The glycolipid of *Halobacterium sodomense*

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The glycolipid of the halophilic archaeobacterium *Halobacterium sodomense* has been analysed by spectroscopic methods including ¹³C NMR to establish structural details, and has been shown to be a sulphated C₂₀,C₂₀ mannosyl glucosyl diether glycerol. This structure differs from all other known halobacterial glycolipids in having a sulphate substitution at C-2 of the mannose residue, and in being an α-D-(1→4)-linked disaccharide.

Introduction

The taxonomy of the red archaeobacterial halophiles (halobacteria) is based largely on chemotaxonomic markers, rather than standard biochemical tests (Grant & Larsen, 1989a). In particular, the use of polar lipid composition to assign isolates to particular groups of genera has proved of considerable value. All halobacteria examined to date contain C₂₀,C₂₀ (diphytanyl), C₂₀,C₂₅ (phytanyl-sesterterpanyl) or C₂₅,C₂₅ (disesterterpanyl) diether analogues of phosphatidyl glycerol (PG) and phosphatidyl glycerol phosphate (PGP). The presence or absence of phosphatidyl glycerol sulphate (PGS) and the structure(s) of any glycolipid(s) present define groups that are supported by 16S rRNA/DNA hybridization (Ross & Grant, 1985). Thus, *Natronobacterium* and *Natronococcus* spp. lack PGS and glycolipids, *Halobacterium* spp. are characterized by sulphated forms of triglycosyl (S-TGD-1) and tetraglycosyl (S-TeGD) lipids, *Haloferax* spp. by a sulphated diglycosyl lipid (S-DGD-1) and *Haloarcula* spp. by a non-sulphated triglycosyl lipid (TGD-2) (Grant & Larsen, 1989b, c; Larsen & Grant, 1989; Grant, 1989a, b). All of the glycolipids characterized to date are derived from a basic α-D-mannosyl α(1→2)-α-D-glucosyl diphytanyl glycerol, by substitution of sugar or sulphate groups at the C3 or C6 positions of the mannose residue (Fig. 1).

The type strains of the organisms named as *Halobacterium saccharovorum*, *Halobacterium trapanicum* and

Halobacterium sodomense have all been considered in the past to be members of the genus *Halobacterium* as defined at that particular time. However, 16S rRNA/DNA hybridization studies have shown that these strains comprise a relatively diverse taxon distinct from the genus *Halobacterium* as currently defined (Ross

Glycolipids		
	R ₁	R ₂
DGD-1	H	OH
S-DGD-1	SO ₃ H	OH
TGD-1	β-Galp	OH
TGD-2	β-Galp	OH
S-TGD-1	3-SO ₃ H-β-Galp	OH
TeGD	β-Galp	O-α-Galp
S-TeGD	3-SO ₃ H-β-Galp	O-α-Galp

Fig. 1. Structural relationships of glycolipids based on α-D-mannosyl α(1→2)-α-D-glucosyl diphytanyl glycerol.

Abbreviations: FAB, fast atom bombardment; PG, phosphatidyl glycerol; PGP, phosphatidyl glycerol phosphate; PGS, phosphatidyl glycerol sulphate.

& Grant, 1985; Larsen & Grant, 1989). These halobacteria also comprise the only group showing *dam* methylation of DNA (Lodwick *et al.*, 1986). Immunological evidence does not support any close relationship between *Hb. saccharovorum* and the type species of the genus *Halobacterium* (Conway de Macario *et al.*, 1986) and these organisms also have different classes of fructose biphosphate aldolases (Dhar & Altekar, 1986). However *Hb. saccharovorum*, *Hb. sodomense* and *Hb. trapanicum* are not particularly closely related, since *Hb. saccharovorum* shows only 45% DNA/DNA homology with *Hb. sodomense* and 52% homology with *Hb. trapanicum* (Ross & Grant, 1985). Accordingly, these three organisms are treated as *species incertae sedis* in the latest edition of Bergey's Manual (Larsen & Grant, 1989), where it is considered premature to activate the suggestion of Grant & Ross (1986) that these organisms merit reclassification as separate species within a new genus.

There has also been some doubt over the polar lipid composition of these organisms, in particular the structure of the single glycolipid possessed by each strain. Recently, the glycolipid of *Hb. saccharovorum* has been shown to be S-DGD-1 (Lanzotti *et al.*, 1988). The glycolipid of *Hb. trapanicum* co-chromatographs with that of *Hb. saccharovorum* in several solvent systems (unpublished results), but the glycolipid of *Hb. sodomense* undoubtedly shows different chromatographic behaviour (Torreblanca *et al.*, 1986).

We report here the detailed characterization of the glycolipid of *Hb. sodomense*.

Methods

Strain and culture conditions. The type strain of *Hb. sodomense* (ATCC 33755) was grown in liquid shake culture in the medium described by Oren (1983). Cells were harvested in the late exponential phase of growth by centrifugation, washed with a basal salt solution (the growth medium without organic components) and lyophilized.

Extraction and isolation of lipids. Lyophilized cells (6 g) were extracted continuously by Soxhlet for 12 h with CHCl_3 /methanol (1:1, v/v) and then with methanol/ H_2O (1:1, v/v). The extracts were pooled and evaporated under vacuum. Total lipid extract (700 mg), dissolved in 20 ml CHCl_3 /methanol/ H_2O (65:25:4, by vol.), was mixed with 5 g silica gel (Kieselgel 70–230 mesh, Merck) and dried; the dry material was added to the top of silica gel column (40 cm $\times$ 10 mm, i.d.). The column was eluted with 3 l of a step gradient of 0–50% (v/v) methanol in CHCl_3 in 5% increments (250 ml for each gradient step). All the lipids eluted were further purified by preparative TLC in CHCl_3 /methanol/ H_2O (65:25:4, by vol.).

Degradation procedures. Solvolysis of glycolipid was done in dioxane/pyridine (1:1, v/v) at 100 °C for 2 h. After cooling, H_2O was added and the solution extracted with butan-1-ol. The butanolic phase was washed with H_2O and dried.

Acid hydrolysis was carried out in 2 M-HCl at 100 °C for 6 h. Hydrolysed products, after drying, were extracted with CHCl_3 and the

residue was dissolved in H_2O . The CHCl_3 - and H_2O -soluble fractions were examined chromatographically.

Permethylation was carried out using the procedures of Wagner & Seligmann (1973).

Acid methanolysis of permethylated glycolipid was done in 1 M-HCl in methanol at 100 °C for 3 h.

Chromatographic procedures. TLC was performed on 0.25 mm layers of silica gel F254 (Merck) activated at 100 °C for 2 h. Solvents included: CHCl_3 /methanol/ H_2O (65:25:4, by vol.) and CHCl_3 /methanol/ CH_3COOH / H_2O (85:22.5:10:4, by vol) for complex lipids; CHCl_3 /diethyl ether (9:1, v/v) for 2,3-di-*O*-phytan-1-yl-sn-glycerol; butan-1-ol/ CH_3COOH / H_2O (60:20:20, by vol.) for glucose and mannose; CHCl_3 /methanol (97:3, v/v) for permethylated sugars. Compounds were detected either by exposure to I_2 vapour, or by spraying with 0.1% $\text{Ce}(\text{SO}_4)_2$ in 1 M- H_2SO_4 followed by heating at 150 °C for 5 min. Specific reagents included the Dittmer and Lester reagent for phospholipids and α -naphthol/ H_2SO_4 for glycolipids (Kates, 1986).

HPLC separation of the hexoses was done on an NH_2 -bonded reverse-phase Carbohydrate Analysis column (30 cm $\times$ 3.9 mm i.d.) (Waters Associates) using as solvent $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (80:20, v/v) at a flow rate of 1 ml min⁻¹ with a Waters Associates apparatus; eluents from the column were detected by differential refractometry.

GLC of permethylated sugar was carried out on a glass column (2 mm $\times$ 2 m) packed with 20% butan-1,4-diol succinate on Chromosorb WAW, with a column temperature of 155 °C and N_2 (1 kg cm⁻²) as carrier gas (Yahara *et al.*, 1977).

Mass spectrometry (MS). A Kratos MS-50 instrument equipped with a Kratos fast atom bombardment (FAB) source was used. The positive FAB mass spectra were obtained by dissolving the samples in a glycerol matrix, adding 0.1% sodium acetate in methanol to the probe prior to bombardment with argon atoms of kinetic energy equivalent to 2–6 keV. The negative FAB mass spectra were obtained as described for the positive FAB, without the addition of sodium acetate.

NMR spectroscopy. Samples for NMR spectroscopy were prepared by dissolving the lipid in 0.5 ml $\text{C}^2\text{HCl}_3/\text{C}^2\text{H}_3\text{O}^2\text{H}$ (7:3, v/v) or deuterobenzene- $\text{d}_6/\text{C}^2\text{H}_3\text{O}^2\text{H}$ (1:1, v/v).

All the ^{13}C NMR spectra were run at 125 MHz on a Bruker WH-500 spectrometer at room temperature, using trimethylsilane as an internal standard. Analyses of multiplicities were achieved by DEPT experiments performed using polarization transfer pulses of 90° and 135°, respectively, obtaining in the first case only signals for methine groups and in the other case positive signals for methine and methyl groups and negative signals for methylene groups.

IR spectra. IR spectra were recorded at room temperature using an FT-IR Bio-Rad spectrophotometer.

Optical rotations. Optical rotations were measured at room temperature using a Perkin Elmer 141 polarimeter.

Results and Discussion

All the lipids eluted from the silica gel column were further purified by preparative TLC and identified by analytical TLC in comparison with authentic samples and by specific staining tests.

The complex lipids of *Hb. sodomense* were PG (13% of the total lipid extract), PGP (60%), PGS (3%) and an unknown glycolipid (22%).

The glycolipid eluted from the silica gel column with CHCl_3 /methanol (80:20, v/v) had an R_F of 0.35 in

CHCl_3 /methanol/ H_2O (65:25:4, by vol.), different from that of the glycolipid of *Hb. saccharovorum* (0.4) (Lanzotti *et al.*, 1988). It responded to sugar reagent and had an $[\alpha]_D$ value of 26.4 in CHCl_3 /methanol (1:1, v/v).

The IR spectrum of the compound in liquid film was similar to that reported for the glycolipid of *Hb. saccharovorum*, particularly a broad band at 1250 cm^{-1} (S=O group) and an absorption band at 830 cm^{-1} (S—O—C) indicating the presence of sulphate.

Positive FAB mass spectra of the compound gave a molecular ion peak at m/z 1078 ($\text{M}^+ + \text{Na}^+$) while that of the compound obtained after solvolysis in dioxane/pyridine gave a molecular ion at m/z 999 ($\text{M}^+ + \text{Na}^+$), thus establishing that the glycolipid possessed one sulphate group. Negative FAB mass spectra of the glycolipid confirmed these results, showing a molecular ion at m/z 1055 ($\text{M} - \text{H}^+$).

Acid hydrolysis of the desulphated derivative gave 2,3-di-*O*-phytanyl-sn-glycerol in the CHCl_3 phase and glucose and mannose in the H_2O phase. The sugars and isopranoid diether were identified in TLC, HPLC and ^{13}C NMR by comparison with authentic samples. The absolute configuration of the two hexoses was established, after HPLC purification, by determination of their rotation ($[\alpha]_D = +52^\circ$ for glucose and $+15^\circ$ for mannose). A 1:1 molar ratio of the two hexoses was confirmed by integration of the HPLC peak areas against known concentrations.

Signals of the anomeric protons, in the ^1H NMR spectra of the glycolipid, at δ 4.78 (d, $J = 1\text{ Hz}$, glucose) and at δ 4.73 (d, $J = 1\text{ Hz}$, mannose) and the value of $^3J_{\text{H}_1-\text{H}_2}$ indicate an α configuration for the two hexoses. These data were consistent with the value of the chemical shift of the anomeric carbons in the ^{13}C NMR spectra runs in deuterobenzene- $\text{d}_6/\text{C}^2\text{H}_5\text{O}^2\text{H}$ (1:1, v/v): 97.30 for mannose and 94.14 for glucose. In Table 1 are reported the assignments of the signals in the ^{13}C NMR spectrum of the glycolipid. Most of the signals were assigned by comparison with signals derived from the closely related glycolipid of *Hb. saccharovorum* (Lanzotti *et al.*, 1988). The two signals at 61.5 and 61.8 assigned to the C6 of the glucosyl and mannosyl residues, respectively, indicate that sulphate in this glycolipid is not linked at C6 of the mannosyl moiety as reported for the glycolipid of *Hb. saccharovorum*. However, other signals of the carbohydrate moieties are tentatively assigned and could be reversed.

Permethylation of intact sulphated glycolipid and of its desulphated derivative and successive acid methanolysis gave methyl 2,3,6-trimethyl glucose in both cases, and methyl 3,4,6-trimethyl mannose for sulphated glycolipid and methyl 2,3,4,6-tetramethyl mannose for its desulphated derivative.

These data confirm an α -D-mannopyranosyl-(1 $\rightarrow$ 4)- α -

Table 1. ^{13}C NMR assignments of the sulphated glycolipid of *Hb. sodomense*

Position	Carbon atom	Chemical shift (δ , p.p.m.)*
Isopranoid chains	1	68.5
	1a	70.7
	2	37.1
	2a	37.4
	3	30.5
	3a	30.6
	4, 6, 8, 10, 12	38.2
	5	24.9
	7, 11	33.2
	9	24.8
	13	25.2
	14	40.0
	15	28.5
	16	20.0
	17, 18	20.1
	19, 20	22.9
Glycerol	1	71.5
	2	78.4
	3	69.6
Glucosyl	1	96.9 (97.14)
	2	72.0
	3	72.2
	4	77.1
	5	70.5
	6	61.5
Mannosyl	1	96.9 (97.30)
	2	77.6
	3	71.8
	4	67.7
	5	73.4
	6	61.8

* Chemical shifts in parentheses refer to spectra performed in deuterobenzene- $\text{d}_6/\text{C}^2\text{H}_5\text{O}^2\text{H}$.

D-glucopyranosyl sequence and indicate that sulphate is linked to the C2 of the mannose, establishing the structure of the glycolipid as 2,3-di-*O*-phytanyl-1-*O*-[mannopyranosyl-1(2-sulphate)- α -D-(1 $\rightarrow$ 4)-glucopyranosyl- α -D]-sn-glycerol (Fig. 2).

This is the first report of a halobacterial glycolipid that is not derived from the core mannosyl α -(1 $\rightarrow$ 2)-glucosyl diphytanyl glycerol described by Kamekura & Kates (1989). The glycolipid is unusual on two counts: firstly, it is a mannosyl α -(1 $\rightarrow$ 4)-glucosyl diphytanyl glycerol sulphate; secondly, the sulphate residue is linked to the C2 of the mannose residue rather than the C3 or C6 (Kamekura & Kates, 1989). We propose to designate this lipid as S-DGD-3 to distinguish it from the S-DGD-1 glycolipid of *Hb. saccharovorum* and other strains. An uncharacterized unsulphated glycolipid designated GL-1 by Kamekura & Kates (1989), possessed in trace amounts by *Haloarcula* spp., has in the past been referred

Fig. 2. 2,3-di-*O*-phytanyl-1-*O*-[mannopyranosyl-(2-sulphate)- α -D-(1 $\rightarrow$ 4)-glucopyranosyl- α -D]-sn-glycerol (S-DGD-3).

to as DGD-2 (Torreblanca *et al.*, 1986; Grant & Larsen, 1989*a*). This glycolipid may have the same structure as the unsulphated form of S-DGD-3. Detailed analyses will be necessary to settle this point and to establish if S-DGD-3 is different from the uncharacterized sulphated glycolipid, designated S-DGD-2, isolated from another halobacterium described by Kamekura & Kates (1989).

These results reinforce the idea that *Hb. sodomense* is distinct from *Hb. saccharovororum*, although both have similar polar lipid profiles (PG, PGP, PGS, glycolipid) distinct from any of the other halobacterial groups (Grant & Larsen, 1989*a*). There is no doubt that *Hb. sodomense* and *Hb. saccharovororum* (probably together with *Hb. trapanicum*) should be reclassified. The real question is whether they together constitute a taxon at genus level, or whether *Hb. sodomense* is sufficiently different to merit separate reclassification at that level. In view of the weight given to lipid composition and structure in the current classification (Grant & Larsen, 1989*a*), separate status for *Hb. sodomense* merits serious consideration. It is of interest that in the recent description of a psychrophilic halobacterium (Franzmann *et al.*, 1988), oligonucleotide catalogue data indicated that *Hb. saccharovororum* was its closest relative amongst those halobacteria so far catalogued, whereas *Hb. sodomense* was much less closely related although more closely related than selected examples from the genera *Haloarcula*, *Haloferax*, *Halococcus*, *Natronococcus* and *Halobacterium sensu stricto*.

We are currently attempting to sequence the 16S rRNA of *Hb. sodomense*, with the aim of establishing its taxonomic ranking unequivocally.

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