	2	2	10	2	5	26	6	5	35	5	2
	IV	—	—	—	—	—	—	—	11	46	11	32

the lipid described for the type strain of *M. fortuitum* (Gautier *et al.*, 1992). Our results also confirm that this glycolipid is widely distributed in the species, thus representing a useful chemical taxonomic tool, like its homologous 2,3,4-tri-*O*-acyltrehalose, in the differentiation of members of the *M. fortuitum* complex (*M. fortuitum*, *M. peregrinum*, *M. abscessus*, *M. chelonae*) that contain specific glycolipid profiles (Hamid *et al.*, 1993b; López-Marín *et al.*, 1991; Tsang *et al.*, 1984). DAT is an amphipathic molecule because the two acyl substituents occupy the same glucosyl residue. In accordance with the molecular models of the cell envelope of mycobacteria (McNeil & Brennan, 1981; Minnikin, 1982, 1991) DAT could be located in the cell wall, interacting with the hydrophobic part of the mycoloyl-arabinogalactan, or even in the plasma membrane.

The overall structure of *M. fortuitum* DAT is strikingly similar to that of *M. tuberculosis* (Baer, 1993; Besra *et al.*, 1992a; Lemassu *et al.*, 1991). However, it is also evident that branched fatty acyl groups are structurally more complex in the latter species, where the presence of mixtures of straight-chain fatty acyl substituents and mycosanoyl, mycolipanoyl and phthienoyl groups has been documented (Baer, 1993; Besra *et al.*, 1992a; Lemassu *et al.*, 1991; Minnikin *et al.*, 1985). Resolution of DAT from *M. tuberculosis* by TLC yielded four families of glycolipids: the major ones were resolved by RPTLC (Besra *et al.*, 1992a). A glycolipid (DAT 1a) obtained in such a way, was characterized as a DAT in which octadecanoyl was located at position 2 and mycosanoyl (2,4-dimethyl docosanoyl) was at position 3 of the same glucosyl residue (Besra *et al.*, 1992a). From the fast-atom bombardment mass spectra (Baer, 1993; Lemassu *et al.*, 1991) and lipid analysis of other resolved DATs (Besra *et al.*, 1992a), it seems that the overall fatty acyl distribution in DATs of *M. tuberculosis* is similar to that of DAT 1a, that is one straight-chain acyl group and one branched-chain acyl group per molecule.

Taking into account the data of Table 2 it can be suggested that in *M. fortuitum*, unlike *M. tuberculosis*, three different combinations of fatty acyl substituents are present in its DATs: (1) straight-chain plus straight-chain (DAT-Ia), (2) branched-chain plus branched-chain (DAT-Ib, DAT-II and DAT-IV), and (3) straight-chain plus branched-chain (DAT-III) (note that the linear to branched acyl group ratio is approximately one). DAT-III is the principal and could be considered the more closely related to DATs of *M. tuberculosis*. At present, however, it is not known if DAT-III follows the same pattern of substitution as that found in DAT 1a.

DATs from *M. tuberculosis* have been envisaged as simplified versions of lipo-oligosaccharides (LOS) in which an acylated trehalose is also an integral part of the molecule (Brennan, 1989). Both LOS and DATs are present in several strains of *M. tuberculosis* (Lemassu *et al.*, 1992), but similar evidence is not thus far available for *M. fortuitum*. However, in a recent investigation (Besra *et al.*, 1992b), a tetra-*O*-acyl triglucosyl glycolipid has been described in the latter species. The structure of the acyl substituents suggests a relation between this compound

and the DATs and triacyltrehaloses previously characterized in *M. fortuitum* (Gautier *et al.*, 1992; Hamid *et al.*, 1993a; Sempere *et al.*, 1993), although clearly more extended analyses are required to clarify if LOS and DATs coexist in this species.

Another point arising from this work is the possible antigenicity of the DATs of *M. fortuitum*. As mentioned, these compounds present an overall structure related to the strong antigenic DAT of *M. tuberculosis* (Papa *et al.*, 1989; Ridell *et al.*, 1992). Acyltrehaloses from *M. fortuitum* have been shown to be antigenic (Hamid *et al.*, 1993a) and the relative structural simplicity of DAT raises the possibility of its chemical synthesis for use in serodiagnosis (Baer & Wu, 1993; Wallace & Minnikin, 1993).

ACKNOWLEDGEMENTS

This work was supported by contract FISS 93/0463, Ministerio de Sanidad y Consumo, Spain. Thanks are due to Dr M. Gacto (Departamento Genética y Microbiología, Universidad de Murcia, Spain) for the revision of the manuscript.

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Received 20 December 1993; revised 21 February 1994; accepted 14 March 1994.