

Lipids of extremely halophilic archaeobacteria from saline environments in India: a novel glycolipid in *Natronobacterium* strains

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Several strains of extremely halophilic archaeobacteria, both non-alkaliphilic and alkaliphilic, including *Halobacterium*, *Haloferax* and *Natronobacterium* species, were isolated from salt locales in India. The major phospholipids in these strains were the C₂₀-C₂₀-glycerol diether analogues of phosphatidylglycerolmethylphosphate (PGP-Me), phosphatidylglycerol (PG) and phosphatidic acid (PA). In addition, the *Halobacterium* strains possessed the characteristic glycolipids, sulfated triglycosyl and tetraglycosyl diethers (S-TGD-1 and S-TeGD, respectively) and the unsulfated triglycosyl diether (TGD-1); and the *Haloferax* strains had the characteristic sulfated and unsulfated diglycosyl glycerol diethers (S-DGD-1 and DGD-1, respectively). The PGP-Me, and PG components of the haloalkaliphiles each occurred as two molecular species with C₂₀-C₂₀' and C₂₀-C₂₅' (isoprenoid) glycerol diether lipid cores. In contrast to previous reports of the absence of glycolipids in natronobacteria, the *Natronobacterium* strains from India were found to contain small amounts of a novel glycolipid identified as glucopyranosyl-1 → 6-glucopyranosyl-1 → 1-glycerol diether (DGD-4). The lipid cores of DGD-4 also contained mainly unhydroxylated or hydroxylated C₂₀-C₂₀' C₂₀-C₂₅ and C₂₅-C₂₅ molecular species with unsaturated (isoprenoid) chains. Hydroxylated lipid cores have previously been identified only in some methanogenic archaeobacteria.

Keywords: archaeobacteria, lipids, *Natronobacterium*, *Halobacterium*, *Haloferax*

INTRODUCTION

Comparative study of the polar lipid composition of extremely halophilic archaeobacteria, both non-alkaliphilic (*Halobacterium*, *Haloarcula*, *Haloferax* and *Halococcus*) and alkaliphilic (*Natronobacterium* and *Natronococcus*) species, has proved to be a useful chemotaxonomic tool for this group of archaea (Torreblanca *et al.*, 1986; Grant & Larsen, 1989; Kates, 1993a, b). All

strains of extreme halophiles examined possess ether-linked lipids based on the lipid core 2,3-di-*O*-phytanyl-*sn*-glycerol (C₂₀-C₂₀-diether) (Kates, 1978, 1990; 1993a, b). Extreme haloalkaliphiles also possess 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol (C₂₅-C₂₀-diether) and 2,3-di-*O*-sesterterpanyl-*sn*-glycerol (C₂₅-C₂₅-diether) lipid cores (De Rosa *et al.*, 1982, 1983), and the former of these two lipids has also been reported in some halococci (Moldoveanu *et al.*, 1990).

Abbreviations: CI-MS, chemical ionization mass spectrometry; DGD, diglycosyl diphytanyl-glycerol diether; DGD-4 (GL-1), novel diglycosyl-glycerol diether in *Natronobacterium* strains; FAB-MS, fast atom bombardment mass spectrometry; PA, phosphatidic acid (diether analogue); PG, phosphatidylglycerol (diether analogue); PGP, phosphatidylglycerolmethylphosphate (diether analogue); PGP-Me, phosphatidylglycerolmethylphosphate (diether analogue); PGS, phosphatidylglycerosulfate (diether analogue); S-TGD-1, sulfated triglycosyl diphytanyl-glycerol diether; S-TeGD, sulfated tetraglycosyl diphytanyl-glycerol diether.

The distinctive features of polar lipid composition within the extreme halophiles are: (i) the presence of PGP-Me (Kates *et al.*, 1993) and PG as major phospholipids (Torreblanca *et al.*, 1986; Grant & Larsen, 1989; Kates, 1993b); (ii) the presence of PGS in *Halobacterium* and *Haloarcula* species and its absence in *Haloferax*, *Halococcus*, *Natronobacterium* and *Natronococcus* species (Tindall *et al.*, 1984; Ross *et al.*, 1985; Torreblanca *et al.*, 1986; Grant & Larsen, 1989; Kates, 1993b); (iii) correlation of glycolipid

composition with generic classification of non-alkaliphilic archaea (Torreblanca *et al.*, 1986; Grant & Larsen, 1989; Kates, 1990, 1993a, b); (iv) the absence of glycolipids in haloalkaliphiles (Tindall *et al.*, 1984; Ross *et al.*, 1985; Grant & Larsen, 1989; Kates, 1990); and (v) the presence of phosphatidyl-1,2-cyclic-glycerophosphate in *Natronococcus*, which distinguishes this genus from *Natronobacterium* (Morth & Tindall, 1985; Lanzotti *et al.*, 1989).

There have been no studies so far to show whether the halophilic archaeobacteria possess hydroxy diether core lipids, which have been reported in methanogens (Ferrante *et al.*, 1988; Sprott, 1992).

Previous studies on the lipids of extreme halophiles from various locales have contributed to the taxonomic assignment of new genera: *Natronobacterium* and *Natronococcus* (De Rosa *et al.*, 1982, 1983, 1988; Tindall *et al.*, 1984), *Haloferax* and *Haloarcula* (Torreblanca *et al.*, 1986; Kates, 1993a, b). We now report on the polar and non-polar lipids of *Natronobacterium* and other halobacterial strains isolated from salt locales in India (Upasani, 1988; Upasani & Desai, 1990). A novel unsulfated glycolipid (DGD-4) has been isolated and identified in the *Natronobacterium* strain SSL1 and detected also in other strains of *Natronobacterium*. The lipid core of this novel glycolipid had mainly C₂₀-C₂₀, C₂₀-C₂₅ and C₂₅-C₂₅-glycerol diethers with unhydroxylated or hydroxylated unsaturated (isoprenoid) chains; hydroxylated lipid cores have previously been found only in some methanogens (Sprott, 1992).

METHODS

Cultures and growth conditions. *Halobacterium* MSW5 (NCMB 2288) and *Haloferax* MSW1 (NCMB 2287, deposited as *Halobacterium*) were isolated from marine salterns at Mithapur, India; *Natronobacterium* SSL1 (ATCC 43988) and SSL6 (ATCC 43987) strains were isolated from Sambhar Salt Lake, India (Upasani, 1988; Upasani & Desai, 1990). *Halobacterium cutirubrum* (NRC 34001) and *Haloferax volcanii* (NRC 2112) were provided by Roger Latta, NRC, Ottawa, and *Natronobacterium gregoryi* (NCMB 2189) was a gift from W. D. Grant, University of Leicester, UK.

Halobacterium and *Haloferax* strains were grown in the media of Larsen (1981) and Mullakhanbhai & Larsen (1975), respectively. The *Natronobacterium* cultures were grown in the medium of Brown (1963) as described by Tindall *et al.* (1980), but using lower magnesium sulfate and sodium chloride concentrations; its composition was (g l⁻¹): bacteriological peptone (Oxoid) 10.0; trisodium citrate 3.0; MgSO₄ (anhydrous) 2.5; KCl 2.0; Na₂CO₃ (anhydrous) 16.0; NaCl 225; pH 9.5. Inorganic and organic components were autoclaved separately. When the Oxoid peptone was replaced by Bacto-peptone (Difco) no growth occurred. Proteose peptone (Difco) or a combination of proteose peptone (Difco; 7.0 g l⁻¹) with yeast extract (Difco; 3.0 g l⁻¹) supported good growth of the haloalkaliphilic strains.

All cultures were grown in 1.5 l batches of medium, inoculated with 1–2% (v/v) of a 5–7 day inoculum, at 38 °C ± 1 °C on an environmental shaker (100–110 r.p.m.). The non-alkaliphilic and alkaliphilic cultures were harvested after 5 and 8 d, respectively. The slower-growing *Natronobacterium* SSL6 strains were grown for 10–12 d. The wet cell mass obtained by centrifugation at 10000 r.p.m. for 10–15 min at 10 °C was

washed twice with the inorganic salts solution of the respective growth medium.

Extraction of lipids. The washed cell pellets of the extreme halophiles and haloalkaliphiles were resuspended in the inorganic salts solution of the respective medium, and total lipids were extracted by the method of Bligh & Dyer (1959) as modified by Kates (1986). Polar lipids were separated from non-polar components by acetone precipitation (Kates, 1986).

Chromatography. Thin-layer chromatography (TLC) of polar lipids was performed on analytical precoated plates (Whatman K6 silica gel 60A, 0.25 mm thick; preparative, 0.75 mm thick) using single or double development in the following solvent systems: A, CHCl₃/methanol/conc. ammonium hydroxide (65:35:5, by vol.); B, CHCl₃/methanol/acetic acid/H₂O (85:22.5:10:4, by vol.); and C, CHCl₃/MeOH/CH₃COOH/H₂O (85:22.5:8:3, by vol.). The non-polar lipids were separated on analytical precoated plates (Merck silica gel 60H, 0.25 mm thick) using the following solvent systems: D, CHCl₃/methanol (93:7, v/v) for bacterioruberin and monoanhydro-bacterioruberin isomers; E, petroleum ether (b.p. 60–80 °C)/diethyl ether (99:1, v/v) for vitamin MK-8, lycopene and β-carotene; F, petroleum ether (b.p. 60–80 °C)/diethyl ether (99.5:0.5, v/v) for squalenes, β-carotene and lycopene; and G, petroleum ether/ethyl ether (70:30, v/v) for diphytanyl-glycerol. Lipids were detected with the following spray reagents (Kates, 1986): 0.5% α-naphthol/H₂SO₄ for glycolipids, (NH₄)MoO₄/H₂SO₄ for phospholipids; H₂SO₄/C₂H₅OH (1:1, by vol.) followed by charring at 150 °C, or staining with aqueous rhodamine 6G followed by visualization under ultraviolet light 266 nm) for detection of all lipids. Water-soluble products of methanolysis were chromatographed on TLC cellulose plates (Merck DC cellulose, 0.1 mm thick) in solvent H [pyridine/ethyl acetate/H₂O (2:5:5, by vol., upper phase)] for sugars, and in solvent I [1-butanol/acetic acid/H₂O (5:3:1, by vol.)] for phosphate esters. Sugars were detected with the silver nitrate spray reagent and phosphate esters with the phosphate spray reagent (Kates, 1986); they were identified by comparison of their TLC mobilities with those of the corresponding authentic compounds.

Isolation and purification of individual polar lipids. Individual polar lipids were isolated and purified by repeated TLC on silica gel in solvent B for phospholipids and solvent C for glycolipids. Solvent C was effective in completely separating the glycolipid (GL-1) in *Natronobacterium* strain SSL1 from the PGP-Me component. The bands corresponding to individual phospholipids (PGP-Me and PG) were eluted from the silica with CHCl₃/methanol/H₂O (1:1:0.1, by vol.) or CHCl₃/methanol/0.2 M HCl (1:2:0.8, by vol.), and the glycolipid (GL-1) was eluted using CHCl₃/methanol/H₂O (1:1:0.1, by vol.), as described elsewhere (Kates, 1986). The acidic phospholipids (free acid form) were neutralized with methanolic 1 M NH₄OH and precipitated as the ammonium salt forms (Kates, 1986; Moldoveanu *et al.*, 1990). Lipid components were identified by TLC mobilities relative to those of authentic standards (Moldoveanu *et al.*, 1990).

Analytical methods. Phosphorus and total hexose contents of phospholipids and glycolipids, respectively, were determined as described elsewhere (Kates, 1986). Separated lipid components were determined gravimetrically after elution from the plates.

Physical measurements. The purified lipid components were analysed by negative and positive fast atom bombardment mass spectrometry (FAB-MS) with a Kratos Concept 2H mass spectrometer (caesium gun ion source), and by chemical ionization-mass spectrometry (CI-MS), with a VG 7070E mass

Table 1. Lipid content and composition of extremely halophilic archaeobacterial strains from India

Strain	Total lipids (% by wt)*	Polar lipids (% of total lipids)	Non-polar lipids (% of total lipids)	Lipid-P (% of total lipids)	Lipid-hexose (% of total lipids)
<i>Haloferax</i> MSW1	7.5	79	21	3.6	3.3
<i>Halobacterium</i> MSW5	5.2	58	42	2.4	5.3
<i>Natronobacterium</i> SSL1	4.6	85	15	4.7	0.24
<i>Natronobacterium</i> SSL6	8.6	60	38	3.4	0.17
<i>Natronobacterium gregoryi</i>	5.0	83	8	2.6	—

*Based on an estimated dry cell weight being 10% of wet cell weight.

spectrometer (ethyl ether as ionization element). $^1\text{H-NMR}$ and $^{31}\text{P-NMR}$ spectra were measured in CDCl_3 using a Bruker MSL-300 NMR spectrometer. The $^1\text{H-NMR}$ spectrum of purified glycolipid was measured in perdeuterated dimethylsulfoxide (DMSO) as well as in CDCl_3 . Partially methylated alditol acetates of sugars were analysed by GC-MS on a HMW-DB-17 capillary column (30 m), temperature-programmed from 180 °C (at 2 °C min^{-1}) on a Hewlett Packard HP-5985 GC-MS instrument.

Methanolysis of lipids. Acid methanolysis of phospholipids and glycolipids was performed in 2 M HCl in methanol at 75 °C for 5 h (Kates, 1986). The hydrophobic products (petroleum-ether-soluble, mainly diether lipid cores, also some isoprenyl degradation products) were identified by TLC in solvent G and by CI-MS analysis. The methanol/water-soluble products (phosphate esters, sugars) were identified as described elsewhere (Moldoveanu *et al.*, 1990).

For detection of hydroxylated lipid cores, mild acid methanolysis was carried out with 0.5 M methanolic HCl at 50 °C for 16 h (Sprott, 1992), and the products analysed by TLC and CI-MS (Ferrante *et al.*, 1988; Moldoveanu & Kates, 1988).

Permethylolation analysis. Glycolipid (GL-1) was permethylated and the partially methylated sugars obtained after methanolysis were analysed as the partially methylated alditol acetates by GC-MS on a JMW-DB-17 capillary column, as described elsewhere (Moldoveanu *et al.*, 1990; Kates, 1986). The anomeric configuration of the glycosidic linkages was assigned by $^1\text{H-NMR}$ in permethylated DMSO (see Kates, 1990).

RESULTS

Characterization of strains from saline environments in India

The non-alkaliphilic extreme halophiles isolated from marine salterns were separated into two groups on the basis of morphology, salt requirements and biochemical characteristics (Upasani, 1988; Upasani *et al.*, 1991), as follows: group I (MSW1–MSW3), *Haloferax* strains; and group II (MSW4–MSW6), *Halobacterium* strains. The rod-shaped alkaliphilic strains (SSL1–SSL6) were assigned to the genus *Natronobacterium* (Upasani & Desai, 1990).

Strain SSL1 had a faster growth rate and higher cell-mass yield than any of the other haloalkaliphiles, thus providing sufficient material for isolation and unambiguous chemical identification of the major lipid components of this strain.

Lipid composition

The cellular content of total lipids for the strains examined was in the range 5–8% of dry cells (Table 1). Lipid-P (lipid-associated phosphorus) amounted to 2.4–3.6% of total lipids for all the strains except *Natronobacterium* SSL1, which had a higher lipid-P content (4.7%), probably due to its lower content of non-polar lipids (Table 1, see below). Total lipid-associated hexose amounted to 3–5% of total lipids for the non-alkaliphilic strains and about 0.2% of total lipids for the *Natronobacterium* strains. The presence of lipid-hexose in the *Natronobacterium* strains suggested that these strains do contain small amounts of glycolipids, which have not been found previously in natronobacteria.

Polar lipids accounted for 79–85% of total lipids except for strains MSW5 and SSL6, which had only 58–60% polar lipids (Table 1). The content of non-polar lipids plus pigments accounted for the remainder of the lipids and was unusually high in the strains MSW5 and SSL6, amounting to about three times the value reported for halobacterial strains previously studied (Kamekura & Kates, 1988).

Non-polar lipids. TLC of the non-polar (acetone-soluble) lipids in solvents D, E and F showed the presence in all strains examined of C30 isoprenoids (squalene, dihydro-squalene and tetrahydro-squalene), C40 carotenoids (carotene and lycopene) and C50 carotenoids (bacterioruberins and anhydrobacterioruberins), as well as retinal and vitamin MK-8. However, the relative proportions of these components varied among these strains. The strains of Indian extreme halophiles and haloalkaliphiles appear to be similar to other known halobacteria with respect to the presence of isoprenoid compounds and carotenoid pigments (Kamekura & Kates, 1988).

Table 2. TLC mobilities and polar lipid composition in extremely halophilic archaeobacteria from Indian salterns

Single TLC development was in one of two solvent systems: A, CHCl₃/methanol/conc. ammonium hydroxide (65:35:5, by vol.); C, CHCl₃/methanol/acetic acid/H₂O (85:22:5:8:3, by vol.). TLC plates were stained for phospholipids and glycolipids as described in Methods. tr, trace.

Lipid component	R _F value in solvent:		Presence (+) or absence (-) of lipid component in Indian strains:				Presence (+) or absence (-) of lipid component in reference species:		
	A	C	MSW1	MSW5	SSL1	SSL6	<i>Haloferax volcanii</i>	<i>Halobacterium cutirubrum</i>	<i>Natronobacterium gregoryi</i>
PL-1 (unidentified)	0.02	—	—	—	tr	tr	—	—	—
S-TeGD	0.038	—	—	+	—	—	—	+	—
S-TGD-1	0.13	—	—	++	—	—	—	++	—
TGD-1	0.15	—	—	+	—	—	—	+	—
S-DGD-1	0.23	0.32	++	—	—	—	++	—	—
DGD-1	0.28	0.51	+	—	—	—	+	—	—
GL-1									
C ₂₀ -C ₂₀	0.20	0.39	—	—	+	+	—	—	+
C ₂₀ -C ₂₅	0.20	0.44	—	—	+	+	—	—	+
PL-2 (unidentified)	—	0.37	—	—	+	tr	—	—	+
PGP-Me									
C ₂₀ -C ₂₀	0.20	0.25	+++	+++	+++	+++	+++	+++	+++
C ₂₀ -C ₂₅	0.22	0.28	—	—	++	++	—	—	++
PGS	0.30	—	—	+	—	—	—	+	—
PG									
C ₂₀ -C ₂₀	0.60	0.77	++	++	++	++	++	++	++
C ₂₀ -C ₂₅	0.63	0.82	—	—	++	++	—	—	++
GL-2 (unidentified)	—	0.80	—	—	+	—	—	—	+
PA	0.10	0.96	tr	tr	+	+	tr	+	+

Polar lipids. The polar lipids of the Indian strains of extreme halophiles were readily separated by TLC in solvents A, B or C and were identified by comparison of their mobilities to those of the lipids in the reference strains of *Haloferax*, *Halobacterium* and *Natronobacterium* (Table 2), and by NMR and mass spectrometry as described below. The C₂₀-C₂₀ and C₂₀-C₂₅ molecular species of PGP-Me, PG and GL-1 were best-resolved by TLC in solvent C, and were identified by FAB-MS as described below.

The non-alkaliphilic strains of group I (e.g. strain MSW1) showed the presence of S-DGD-1, DGD-1, and C₂₀-C₂₀ species of PGP-Me and PG, and the absence of PGS, characteristic of *Haloferax* strains (Torreblanca *et al.*, 1986; Kates, 1993), whereas those of group II (e.g. strain MSW5) showed the presence of S-TeGD, S-TGD, TGD-1, and C₂₀-C₂₀ species of PGP-Me, PGS and PG, characteristic of *Halobacterium* strains (Torreblanca *et al.*, 1986; Kates *et al.*, 1993; Kates, 1993a, b) (Table 2).

In contrast, the lipids of the haloalkaliphilic strains (e.g. strain SSL1) contained both C₂₀-C₂₀ and C₂₀-C₂₅ species of PGP-Me and PG, and lacked any PGS, as reported previously for *Natronobacterium* strains (Tindall *et al.*, 1984; Morth & Tindall, 1985; De Rosa *et al.*, 1988) (Table 2). Surprisingly, however, an unidentified glycolipid (GL-1) was clearly detected on TLC plates of the polar lipids of

alkaliphilic strains SSL1 and SSL6 by staining with the α -naphthol sugar reagent (Table 2). This observation is in contrast to all previously reported analyses of haloalkaliphilic lipids, which indicated the absence of glycolipids in known haloalkaliphiles (Tindall *et al.*, 1984; Morth & Tindall, 1985; De Rosa *et al.*, 1988; Upasani & Desai, 1990). GL-1 also occurred as both C₂₀-C₂₀ and C₂₀-C₂₅ molecular species. It is of interest that a glycolipid spot with the same mobility as GL-1 could be detected on TLC plates of the polar lipids of the reference strain *N. gregoryi* when a high lipid load was applied (Table 2).

Quantitative composition (% by wt) of the polar lipid components of *Natronobacterium* strain SSL1 was found to be as follows: PGP-Me was the major lipid (approx. 72%, by wt) followed by PG (22%) and the novel glycolipid (GL-1, 6%). Small amounts (< 1%) of PA and two unidentified phospholipids (PL-1 and PL-2), and traces of another unidentified glycolipid (GL-2) were also present. PL-1, PL-2 and GL-2 were not further investigated.

Identification of polar lipids in *Natronobacterium* strain SSL1

Phosphatidylglycerolmethylphosphate (PGP-Me). The PGP-Me component was separated into two molecular species by preparative TLC in solvent system C (Table 2),

and each isolated molecular species was subjected to negative FAB-MS and identified as the C_{20} - C_{20} (slower moving) species: $[M-H]^-$, m/z 899.7; $[M+Na-H]^-$, m/z 921.6 (calculated $M = 900$); and the C_{20} - C_{25} (faster-moving) species: $[M-H]^-$, m/z 969.7; $[M+Na-H]^-$, m/z 991.7 (calculated $M = 970$). Identification of these molecular species was confirmed by CI-MS of the corresponding lipid cores (diphytanylglycerol diethers) obtained after acid methanolysis, showing $[M+1]^+$ ion peaks with m/z 653 and 723, as expected for the C_{20} - C_{20} ($M = 652$) and C_{20} - C_{25} ($M = 722$) diethers, respectively. The C_{20} - C_{20} species (slower moving) was present in higher proportion than the C_{20} - C_{25} species (faster moving), in contrast to the lower proportions of the C_{20} - C_{20} species found in other natronobacteria, but similar to the findings for *Natronococcus occultus* (De Rosa *et al.*, 1988).

The 1H -NMR spectrum of PGP-Me(NH_4)₂ (in $CDCl_3$) showed diagnostic signals that were essentially identical to those reported previously (Moldoveanu *et al.*, 1990; Kates *et al.*, 1993). The ^{13}C -NMR spectrum of PGP-Me was identical to that reported previously and clearly showed the presence of the P-O- CH_3 signal (doublet) at 53 p.p.m. (Kates *et al.*, 1993; Stewart *et al.*, 1988).

The water-soluble products of methanolysis were identified as glycerol diphosphate by TLC on cellulose plates in solvent I (Moldoveanu *et al.*, 1990), as expected for PGP-Me.

Phosphatidylglycerol (PG). As described for PGP-Me, the PG component was also separated into two molecular species by TLC in solvent C (Table 2), and these were identified by FAB-MS as the C_{20} - C_{20} and C_{20} - C_{25} molecular species of PG: $[M-H]^-$, m/z 805.7 and 875.8, respectively; calculated $M = 806$ and 876, respectively. The identity of the two molecular species was confirmed by CI-MS analysis of the lipid cores, as described above for PGP-Me. As was observed for PGP-Me, the C_{20} - C_{20} molecular species of PG appeared to be present in higher proportion than the C_{20} - C_{25} species, in contrast to the proportions reported for other natronobacteria, but similar to those reported for *Natronococcus occultus* (De Rosa *et al.*, 1988).

The water-soluble products of acid methanolysis contained glycerophosphate, as expected for PG. The 1H -NMR spectrum showed the same signals for the phytanyl ether linked chains, for glycerol CH-O and CH_2 -O groups and for CH_2 -O-P groups (but not for P-O- CH_3 groups) as in the spectrum of PGP-Me (Kates *et al.*, 1993); a signal at 3.63 p.p.m. (CH_2 -OH in glycerol) was present, as expected for PG.

Phosphatidic acid (PA). This phosphorus-positive minor component was identified on the basis only of its TLC mobilities in solvents A and C (Table 2), and was not further characterized because of insufficient material.

Novel diglucosylglycerol diether. This novel glycolipid (GL-1) could not be completely separated from PGP-Me by TLC in solvents A or B, but could be isolated in pure

form by preparative TLC in solvent C, which was designed specifically for this purpose.

Acid methanolysis of GL-1 yielded only glucose as the water-soluble product and diphytanylglycerol as the hydrophobic product, identified by TLC in solvents H (cellulose plates) and G (silica gel plates), respectively. Quantitative sugar analysis and gravimetric estimation of the diether component gave a molar ratio of glucose:diether of 2:1, showing that GL-1 was a diglucosyl diether (designated DGD-4). Permethylation analysis of GL-1 yielded two partially methylated sugars, shown by GC-MS analysis of their alditol acetates (retentions relative to 1,5-diacetyl-2,3,4,6-tetramethyl-D-glucitol: 1.00 and 1.33) to be 2,3,4,6-tetramethylglucose and 2,3,4-trimethylglucose, respectively. The two glucose units are thus linked 1 → 6.

The anomeric configuration of each glucose unit was studied by 1H -NMR, which showed signals for the presence of both α - and β -glucosidic linkages at 4.125–4.150 p.p.m. (doublet, J , 7.5 Hz; anomeric H of methyl- β -glucoside, 4.35 p.p.m., J , 7.5) and at 4.613–4.625 p.p.m. (doublet, J , 3.75; anomeric H of methyl- α -glucoside, 4.68 p.p.m., J , 3.5) (see data in Kates, 1990). The fact that the 4.62 p.p.m. signal showed essentially no shift relative to that of α -methylglucoside, whereas the 4.13 p.p.m. signal was shifted downfield from the β -methylglucoside signal suggests that the terminal glucose is β -linked and the internal glucose attached to 2,3-diphytanyl-*sn*-glycerol is α -linked. Attempts to determine the anomeric configuration by the action of specific glucosidases were unsuccessful since no hydrolysis products were observed after incubation of GL-1 with these enzymes.

Negative FAB-MS of GL-1 showed the presence of two molecular ion peaks, $[M-H]^-$, with m/z 967.8 and 1037.6, corresponding to the C_{20} - C_{20} and the C_{20} - C_{25} molecular species with four double bonds each (calculated M , $976 - 8H = 968$, and $1046 - 8H = 1038$, respectively). This suggests that one of the lipid core chains may be isoprenoid rather than isopranoid. Ion peaks, $[M-H]^-$, were also observed at m/z 989.7, 1057 and 1127, corresponding to hydroxylated and monounsaturated C_{20} - C_{20} -DGD-4 ($M = 990$), hydroxylated and diunsaturated C_{20} - C_{25} -DGD-4 ($M = 1058$) and hydroxylated and diunsaturated C_{25} - C_{25} -DGD-4 ($M = 1128$), respectively (see Ferrante *et al.*, 1988; Sprott, 1992). The presence of these hydroxylated and unsaturated species was corroborated by ion fragment peaks at m/z 669.4, 731.6 and 805.5, corresponding to hydroxylated C_{20} - C_{20} -diether ($M = 668$), hydroxylated and unsaturated (three double bonds) C_{20} - C_{25} -diether ($M = 732$) and hydroxylated and monounsaturated C_{25} - C_{25} -diether ($M = 806$), respectively.

The presence of saturated, unsaturated and hydroxylated lipid cores was confirmed by CI-MS of the intact GL-1 and of its hydrophobic products of mild acid methanolysis (Moldoveanu *et al.*, 1990; Ekiel & Sprott, 1992). The following CI-MS ion peaks, $[M+1]^+$, were observed for the intact GL-1 (m/z): 653 (C_{20} - C_{20} -diether; calculated

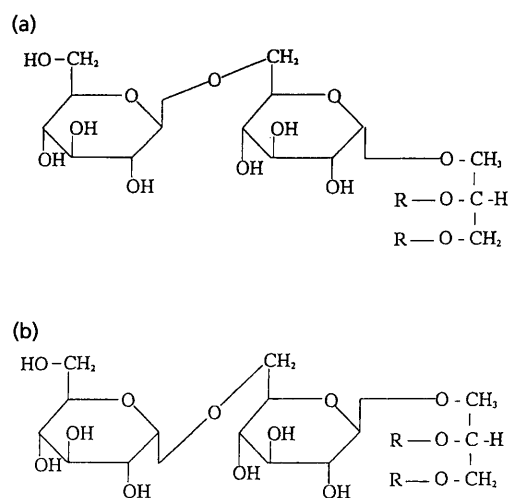


Fig. 1. Possible structures of glycolipid GL-1 (DGD-4) from *Natronobacterium* strain SSL1: (a) (most probable), glucopyranosyl- β 1 \rightarrow 6-glucopyranosyl- α 1 \rightarrow 1-glycerol diether; (b) glucopyranosyl- α 1 \rightarrow 6-glucopyranosyl- β 1 \rightarrow 1-glycerol diether. R = C₂₀ and C₂₅ isoprenoid, isoprenoid or hydroxyisoprenoid chains.

$M = 652$); 723 (C₂₀-C₂₅-diether; calculated $M = 722$); 373, 355 (monophytanylglycerol, C₂₀-MPG; calculated $M = 372$; $M - 18 = 354$); 443, 425 (C₂₅-MPG; calculated $M = 442$; $M - 18 = 424$). The hydrophobic products of acid methanolysis showed ion peaks with m/z 311 (methoxyphytol and methoxyisophytol, calculated $M = 310$); 297 (phytol, calculated $M = 296$); 281 (phytene, calculated $M = 280$); 681 (monounsaturated methoxy-C₂₀-C₂₀-diether; $M = 680$); 753 (methoxy-C₂₀-C₂₅-diether; $M = 752$). All of these peaks are diagnostic of hydroxylated or unhydroxylated isoprenoid chains, which are readily cleaved from the diether lipid cores, and of the hydroxylated lipid cores themselves (Ferrante *et al.*, 1988; Moldoveanu & Kates, 1988; Moldoveanu *et al.*, 1990; Ekiel & Sprott, 1992). Parent ion peaks, $[M + H]^+$, of the intact diether lipid cores were also present at m/z 653 (saturated C₂₀-C₂₀-diether; calculated $M = 652$); 669 (HO-C₂₀-C₂₀-diether; calculated $M = 668$); 717 (unsaturated C₂₀-C₂₅-diether; calculated for three double bonds; $M = 716$); 732 (unsaturated OH-C₂₀-C₂₅-diether; calculated for three double bonds, $M = 732$); and 789 (diunsaturated C₂₅-C₂₅-diether; calculated $M = 788$).

The presence of unsaturated isoprenyl chains in GL-1 was further supported by its ¹H-NMR spectrum which showed signals at 1.62 p.p.m. for allylic methyl groups, at 2.08 p.p.m. for allylic methylene groups, and at 5.0 p.p.m. for olefinic isoprenoid protons (Moldoveanu *et al.*, 1990).

The data thus show that the most probable structure of the new glycolipid DGD-4 is as shown in Fig. 1(a): glucopyranosyl- β 1 \rightarrow 6-glucopyranosyl- α 1 \rightarrow 1-glycerol diether. The alternative structure, glucopyranosyl- α 1 \rightarrow 6-glucopyranosyl- β 1 \rightarrow 1-glycerol diether (Fig. 1b), is less likely, but has not yet been eliminated. The diether core of GL-1 contains C₂₀-C₂₀, C₂₀-C₂₅ and C₂₅-C₂₅,

saturated, unsaturated and/or hydroxylated molecular species; the C₂₀-C₂₀ and C₂₀-C₂₅ species are present in approximately equal amounts, while the C₂₅-C₂₅ species is present only in low amounts.

DISCUSSION

The extreme-halophile phenotype encompasses three different classes of archaeal membranes: the exclusively C₂₀-C₂₀ bilayer type found in non-alkaliphilic halophiles (Ross *et al.*, 1981; Kates, 1978, 1993a, b); a 'zip'-type membrane with about equal proportions of C₂₀-C₂₀ and C₂₀-C₂₅ core lipids in haloalkaliphiles and halococci (De Rosa *et al.*, 1982, 1983; Moldoveanu *et al.*, 1990); and the mixed bilayer/'zip'-type composed of major proportions of C₂₀-C₂₅ core lipid with lesser amounts of the C₂₀-C₂₀ and C₂₅-C₂₅ species as in *Natronobacterium* strains, or major proportions of the C₂₀-C₂₀ species and lesser amounts of the C₂₀-C₂₅ and C₂₅-C₂₅ species as in *Natronococcus* strains (De Rosa *et al.*, 1983, 1988). The relative proportions of each of the three molecular species of lipid cores and the resulting bilayer type is dependent largely on genetic and phenotypic factors and on growth conditions of the extreme halophiles (Tindall, 1985; Morth & Tindall, 1985). These factors may account for the fact that the lipids of the Indian strains of *Natronobacterium* examined here are more similar to those of *Natronococcus* strains with respect to their core lipid profile than to those of *Natronobacterium* studied previously (De Rosa *et al.*, 1988).

The results of the present survey of archaeobacteria from saline environments in India show that, on the basis of their polar lipid composition, the non-alkaliphilic halobacteria from marine salterns at Mithapur comprise two groups belonging to the genera *Haloferax* (group I, strains MSW1-MSW3) and *Halobacterium* (group II, strains MSW4-MSW6), respectively. The haloalkaliphilic strains (SSL1-SSL6) from Sambhar Salt Lake brines clearly belong to the genus *Natronobacterium*, but their lipid composition differs somewhat from that reported previously for *N. gregoryi* and other haloalkaliphiles (Ross *et al.*, 1985; Grant & Larsen, 1989) in that a novel glycolipid (DGD-4) was detected, possessing hydroxylated and unsaturated lipid cores. A glycolipid with the same TLC mobility as DGD-4 was also detected in the type strain *N. gregoryi*. It should be noted that unsaturated (isoprenoid) chains have been identified previously in polar lipids of *Halococcus* species from Spain (Moldoveanu *et al.*, 1990) and in lipid biosynthetic intermediates in *H. cutirubrum* (Moldoveanu & Kates, 1988), but hydroxylated lipid cores have previously only been reported in methanogen species of archaeobacteria (Sprott, 1992). It is possible that hydroxylated diether lipid cores may be generally distributed in extreme halophiles and have been missed because of the relatively harsh hydrolytic procedures used in their isolation (Ekiel & Sprott, 1992; Sprott, 1992). However, it is not clear what function there would be for the presence of a hydroxyl group at the 3' position of the phytanyl group in the lipid core of the glycolipid DGD-4, which would make the lipid core molecule less

hydrophobic and also less thermostable and more susceptible to hydrolytic breakdown.

Apart from having hydroxylated and unsaturated diether lipid cores, the glycolipid DGD-4 is further unique in having a $\beta 1 \rightarrow 6$ -linked diglucosyl sugar moiety rather than the $\alpha 1 \rightarrow 2$ -linked mannosyl-glucosyl group (with sugar or sulfate substitution at C-3 and/or C-6 of the mannose residue) found in glycosyl diethers of all extreme halophiles examined so far (see Kushwaha *et al.*, 1982; Torreblanca *et al.*, 1986; Kates, 1990, 1993a, b), with the following exceptions: (i) the mannosyl-2-sulfate- $\alpha 1 \rightarrow 4$ -glucosyl diether in *H. sodomense* (Trincone *et al.*, 1990); (ii) the mannosyl-2-sulfate- $\alpha 1 \rightarrow 2$ -glucosyl diether in *H. trapanicum* (Trincone *et al.*, 1993); and (iii) the mannosyl-2,6-disulfate- $\alpha 1 \rightarrow 2$ -glucosyl diether in an extreme halophile (strain 172) from Japan (Matsubara *et al.*, 1994).

However, the $\beta 1 \rightarrow 6$ -diglucosyl structure in DGD-4 is even more unusual, since it bears a striking resemblance to the $\beta 1 \rightarrow 6$ -diglucosyl(gentiobiosyl)diether glycolipid (hydroxylated or unhydroxylated) present in several species of methanogens (Sprott, 1992). The presence in haloalkaliphiles of this unusual 'methanogen-like' glycolipid (DGD-4) raises the question whether the *Natronobacteria* from Indian saline environments, or in general, may have an evolutionary relation to the methanogens (see Kates, 1993a, b). Further surveys of glycolipids in haloalkaliphiles, and perhaps also halophilic methanogens, some species of which have recently been found (Paterek & Smith, 1985), may be of interest in this connection.

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