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POLAR LIPIDS OF AN EXTREMELY HALOPHILIC BACTERIAL STRAIN (R-4) ISOLATED FROM SALT PONDS IN SPAIN

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The lipids of an extremely halophilic bacterium, strain R-4, isolated from the salt ponds in Spain at Alicante, were found to contain 93% polar lipids and 7% non-polar lipids. Four major polar lipids were detected, all derivatives of 2,3-di-*O*-phytanyl-*sn*-glycerol: (i) a novel glycolipid sulfate, 2,3-di-*O*-phytanyl-1-*O*-[α-D-mannopyranosyl-6′-sulfate-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol, (ii) phosphatidylglycerol, (iii) phosphatidylglycerophosphate, (iv) a glycolipid, 2,3-di-*O*-phytanyl-1-*O*-[α-D-mannopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol. Traces of four other phospholipids and one glycolipid were also detected.

Introduction

Previous studies of the lipids of extremely halophilic bacteria have been carried out mainly on *Halobacterium cutirubrum*, *Halobacterium halobium* and *Halobacterium marismortui* [1–3]. These organisms contain unusual lipids [1] which are derived exclusively from 2,3-di-*O*-phytanylglycerol ether. The polar lipid components in *H. cutirubrum* and *H. halobium* [1] consist mostly of a glycolipid sulfate (galactosylsulfate-mannosyl-glucosyl diphytanyl ether) and the diphytanylglycerol ether analogues of phosphatidylglycerol, phosphatidylglycerophosphate and phosphatidylglycerosulfate. *H. marismortui* [2], which inhabits the Dead Sea, contains mainly the diphytanylglycerol ether analogs of phosphatidylglycerol, phosphatidylglycerophosphate, phosphatidylglycerosulfate and a glycotriaosyl diether (glucosyl-mannosyl-glucosyl diphytanylglycerol ether) but no glycolipid sulfate. Recently, a new glycolipid sulfate [galactopyranosyl sulfate-mannopyranosyl-(← galactofuranosyl)-glucopyranosyl diphytanylglycerol ether] has been reported in *H. cutirubrum* [3]. The occurrence of phytanyl diether lipids has also been reported in methane-producing bacteria [4–7].

Recently, a new strain of an extremely halophilic bacterium (strain R-4) was isolated from salt flats in Spain at Alicante by Rodriguez-Valera et al. [8,9]. On the basis of physiological and biochemical data, the above authors claimed that this strain is significantly different from known species of *Halobacterium* [8,9]. Therefore, it was of interest to examine the lipids of this new strain R-4 for comparison with other halobacteria.

Materials and Methods

Materials

Standard partially methylated alditol acetates (2,3,4,6-tetra-*O*-methyl glucose; 2,3,4,6-tetra-*O*-methyl mannose; 3,4,6-tri-*O*-methyl glucose; 2,3,4-tri-*O*-methyl mannose) were gifts from Dr. M.B. Perry, National Research Council, Ottawa. Silica gel G for thin-layer chromatography was purchased

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from Brinkman Instruments (Canada) Ltd. Methyl iodide was obtained from J.T. Baker Chemical Co. All solvents were distilled before use

Organism and growth conditions

A typical strain (R-4) was isolated from seawater evaporation ponds near Alicante, Spain, and deposited in the Czechoslovak Collection of Microorganisms, No. CCM3361. The cells were grown at 38°C in a medium [8,9] composed of inorganic salts (% w/v: NaCl, 19.4; $MgCl_2$, 1.6; $MgSO_4$, 2.4; $CaCl_2$, 0.1; KCl, 0.5; $NaHCO_3$, 0.02; NaBr, 0.05; yeast extract, 0.5; pH, 7.3), in 10-l batches in a 15-l cylindrical glass flask, which was stirred magnetically, and aereated through a sparger. The cultures were grown for 4 days, harvested by centrifugation and washed twice with 25% NaCl solution at the centrifuge. Harvested cells were then extracted for lipids as described below.

Chromatography

Thin-layer chromatography (TLC) of polar lipids was performed on silica gel G layers (analytical, 0.25 mm thick; preparative, 0.75 mm thick) using the following solvent systems: A, $CHCl_3/MeOH/CH_3COOH/H_2O$ (85:20:10:4, v/v, double development) for the separation of phospholipids and glycolipids; B, petroleum ether/diethyl ether/acetic acid (60:40:1, v/v) for the separation of phytanylglycerol diether.

Lipids were detected by the following spray reagents [10]: $(NH_4)_2MoO_4/HClO_4$ for phosphatides; 0.5% α-naphthol/H_2SO_4 for glycolipids; H_2SO_4/C_2H_5OH (1:1, v/v) followed by charring and 0.005% aqueous Rhodamine 6G followed by visualization under ultraviolet light for detection of all lipids.

Water-soluble products were examined by paper chromatography on Whatman No. 1 paper in solvent system C, pyridine/ethyl acetate/water (2:5:5, upper phase) for sugars and phosphate esters by ascending chromatography. The developed chromatograms were stained with alkaline $AgNO_3$ stain for sugars and sulfosalicylic acid/$FeCl_3$ reagent for phosphate esters [10].

Permethylated glycolipid sulfate and glycolipids were separated on silica gel G in solvent system D, petroleum ether/diethyl ether (5:20, v/v), and solvent system E, petroleum ether/diethyl ether (15:35, v/v), respectively.

Partially methylated alditol acetates of sugars were analysed by GLC on a 3% SP-2340 on 100/120 chromosorb WAW. The column temperature was programmed at 1°C per min from 200 to 240°C.

Analytical methods

Phosphorus was determined by the method of Allen [11] (see Ref. 10) and total hexose content of glycolipids by a modified phenolsulfuric acid method [10,12] using either glucose or a mixture (1:1, w/w) of glucose and mannose as standard.

Physical measurements

Infrared spectra of lipids were measured in KBr pellets using a Unicam SP 1000 infrared Spectrophotometer. Optical rotations were measured at 24°C at 589 nm with a Perkin-Elmer polarimeter, model 141, with digital readout. ^{1}H-NMR spectra were measured in C^2HCl_3 containing tetramethyl silane as an internal standard, using a Varian HA-100 NMR spectrometer. Mass spectra were recorded on a Finnigan 3100 D quadrupole mass spectrometer with a 6000 data system on line, after GLC on a 1.5 m column (2 mm inner diameter) of 3% SP-2340, temperature programmed from 180 to 240°C at 6°C/min.

Extraction and purification of lipids

Total lipids were extracted from harvested cells by the method of Bligh and Dyer [13] as modified for extremely halophilic bacteria [10]. Polar lipids were freed from non-polar components by acetone precipitation [14]. Individual polar lipids were purified by repeated preparative TLC on silica gel G in solvent A for all polar lipids in this organism. Phospholipids and glycolipid sulfate were converted to their ammonium salt form as described elsewhere [7].

Methanolysis of lipids

Acid methanolysis of lipids using 2.5% HCl (gas) in methanol was performed as described elsewhere [10]. The petroleum ether-soluble material was identified as diphytanyglycerol ether [1]. Sugar methyl glycosides in the methanol/water phase were converted to free sugars [15] and

analysed by paper chromatography in solvent C and by GLC of their trimethylsilyl derivatives [16].

Partial methanolysis of glycolipid sulfate and glycolipid was carried out in a mixture of chloroform/0.25 N HCl (gas) in methanol (1:1, v/v) at 60°C. The progress of methanolysis was monitored by microslide TLC in solvent A. After 1 h all of the glycolipid sulfate was hydrolysed to equal amounts of glycodiosyl diether (R_F 0.3) and monoglycosyl diether (R_F 0.81). The above hydrolysis products were isolated by TLC in solvent A (single development).

Permethylation analysis

Glycolipid sulfate (24 mg) was converted to the free acid and methylated with CH_3I (5 ml) and Ag_2O (50 mg) as described elsewhere [7,17,18]. The reaction, monitored by microslide TLC in solvent D, was complete in 6 h. The main product (R_F 0.85) was purified by TLC in solvent D and eluted with acetone (yield, 4 mg; the low yield is due to the instability of the glycolipid sulfate to the permethylation treatment). Glycolipids (3–6 mg) were also permethylated as described above and the products were purified by TLC in solvent E (R_F 0.78 for monoglycosyl diether, R_F 0.38 for glycodiosyl diether).

Alditol acetates of partially methylated sugars were prepared from the permethylated lipids by a modified method of Yang and Hakomori [19] as described elsewhere [7].

Determination of anomeric configuration

Anomeric configuration of glycolipids was determined by the method of Laine and Renkonen [20].

Results

Lipid composition

Growth of strain R-4 under the conditions given above yielded 4.3 g of cellular protein per 10 l culture; total lipid content was about 11% on a cell protein basis. Acetone precipitation indicated 93% by weight of the lipids were polar and 7% nonpolar. TLC of the polar lipids revealed the presence of six components: two major and four minor phospholipids; one major glycolipid and two minor glycolipids (Fig. 1).

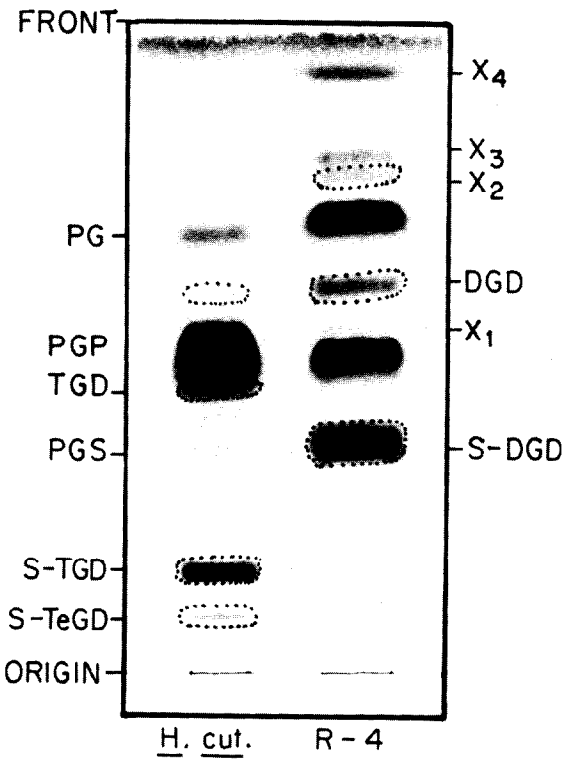

Fig. 1. Thin-layer chromatogram of polar lipids of *H. cutirubrum* and extreme halophile strain R-4. Solvent: chloroform/methanol/acetic acid/water (85:22.5:10:4; double development). The chromatogram was stained with α-naphthol/H_2SO_4 (dotted spots are sugar-positive) and then charred. PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGS, phosphatidylglycerosulfate; DGD, glycodiosyl diether; S-DGD, sulfated glycodiosyl diether; S-TDG, sulfated glycotriaosyl diether; S-TeGD, sulfated glycotetraosyl diether. All components are derivatives of 2,3-di-*O*-phytanyl-*sn*-glycerol. X_1, X_2, X_3 and X_4 are unidentified components.

TLC of the two major phospholipids in different solvents indicated that they were phosphatidylglycerol and phosphatidylglycerophosphate. These identifications were confirmed by elemental analysis (Table I) and infrared spectroscopy [1]. The minor phospholipids could not be identified due to insufficient material.

Identification of glycolipid sulfate, a glycodiosyl diether sulfate

This component is one of the three major polar lipids of strain R-4, accounting for about 29% (by wt.) of total polar lipids (Table II). The TLC pure

TABLE I

ELEMENTAL ANALYSIS OF PHOSPHOLIPIDS

Values for phosphatidylglycerol were calculated for $C_{46}H_{100}O_9PN$ (monoammonium salt, monohydrate), those for phosphatidylglycerophosphate were calculated for $C_{46}H_{103}O_{12}P_2N_2$ (diammonium salt, monohydrate).

	Phosphatidyl-glycerol		Phosphatidyl-glycerophosphate	
	Found	Calcd.	Found	Calcd.
Molecular weight	–	842.3	–	938.5
C (%)	65.3	65.60	59.08	58.87
H (%)	11.76	11.97	10.76	11.09
P (%)	3.48	3.68	6.45	6.60
N (%)	1.55	1.66	3.09	2.99
N/P, atomic ratio	1.08	1.00	1.10	1.00
$[\alpha]_D$				
Sodium salt [a]	+3.8°	–		
Ammonium salt	0.0°	–		

[a] Phosphatidylglycerol from *H. cutirubrum* [1] (sodium salt, +3.46° and ammonium salt, 0.0°).

product was a colorless solid giving a positive test for sugar. The infrared spectrum of its ammonium salt showed absorption bands at: 3420 cm^{-1} (OH groups); 2970, 2940, 2880, 1470 cm^{-1} (CH_3 and CH_2 groups); 1385 and 1370 cm^{-1} (a doublet, isopropyl group); 1250 cm^{-1} (a broad band, S=O); 830 cm^{-1} (a weak band, S-O-C); 1100 cm^{-1} (C-O-C groups); 1070 cm^{-1} (alcoholic C-O). No carboxylic or ester bands were present. This spectrum was similar to those of other glycolipid sulfates isolated from *H. cutirubrum* [3,15]. The presence of sulfur was also confirmed by its elemental analyses (Table III).

Acid methanolysis of this lipid yielded *sn*-2,3-di-*O*-phytanylglycerol, which was identical to an authentic standard [1]. The hexose content was 34% and the hexose/diether mole ratio was 2:1 (Table III). Analysis of the methanol/water phase showed the presence of glucose and mannose in an equimolar ratio, thus indicating a mol ratio of diether/glucose/mannose of 1:1:1. The mol ratio of sulfur to diether of 1:1 (Table III) indicated that the glycolipid was monosulfated.

Partial acid methanolysis of this glycolipid sulfate resulted in the formation of a glycodiosyl diether containing glucose and mannose in equimolar ratio and a monoglycosyl diether which contained only glucose. The sequence of hexoses in GLS was, therefore, mannose-glucose-diether.

Permethylation analysis was then used to determine the linkage positions between the sugars and also between the sulfate and the sugars. GC-MS analysis [21] of the alditol acetates of the partially methylated sugars derived from per-

TABLE II

POLAR LIPID COMPOSITION OF STRAIN R-4 COMPARED TO *H. CUTIRUBRUM* AND *H. MARISMORTUI*

Data for *H. cutirubrum* and *H. marismortui* are taken for comparison from Ref. 2. n.d., not determined.

Polar lipids	Lipid composition (mol%)		
	Strain R-4	*H. cutirubrum*	*H. marismortui*
Phosphatidylglycerol	44	4	11
Phosphatidylglycerophosphate	30	70	62
Phosphatidylglycerosulfate	–	4	17
Glycodiosyl diether sulfate	25	–	n.d.
Glycotriaosyl diether sulfate	–	21	–
Glycodiosyl diether	–	n.d.	n.d.
Glycotriaosyl diether	–	trace	11
Unidentified lipids	<1	–	<1
Number of negative charges per mol ionic lipid [a]	1.3–1.6	1.7–2.4	1.7–2.3

[a] The first and second values given are calculated on the basis of 2 and 3 negative charges per molecule of phosphatidylglycerophosphate, respectively.

TABLE III

ANALYTICAL DATA FOR GLYCOLIPIDS

	Glycodiosyl diether sulfate		Glycodiosyl diether		Monoglycosyl diether	
	Found	Calcd. [a]	Found	Calcd. [b]	Found	Calcd. [c]
Molecular weight	–	1091	–	977.5	–	815.3
C (%)	60.21	60.49	–	67.58	–	71.78
H (%)	10.19	10.36	–	11.14	–	11.93
S (%)	3.07	2.93	–	–	–	–
N (%)	1.50	1.28	–	–	–	–
Hexose (%)	34.3	33	35.50	36.86	21.50	22.09
Diether (%)	58.00	59.76	65.10	66.82	78.40	80.11
Hexose/diether mole ratio	2.14	2.00	1.97	2.00	0.99	1.00
S/diether, mol ratio	0.92	1.00	–	–	–	–
$[\alpha]_D$ (in degrees)	+42.8	–	+61.10	–	+43.59	–
M_D (in degrees)	+466.7	+519	+597	+519	+355.4	+365

[a] Calculated for $C_{55}H_{111}O_{16}NS.H_2O$ [22,23].
[b] Calculated for α-Man*p*-α-Glc*p*-diether ($C_{55}H_{108}O_{13}$).
[c] Calculated for α-Glc*p*-diether ($C_{49}H_{98}O_8$).

methylated glycolipid sulfate showed the presence of 3,4,6-trimethyl glucose and 2,3,4-trimethyl mannose (Table IV). This showed that sulfate was attached to the terminal sugar, mannose, and not to the internal hexose, glucose. If sulfate were attached to internal glucose, then permethylated GLS would have yielded a dimethyl glucose and a tetramethyl mannose. Furthermore, permethylated glycodiosyl diether, obtained by desulfation of GLS, yielded 3,4,6-trimethylglucose and 2,3,4,6-tetramethylmannose. These results indicated that the terminal mannose is attached to the internal

TABLE IV

GAS-LIQUID CHROMATOGRAPHY OF PARTIALLY METHYLATED ALDITOL ACETATES FROM PERMETHYLATED GLYCOLIPIDS

2,3,4,6-Tetramethylgalactose and 2,3,4-trimethylmannose were prepared by permethylation of triglycosyl diether from *H. cutirubrum*

	Peak	Relative retention time	Peak area ratio
Diglycosyl diether sulfate	D	1.67	1.00
	E	1.90	0.94
Diglycosyl diether (from diglycosyl diether sulfate)	A	0.94	0.98
	D	1.68	1.00
Monoglycosyldiether	B	1.00	–
Standards			
2,3,4,6-Tetramethylmannose	A	0.95	
2,3,4,6-Tetramethylglucose	B	1.00	
2,3,4,6-Tetramethylgalactose	C	1.13	
3,4,6-Trimethylglucose	D	1.67	
2,3,4-Trimethylmannose	E	1.91	

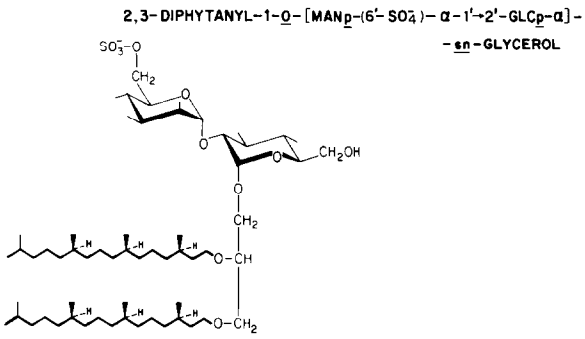

Fig. 2. Chemical structure of sulfated glycodiosyl diether: 2,3-diphytanyl-1-*O*-[Man*p*-(6-SO_4)-α-1 → 2-Glc*p*-α]-*sn*-glycerol.

glucose at position 2 and also that sulfate is attached to mannose at position 6.

Determination of the configuration of the glycosidic linkages in the desulfated glycolipid sulfate by CrO_3 oxidation showed no loss of any hexose residue, indicating that both linkages had the α-configuration. The above conclusions were also supported by the molecular rotation data (Table III).

Thus, the complete structure of the glycolipid sulfate (diglycosyl diether sulfate) in strain R-4 is 2,3-di-*O*-phytanyl-1-*O*-[α-D-mannopyranosyl-6′-sulfate-(1′ → 2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (Fig. 2).

Structure of glycodiosyl diether

This glycolipid had the analytical data (Table III) expected for a diglycosyl diether and the same mobilities as desulfated glycolipid sulfate. Also, it gave essentially the same results with respect to acid methanolysis, CrO_3 oxidation, molecular rotation (Table III) and permethylation analysis (Table IV) as was observed for the desulfated glycolipid sulfate. This glycolipid is thus the naturally occurring desulfated glycodiosyl diether sulfate with the structure: 2,3-di-*O*-phytanyl-1-*O*-[α-D-mannopyranosyl-(1′ → 2′)-*O*-α-D-glucopyranosyl-*sn*-glycerol.

Discussion

This study establishes the structures of all the polar lipids in an extremely halophilic bacterial strain R-4 from Alicante, Spain. The striking features of the polar lipid composition of strain R-4, when compared to that of *H. cutirubrum* and *H. marismortui*, are the presence of a new glycolipid sulfate, an unusually high proportion of phosphatidylglycerol and an apparent lack of phosphatidylglycerosulfate and the glycotriaosyl diether sulfate found in *H. cutirubrum*. The glycolipid sulfate found in *H. cutirubrum* has three hexoses, glucose, mannose and galactose attached to a 2,3-di-*O*-phytanylglycerol ether moiety, with a sulfate group attached to the terminal galactose at position 3, whereas the glycolipid sulfate from strain R-4 has only two sugars, glucose and mannose, with the sulfate attached to the terminal mannose, at position 6. The linkages between the glucose and mannose in both glycolipid sulfates are identical. The apparent lack of phosphatidylglycerosulfate in strain R-4 appears to be compensated by an equivalent increase in the amounts of the glycodiosyl diether sulfate (Table II). Glycotriaosyl diether sulfate and glycotriaosyl diether, found in *H. cutirubrum* and *H. marismortui* respectively, have not been detected in strain R-4. Furthermore, the proportions of phosphatidylglycerol in strain R-4 are far higher than in *H. cutirubrum* or *H. marismortui*. However, the amounts of phosphatidylglycerol and phosphatidylglycerophosphate taken together appear to be almost identical in all the three bacteria. In spite of the above mentioned differences, strain R-4 maintains a value (1.3–1.6), for the number of negative charges per mol ionic lipid, which is comparable to that (1.7–2.4) for *H. cutirubrum* and (1.7–2.3) for *H. marismortui* (Table II). There appears to be considerable variation in the lipid composition of extreme halophiles from different natural sources and further studies on this point are in progress.

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