A family of diacyltrehaloses isolated from *Mycobacterium fortuitum*

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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), sulphotrehaloses (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., 1993; 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,

**Keywords:** *Mycobacterium fortuitum*, glycolipid, acyltrehalose
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 see Sempere et al., 1993). Our study extends this previous finding and, moreover, defines DAT as a mixture of four major compounds varying in type strain (Gautier et al., 1992; Hamid et al., 1993a). A more precise structural elucidation of this molecule is interesting because of its relationship to the antigenic type and combinations of fatty acyl substituents. A more precise structural elucidation of this molecule is interesting because of its relationship to the antigenic

**METHODS**

**Strains and culture conditions.** Strains M-50 (ATCC 6841T), 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 study (Sempere et al., 1993) were reanalysed in the present study. Cells were recovered by filtration and stored overnight at 4 °C in Sauton broth at 35 °C for 10 d.

**Extraction and analysis of lipids.** Cells were recovered by filtration and stored overnight at 4 °C in Sauton broth at 35 °C for 10 d.

**Fractionation of DAT.** Purified DATs from strains M-431, M-855 and M-858 were fractionated by reverse-phase T.l.c. (RPTLC) employing RP-18F254 plates (Merck), 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, using a component of the RP-18 plates presented a similar retention time to that of 2-methyl heptadecene-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 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. $^1$H-$^1$H COSY analysis of DAT of M. fortuitum M-431: chemical shifts of the protons assigned to trehalose

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical shift (p.p.m.)</th>
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<tbody>
<tr>
<td>H-1</td>
<td>5.29</td>
</tr>
<tr>
<td>H-2</td>
<td>4.80</td>
</tr>
<tr>
<td>H-3</td>
<td>5.45</td>
</tr>
<tr>
<td>H-4</td>
<td>5.38</td>
</tr>
<tr>
<td>H-5</td>
<td>3.38</td>
</tr>
<tr>
<td>H-6</td>
<td>3.76</td>
</tr>
<tr>
<td>H-1'</td>
<td>ND</td>
</tr>
<tr>
<td>H-2'</td>
<td>ND</td>
</tr>
<tr>
<td>H-3', H-4', H-5', H-6'</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected (included in the region 3-4-40 p.p.m. in each case).

The 'H 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. ($\text{CH}_2=\text{CH}$), 2.04 p.p.m. ($\text{CH}_2=\text{CH}==\text{CH}$), 5.26 p.p.m. ($\text{CH}_2==\text{CH}$) and 6.65 p.p.m. ($\text{CH}_2=\text{CH}==\text{CH}$). 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 $^1$H-$^1$H 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 Hz, and $J_{2,3}$, and $J_{3,4}$ were 9-9 Hz. These data clearly proved an $\alpha,\alpha'$ 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 C-2' to C-5' 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...
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-I 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 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).

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

![Fig. 3. RPTLC (10 x 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.](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>DAT</th>
<th>Fatty acids (molar %)</th>
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<tbody>
<tr>
<td></td>
<td>14:0</td>
<td>16:1</td>
</tr>
<tr>
<td>M-431</td>
<td>I</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>-</td>
</tr>
<tr>
<td>M-855</td>
<td>I</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>-</td>
</tr>
<tr>
<td>M-858</td>
<td>I</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>-</td>
</tr>
</tbody>
</table>
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 amphiphatic 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).

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