

Occurrence of a novel glycolipid, 'trehalose 2,3,6'-trimycolate' in a psychrophilic, acid-fast bacterium, *Rhodococcus aurantiacus* (*Gordona aurantiaca*)

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A new type of glycolipid possessing trehalose and mycolic acids has been identified in the lipids of *Rhodococcus aurantiacus* (*Gordona aurantiaca*), a psychrophilic acid-fast bacterium. Gas chromatographic and gas chromatographic-mass spectrometric analysis of hydrolysis products of the most abundant glycolipid class (GM) (containing approx. 50% of the total lipids) showed that the hydrophilic moiety was composed essentially of trehalose, while the hydrophobic moiety contained C₆₂₋₇₄ polyunsaturated mycolic acids only. Permethylation study revealed that the new glycolipid possessed three mycolic acid residues for each trehalose molecule and was identified as trehalose 2,3,6'-trimycolate.

Trehalose trimycolate *Asymmetric ester* *Mycolic acid* *Cord factor* *Acid-fast bacillus*

1. INTRODUCTION

Mycolic acids (2-alkyl-3-hydroxy long-chain fatty acids) are the most characteristic component of Actinomycetales cell walls, such as Mycobacteria [1,2], Nocardia [3,4] and Corynebacteria [5,6]. They are associated with oligosaccharides such as trehalose or cell wall arabinogalactan and contribute to the unique hydrophobic properties of the cell walls or immunopharmacological activity. *Rhodococcus aurantiacus* (formerly *Gordona aurantiaca*) is a psychrophilic acid-fast bacterium isolated originally from a human sputum and occupying taxonomically an intermediate position between Mycobacteria and Nocardia [7,8]. Previously, we have reported that *R. aurantiacus* (*G. aurantiaca*) possessed highly unsaturated

mycolic acids (up to hexaenoic) ranging from C₆₂ to C₇₈ centering at C₇₂, which are useful for cell wall function or fluidity in a low-temperature environment [9]. Recently, we have examined the lipid composition and revealed that *R. aurantiacus* possessed a large amount of glycolipids containing mycolic acid and trehalose. Here, we describe four glycolipids of *R. aurantiacus* on TLC, two of which have not been reported previously. The most abundant glycolipid (GM) was identified surprisingly as an asymmetric polymycoloyl ester of trehalose.

2. MATERIALS AND METHODS

R. aurantiacus strain 80005 was supplied by Dr M. Tsukamura, National Sanatorium Chubu Hospital (Obu, Japan). The cells were cultivated at 30°C aerobically in a medium containing 1%

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glucose, 0.5% peptone and 0.2% yeast extract, with the pH adjusted to 7.0. Cells were harvested in early stationary phase and the lipids extracted several times with chloroform/methanol (2:1, v/v). Lipids were separated directly on a thin-layer plate of silica gel (Uniplate, Analtec, USA) with solvent system A (chloroform/methanol/acetone, 90:10:6, v/v) or B (chloroform/methanol/acetone/acetic acid, 90:10:6:1, v/v). Visualization was performed with iodine vapour for preparative purposes, Dittmer-Lester reagent for phospholipids or Anthrone reagent for glycolipids, followed by charring of all organic compounds. IR spectra of the isolated glycolipids were recorded with a Japan Spectroscopic (Tokyo) apparatus and mass spectra with a Hitachi GC/MS-M80B apparatus with a data processing system. Mild alkaline hydrolysis was performed with 0.2 N NaOH in chloroform/methanol (1:2, v/v) at room temperature and acid methanolysis with 5% anhydrous HCl-methanol in a sealed tube at 90°C for 14 h. Permethylation was carried out by the method of Deferrari et al. [10] using diazomethane boron trifluoride etherate, followed by reduction with NaBH₄ to form partially methylated alditol acetates. The resultant methylated alditol acetates were identified by the GC/MS system according to Björndal et al. [11].

3. RESULTS AND DISCUSSION

Approx. 6–7% of the crude lipids were extracted from packed cells of *R. aurantiacus* strain 80005. As shown in fig.1, four glycolipids were observed, from top to bottom (fig.1): GL-1, GL-2 (GM), GL-3 and GL-4, after developing the plate with solvent system B. Glycolipids 3 (GL-3) and 4 (GL-4) had the same behavior on TLC as the trehalose dimycolate and trehalose monomycolate from *Mycobacterium bovis* BCG, respectively. On the other hand, glycolipids 1 (GL-1) and 2 (GL-2, GM) did not coincide with any other known glycolipids. The IR spectrum of GM showed a very similar profile to the cord factor (trehalose dimycolate) of *M. tuberculosis* BCG, having absorption bands at 800, 1720, 2800 and 3200–3300 cm⁻¹ and also a characteristic region between 900 and 1450 cm⁻¹. These data suggested that the GM may be a glycolipid similar to cord factor (trehalose dimycolate). Mild alkaline

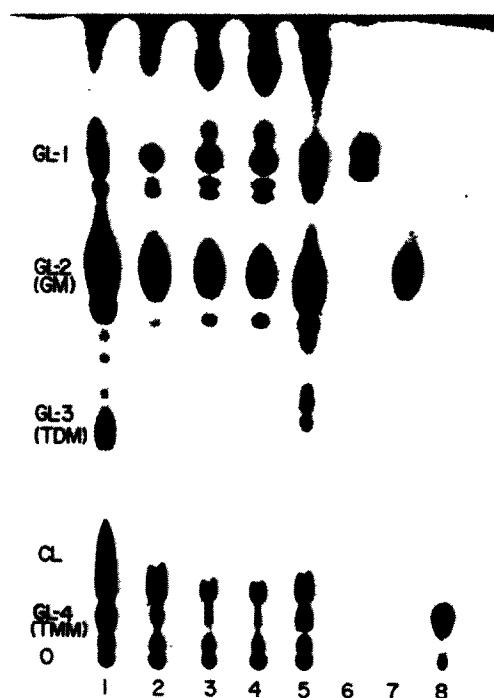


Fig.1. Thin-layer chromatographic separation of glycolipids (GL-1, GL-2, GL-3, GL-4) from *R. aurantiacus* 80005. Lipids were separated on a silica gel plate (Uniplate, Analtec) with a solvent system of chloroform-methanol-acetone-acetic acid (90:10:6:1, v/v) and then visualized by charring at 200°C after spraying with 18 N H₂SO₄. Lanes: 1, *R. aurantiacus* lipids, 3 days growth; 2, 7 days growth; 3, 14 days growth; 4, 21 days growth; 5, *R. aurantiacus* grown at 37°C; 6, isolated GL-1; 7, isolated GL-2 (GM); 8, isolated GL-4. CL, diphosphatidylglycerol (cardiolipin); TDM, trehalose dimycolate; TMM, trehalose monomycolate.

hydrolysis of GM at 25°C for 3 h liberated only fatty acid and a disaccharide.

Only trehalose could be determined in the water-soluble phase after saponification based on the retention times of the trimethylsilyl ether of the product on gas chromatography. This was also confirmed by GC/MS under the same conditions (5% SE-30, 1.5 m × 3 mm Ø, at 200°C). Acid methanolysis of the purified glycolipid (GM) gave only methyl glucoside on gas chromatography. Very mild alkaline hydrolysis of GM with 0.1 N KOH in chloroform-methanol (1:2, v/v) for

Table 1
Characterization of glycolipids from *R. aurantiacus* 80005

Glycolipid	Glycoside after methanolysis	Oligosaccharide after alkaline hydrolysis	Mycolic acid	Permethylation, reduction and acetylation products
GL-2 (GM)	methylglucoside	trehalose	M ₁ + M ₁ ' (C ₆₂₋₇₄)	2,3,4-tri- <i>O</i> -methylacetylglucitol and 4,6-di- <i>O</i> -methylacetylglucitol
GL-3 (TDM)	methylglucoside	trehalose	(M ₁) + M ₁ ' (C ₆₂₋₇₂)	2,3,4-tri- <i>O</i> -methylacetylglucitol

M₁, α -mycolic; M₁', α' -mycolic acid methyl esters on TLC

10–60 min yielded trehalose dimycolate and trehalose monomycolate besides free mycolic acid on TLC. Determination of the numbers and location of the acyl groups on the trehalose moiety were performed by permethylation study with diazomethane and BF₃ ethereal solution. After complete permethylation (checked by IR analysis), the methylated glycolipid was hydrolyzed with alkali (KOH-isopropyl alcohol), methanolized with HCl-methanol and the product then reduced with NaBH₄. The gas chromatographic and mass spectrometric analysis of alditol acetates thus obtained demonstrated that two peaks occurred, identified mass spectrometrically as 2,3,4-tri-*O*-methylacetylglucitol and 4,6-di-*O*-methylacetylglucitol in a ratio of 1:1. A brief summary of the structure analysis is presented in table 1. Therefore, from the data above, it was concluded that the most abundant glycolipid (GM) of *R. aurantiacus* was 2,3,6'-tri-*O*-mycoloyl- α,α (1-1)-trehalose, exclusively.

On the other hand, TLC of the fatty acid methyl esters with a solvent system of *n*-hexane/ether (8:2, v/v) showed that the major component was a hydroxylated fatty acid having the same *R_f* values as that of methyl mycolate of *M. smegmatis*. The mycolic acid structure was determined by GC/MS as the trimethylsilyl ether derivatives of methyl esters, essentially according to [9]. The mycolic acid esters were fully separated into five or six peaks on a glass column of 3 mm \times 0.5 m coated with 1% OV 101 on Gaschrom Q (60–80 mesh), operated at 320°C. Mass spectra of each molecular species of TMS methyl mycolate demonstrated the predominance of C₆₈ or C₇₀ penta- or hexaenoic mycolic acid ranging from C₆₂ to C₇₄, having a C₁₈ or C₂₀ alkyl branch at the 2-position. These spectra

coincided exactly with the mycolic acids of whole cells of *G. aurantiaca* reported in [9]. The mycolic acid composition of GM was determined from the gas chromatographic peak areas and is shown in table 2.

So far, cord factor and related glycolipids have been reported to occur widely as components of the cell walls of Actinomycetes [12–18]. However, in those cases, the mycolic acids are attached essentially to the 6- and/or 6'-position of hexose or the 5-position of pentose (arabinose). This is the first report describing the occurrence and characterization of asymmetric polyester in which the mycolic acids are esterified at the 2- and 3-positions apart from the 6-position of trehalose. This may reflect the taxonomical situation of a unique species of psychrophilic acid-fast bacterium, *R. aurantiacus*, and afford new information on the distribution of

Table 2
Mycolic acid composition of individual glycolipid classes from *R. aurantiacus* 80005

Carbon no.	GL-2 (GaGM)	GL-3 (trehalose dimycolate)	BL (cell wall bound)
62	0.2 ^a	tr	—
64	1.0	5.7	0.1
66	8.2	11.0	1.0
68	26.1	34.4	6.4
70	37.7	39.0	22.5
72	22.8	9.9	38.3
74	4.0	tr	28.0
76	tr	tr	3.8
78	—	—	tr

^a Percent of the total area

acyl trehalose. We are now examining a variety of immunopharmacological activities of polymycoloyl trehaloses differing in acyl numbers.

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