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Polar lipids of non-alkaliphilic *Halococci*

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Until recently, only one species of *Halococcus* has been recognized, namely, *H. morrhuae*, but a large number of extremely halophilic non-alkaliphilic cocci have now been isolated from hypersaline habitats in Spain and classified into four phenons (A–D); one of the phenon D strains has been classified as a new species, *Halococcus saccharolyticus*. Examination of the lipids of *H. saccharolyticus* and four strains of phenons A–C showed the presence in all of them of C_{20}-C_{20} and C_{20}-C_{25} diether molecular species of phosphatidylglycerophosphate (PGP), phosphatidylglycerol (PG) and phosphatidic acid (PA); a monounsaturated isoprenoid C_{20}-C_{20} (phytanyl-phytenyl) species of PGP; a sulfated diglycosyl diphytanylglycerol (S-DGD) with structure 2,3-diphytanyl-1-(6-HSO_3-mannosyl-1-2-glucosyl)-glycerol, which is identical to the S-DGD-1 in *Haloferax mediterranei*; a phosphoglycolipid (P-TGD) tentatively identified as a phytanyl-phytenyl-(H_2PO_3-galactosyl-mannosyl-glycosyl)-glycerol, and two unidentified glycolipids present only in traces. No phosphatidylglycerosulfate (PGS) was detected in any of the strains examined. This pattern of lipids appears to be characteristic of the strains of *Halococcus* from salterns in Spain, but studies of a larger number and variety of *Haloccus* are necessary to establish this conclusion with certainty.

Introduction

In previous studies of the lipids of halococci, only two species were examined: *Halococcus morrhuae* (formerly *Sarcina literalis*) [1,2] and an unidentified *Sarcina* species [1]. Both microorganisms were found by thin-layer chromatography (TLC) to contain the major phospholipid, phosphatidylglycerophosphate (PGP, diphytanyl ether analogue), the minor phospholipid, phosphatidylglycerol (PG, diphytanyl ether analogue), as well as the sulfated triglycosyldiphytanylglycerol (S-TGD-1) and the unsulfated TGD-1, but not phosphatidylglycerosulfate (PGS, diphytanylglycerol analogue). *H. morrhuae* also appears to contain the sulfated tetraglycosyldiphytanylglycerol (S-TeGD) and the unidentified *Sarcina* species contains an unidentified glycolipid.

Based only on these results, the lipids of halococci appeared to be similar to those of halobacteria [3,4], but further studies of a larger variety of halococci were needed to verify this conclusion. In a recent study of halophilic, non-alkaliphilic cocci from several salterns in Spain, 96 strains comprising four phenons were isolated [5]. Examination of the lipids of one of these strains, *Halococcus saccharolyticus* (strain P423 from phenon D) [6], showed a distinctively different lipid pattern from that of the previously studied species [1,2] and from those of halobacteria [3,4].

We have now examined the lipids of *H. saccharolyticus* (phenon D) in greater detail, and have also examined four additional strains of *Halococcus* representing each of the remaining phenons [5], as follows: N-207 and CCM-537 (phenon A), Q-26 (phenon B) and P-1140 (phenon C). We report here the compositional and structural analysis of the lipids of these species of halococci. It is of interest that all of these non-alkaliphilic halococci possess a distinctive pattern of phospholipids (PGP and PG, containing both C_{20}-C_{20} and C_{20}-C_{25} molecular species) and glycolipids (sulfated diglycosyl diphytanylglycerol, S-DGD; a phosphoglycolipid; and two unidentified minor glycolipids), in

Abbreviations: PA, phosphatidic acid; PGP, phosphatidylglycerophosphate; PG, phosphatidylglycerol; PGS, phosphatidylglycerosulfate; S-DGD, sulfated diglycosyldiphytanylglycerol; S-TGD, sulfated triglycosyldiphytanylglycerol; P-TGD, phosphotriglycosyldiphytanylglycerol; TLC, thin-layer chromatography; DPG, diphytanylglycerol; MPG, monophytanylglycerol; GP, glycerophosphate; GBP, glycerolbiphosphate; CI-MS, chemical ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry.

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spite of some similarities to the lipid pattern in the haloalkaliphiles [7] and in *Haloferax* species [8].

Material and Methods

Culture of organisms

Halococcus saccharolyticus [6], strain P-423 (ATCC 49257), obtained from salterns in Spain [5] was grown in 9 l of a standard complex medium for *Halobacterium cutirubrum* [9], supplemented, per liter of medium, with: $MgSO_4 \cdot 7H_2O$, 28.5 g; $MgCl_2$, 18.2 g; and NaBr, 0.58 g [5]. The culture was grown for 7 d (early stationary phase). Other strains (P-1140, N-207, CCM-537 and Q-26, obtained from salterns in Spain [5] were also grown in the supplemented medium but for different incubation periods: 13 d (stationary phase) and 8, 7 and 9 d (early stationary phase), respectively. All cells were harvested by centrifugation and the cells (40 g wet weight) were washed twice and suspended in 200 ml of basal salt solution (KCl, 2 g; $MgSO_4$, 9.8 g; NaCl, 250 g, per liter) and sonicated in 40-ml batches at 4° C for 10 min with a sonic dismembrator (Quigley-Rochester, using the macrotip at 60 W power output with a 30 s interuption every minute.

Lipid extraction

The extraction procedure was based on the method of Bligh and Dyer [10] as modified by Kates [11]. To 200 ml of disrupted cell suspension was added 750 ml of methanol/chloroform (2 : 1, v/v) to give a one-phase solvent system ($CHCl_3$/MeOH/salt solution, 1 : 2 : 0.8, v/v). The suspension was stirred overnight (in the dark) and centrifuged, and the solid residue was reextracted for 5 h, at room temperature with 380 ml of chloroform/methanol/water (1 : 2 : 0.8, v/v). The combined extracts were diluted with 350 ml of chloroform and 350 ml of water to give a two-phased system, which was allowed to separate in a separatory funnel overnight. The chloroform phase was removed and the upper phase was washed twice with 25 ml chloroform. The combined chloroform extracts were diluted with benzene and taken to dryness in a rotary evaporator at 30° C. The residue was dissolved in chloroform/methanol (2 : 1, v/v), and the solution was cleared by centrifugation, diluted with benzene, evaporated to a small volume under nitrogen and stored at −20° C (yield, 80–90 mg total lipid). The cellular residue was re-extracted twice, at 4° C, overnight with 190 ml $CHCl_3$/MeOH/0.2 M HCl (1 : 2 : 0.8, v/v). After centrifugation the combined extracts were converted to two phases as described above. The chloroform phase was taken to dryness and the residue was dissolved in $CHCl_3$/MeOH (2 : 1, v/v), cleared by centrifugation, neutralised with 2 M NH_4OH in MeOH, diluted with benzene and evaporated to a small volume under nitrogen (yield approx. 10 mg). Total yield of combined lipids, 90–100 mg.

Acetone precipitation of polar lipids

A solution of the total combined lipids in a minimum volume of chloroform (1 ml per 100 mg of lipids), was diluted with 10 volumes of dry acetone, mixed well and allowed to stand at −20° C for 48 h. The precipitate of polar lipids was washed twice with small portions of cold acetone and dried in vacuo over KOH. Yield of total polar lipids: approx. 60 mg per 9 l culture (approx. 66% of total lipids).

Chromatography of lipids

Thin-layer chromatography (TLC) of polar lipids was performed on Silica-gel KG layers (analytical, Whatman, 0.25 mm thick, preparative, 0.75 mm thick) using the following solvent systems: (A) $CHCl_3$/MeOH/CH_3COOH/H_2O (85 : 22.5 : 10 : 4, v/v, double development; (B) $CHCl_3$/MeOH/90% CH_3COOH (65 : 4 : 35, v/v); (C) $CHCl_3$/MeOH/NH_4OH (65 : 35 : 5, v/v) for phospholipids and glycolipids; (D) petroleum ether/ethyl ether/CH_3COOH (70 : 30 : 1, v/v) or E, petroleum ether/acetone (95 : 5, v/v, first development) and toluene/acetone (97 : 3, v/v second development) for the separation of non-polar lipids, particularly glycerol diether molecule species [12]; F, $CHCl_3$/MeOH (80 : 20, v/v) for methylated phospholipids or permethylated glycolipids. Lipids were detected by the following spray reagents: 0.5% α-naphthol/H_2SO_4 for glycolipids, $(NH_4)MoO_4/HClO_4$ for phospholipids; H_2SO_4/C_2H_5OH (1 : 1, v/v) followed by charring at 120° C, aqueous rhodamine 6G followed by visualisation under ultraviolet light (266 nm) or I_2 for detection of all lipids [11]. Tentative identification of the components was based on TLC mobilities relative to standards. Water-soluble products of methanolysis were chromatographed on TLC cellulose plates (Merck, DC cellulose, 0.1 mm thick) in solvent G, 1-butanol/acetic acid/water (50 : 30 : 10, v/v) or in solvent H, pyridine/ethyl acetate/water (2 : 5 : 5, upper phase) [9].

Isolation and purification of individual polar lipids

Individual polar lipids were isolated by preparative TLC on Silica-gel G, in solvent B for phospholipids and glycolipids. Each component was finally purified by TLC in solvent A or C (double development) for phospholipids and in solvent B, for glycolipids. The bands corresponding to individual phospholipids (PGP, PG, PGX, PA) (Table I) were eluted from the silica with $CHCl_3$/MeOH/H_2O (1 : 2 : 0.8, v/v) or $CHCl_3$/MeOH/0.2 M HCl (1 : 2 : 0.8, v/v) by the procedure of Bligh and Dyer (see Ref. 11). The individual major glycolipids (GL-2 and GL-4) (Table I) were eluted from the silica using $CHCl_3$/MeOH/H_2O (1 : 1 : 0.1, v/v) and the extracts were diluted with methanol and benz-

ene and brought to dryness in a rotary evaporator. The residual lipids were dissolved in $CHCl_3/MeOH/H_2O$ (1 : 1 : 0.05, v/v) (1 ml per 1–5 mg) and converted to their free acid forms by passing the solution through a small column of Rexyn 101 (H^+ form) which was washed with 10 volumes of $CHCl_3/MeOH$ (1 : 1, v/v) [13]. The free acid forms in the combined eluates were converted to the ammonium salt forms by addition of 2 M methanolic NH_4OH and the eluates were diluted with benzene and brought to dryness under nitrogen [11].

Analytical methods

Phosphorus was determined by the method of Bartlett [14] as modified by Kates [11] and total hexose content of glycolipids by the modified phenol/sulfuric acid method [15] using glucose/mannose (1 : 1) mixture as standard. Separated lipid components were quantitated gravimetrically after elution from the plates as described above. Phospholipids and glycolipids were quantitated by scraping TLC separated spots directly into digestion tubes and analysing for lipid-P [11] and lipid-sugar [15], respectively.

Lipid standards

Standard halobacterial phospholipids (PGP, PG, PGS) and glycolipids (S-TGD, TGD, S-TeGD) were isolated from lipids of *H. cutirubrum* as described elsewhere [13], and the sulfated diglycosyldiphytanylglycerol (S-DGD) was isolated from lipids of *Haloferax mediterranei* as described previously [8]. Phosphatidic acid (PA, diphytanylglycerol analogue) was synthesized as described elsewhere [16].

Physical measurements

The purified lipid components were analysed by negative fast atom bombardment mass spectroscopy (FAB-MS) and by chemical ionisation-mass spectromety (CI-MS), with a digital RL 02 data system and printonic printer. ^{1}H-NMR spectra were measured in $CDCl_3$ or in perdeuterated dimethylsulfoxide. Partially methylated alditols acetates of sugars were analysed by GC-MS on a JMW-DB-17 capillary column (30 m), temperature-programmed from 180 °C at 2 C°/min on a Hewlett Packard HP-5985 GC-MS instrument.

Methylation and permethylation analysis

The ammonium salt of PGP was converted to the free acid form [11] and methylated with diazomethane for 15 min, as described elsewhere [11,17]; the TLC purified tri- and tetramethylated derivatives ($PGP(CH_3)_3$ and $PGP(CH_3)_4$) were analysed by negative FAB-MS and by ^{1}H-NMR.

Acidic glycolipids (GL-2, GL-4) (Table I), ammonium salt forms, were converted to their free acid forms as described above. The residual free acid form (1–5 mg), dried in vacuo over KOH, was permethylated in 5 ml methyl iodide (freshly distilled) and 2 ml of dry benzene in the presence of 100 mg silver oxide, for 18 h, as described elsewhere [11]. The crude product contain-

TABLE I

TLC mobilities and distribution of polar lipids in Halococci

Total lipids extracted from various strains of *Halococcus* were subjected to thin-layer chromatography in solvents A, B and C (see Materials and Methods section); TLC plates were stained with the phosphate reagent or with the sugar reagent (α-naphthol) and then charred with H_2SO_4 at 120 °C. Intensity of spots are indicated by the number of plus (+) signs, based on charring with H_2SO_4.

Lipid component	R_F in solvents [a]			Distribution in strains				
	A	B	C	P-423 [b] (phenon D)	P-1140 (phenon C)	Q-26 (phenon B)	N-207 (phenon A)	CCM-37
Glycolipid (GL-1)	0.05	0.02	0.02	tr	tr	tr	tr	tr
Phosphoglycolipid (GL-2)	0.14	0.04	0.11	+	+	tr	+	+
Glycolipid (GL-3)	0.26	0.12	0.15	tr	tr	tr	tr	tr
Glycolipid (GL-4) (S-DGD)	0.35	0.18	0.35	+ +	+ +	+ +	+ +	+ +
PGP (C_{20}-C_{20})	0.39	0.50	0.23	+ + +	+ +	+ +	+ +	+ + +
PGP (C_{20}-C_{25})	0.44	0.54	0.27	+ +	+ + +	+ +	+ + +	+ +
PGX	0.53	0.61	0.31	+	+	tr	+	+
PG (C_{20}-C_{20})	0.82	0.86	0.66	+	+	tr	+	+
PG (C_{20}-C_{25})	0.85	0.89	0.68	+	+	tr	+	+
PA	0.95	0.99	0.10	+	+	tr	+	+
Neutral lipids + pigments	0.98	1.00	0.90	+ +	+ +	+ +	+ +	+ +

[a] Solvent systems: (A) $CHCl_3/MeOH/CH_3COOH/H_2O$ (85 : 22.5 : 10 : 4, v/v) (double development); (B) $CHCl_3/MeOH/CH_3COOH$ (90%) (65 : 4 : 35, v/v); (C) $CHCl_3/MeOH/NH_4OH$ (65 : 35 : 5, v/v) (double development). Mobilities given for S-DGD, PGP, PG & PA are identical to those of corresponding standards.

[b] *Halococcus saccharolyticus* [5].

ing the permethylated glycolipids was fractionated on a column of silicic acid (Biosil A), eluted with benzene, benzene/diethyl ether (9 : 1, v/v) and benzene/diethyl ether (75 : 25, v/v) [13]. Fully methylated glycolipids were eluted in benzene/diethyl ether (75 : 25, v/v) and subjected to acid methanolysis in 4.5 ml of 2 M methanolic HCl at 75 °C for 5 h. After removing hydrophobic products by petroleum ether extraction, the methanol-water-soluble products were hydrolysed in 1 M HCl at 100 °C for 2 h and deionized by passage through a small column (600 mg) of mixed-bed resin (AG-501 X8D; 25–50 mesh, fully regenerated, Bio-Rad), eluted with MeOH. The partially methylated sugars thus obtained were reduced by treatment with 20 mg of sodium borohydride for 16 h, brought to dryness under nitrogen (with addition of glacial acetic acid and MeOH), dried over phosphorus pentoxide and acetylated with 1 ml acetic anhydride at 105 °C [18]. The partially methylated alditol acetates were analysed by GC-MS as described in the Materials and Methods section.

Methanolysis of lipids

Acid methanolysis of phospholipids and glycolipids was performed, as described elsewhere [11], in 4.5 ml of 2 M HCl in methanol, at 75 °C for 5 h. The hydrophobic products (petroleum ether-soluble) were identified by TLC and CI-MS analysis. The methanol-water-soluble products were hydrolysed in aqueous 1 M HCl at 100 °C for 2 h, the HCl was removed by evaporation on a rotary evaporator and the residue was vacuum dried in a desiccator over KOH and identified by TLC on cellulose plates in solvent G and H (see above).

Results and Discussion

Growth of Halococci and lipid contents

Of the five strains of halococci examined here, the fastest growing strain was CCM-537 (phenon A), followed by strains P-423 (*H. saccharolyticus*, phenon D), and N-207 (phenon A); strains Q-26 (phenon B) and P-1140 (phenon C) were both much slower growing. Incubation periods were adjusted so that cells were harvested in their respective early stationary phases, except for strain P-1140 which was harvested in the stationary phase.

It should be noted that the lipids and pigments of these strains were much more difficult to extract than those of halobacteria, requiring two more extractions with an acidified solvent system to recover further lipid material. Even after this acidic extraction small to trace amounts of lipids (mostly glycolipids) and orange pigments still remained and could be recovered by a third extraction with acidified solvent. Thus, part of the glycolipids and pigments appear to be covalently bound to cellular material and are released only after acid treatment.

The total lipids (including pigments) in *H. saccharolyticus* accounted for about 5% by weight of cellular protein, indicating a lipid content of about half that in halobacteria [4]. Polar lipids accounted for about 66% of the total lipids and non-polar lipids + pigments for about 34%; the non-polar + pigment content is about 3-times that in halobacterial lipids [4]. The lipid-P content of the total lipids was 3.2% and that of the total polar lipids was about 4.1–4.5%; these values are similar to those for halobacteria [4]. The lipid-sugar content of the total polar lipids was 13.5%, indicating a fairly high content of glycolipids.

Lipid contents of the other strains of *Halococcus* were similar to that in *H. saccharolyticus*, except for strain Q-26 which had a somewhat lower lipid content; this may be due to the greater difficulties encountered in lipid extraction from this strain.

Polar lipid composition

Thin-layer chromatographic plates, run in solvent systems A, B or C (R_F values are given in Table I) and stained with the phosphate and sugar stains showed a similar if not identical distribution of polar lipids in all five strains of *Halococcus* examined (Table I). The major phospholipids in all strains were PGP (C_{20}-C_{20} and C_{20}-C_{25} molecular species), PG (C_{20}-C_{20} and C_{20}-C_{25} molecular species), and small amounts of an unidentified phospholipid (PGX) and of phosphatidic acid (PA, diphytanyl ether form). All strains also contained one major glycolipid (GL-4) and three minor glycolipids (GL-1, GL-2 and GL-3), one of which (GL-2) was a phosphoglycolipid (Table I).

Quantitative analysis carried out on the TLC separated lipids of *H. saccharolyticus* (Table II) showed

TABLE II

Quantitative polar lipid composition of H. saccharolyticus

Polar lipid components were separated by TLC in solvent B, weighed and analysed for lipid-P and lipid sugar. Mol% data are given as means ± mean deviations for two separate analyses, calculated on the basis of 1 atom P in PG and PA, 2 atoms P in PGP and PGX, 2 mol sugar in GL-4 and 3 mol sugar in GL-1 and GL-2.

Lipid component	Lipid composition	
	% by wt of polar lipids	mol% of polar lipids
Glycolipid GL-1 / Phosphoglycolipid GL-2	7.7	3.8 ± 0.6
Glycolipid GL-3	tr	tr
Glycolipid GL-4 (S-DGD)	22.2	19.5 ± 3.0
PGP (total	52.6	56.7 ± 9.8
PX	5.1	2.9 ± 1.1
PG (total	6.4	13.9 ± 3.8
PA	6.0	3.1 ± 2.1

TABLE III

Mass spectrometry (negative FAB-MS) ion peaks of polar lipid components of H. saccharolyticus

Component	Molecular species	Calcd. M	$[M-1]^-$	$[M-NH_4]^-$	$[M-2NH_4]^-$ +H	$[M-2CH_3]^-$	$[M-2CH_3]^-$ +H	$[M-X]$	X [a] (mass)
$PGP(NH_4)_2$	C_{20}-C_{20}	920	919	901	885	–	–	731	$GP(NH_4)_2$(189)
	C_{20}-C_{25}	990	–	972	–	–	–	801	$GP(NH_4)_2$(189)
$PGP(CH_3)_3$	C_{20}-C_{20}	928	–	–	–	913	–	745	$GP(CH_3)_2$(183)
								731	$GP(CH_3)_2$ +CH2 (197)
$PGP(CH_3)_4$	C_{20}-C_{20}	942	–	–	–	927	913	745	$GP(CH_3)_3$(197)
								731	$GP(CH_3)_3$ +CH_3 (213)
$PG(NH_4)$	C_{20}-C_{20}	823	–	805	–	–	–	731	(Gro-OH) +NH_4 (92)
	C_{20}-C_{25}	893	–	875	–	–	–	801	(Gro-OH) +NH_4 (92)
$PA(NH_4)_2$	C_{20}-C_{20}	766	–	–	731	–	–	–	–
	C_{20}-C_{25}	836	–	–	801	–	–	–	–
$PGX(NH_4)_2$	C_{20}-C_{20}	918	–	899	–	–	–	731	$GP(NH_4)_2$(189)
	C_{20}-C_{25}	988	–	969	–	–	–	–	–
S-DGD(NH_4) (GL-4)	C_{20}-C_{20}	1073	–	1055	–	–	–	757	Phy+NH_4(315)
								403	DPG+NH_4(670)
S-DGD(CH_3)$_7$	C_{20}-C_{20}	1154	–	–	–	1140	1127	503	DPG (651)
P-TGD(NH_4)$_2$ (GL-2)	C_{20}-C_{20}	1250	–	(1232)	–	–	–	619/617	DPG−2H −OH (633)
								601	DPG−2H+OH −NH_3 (649)
								583	DPG−2H+OH −2NH_3(667)

[a] Abbreviations: GP, glycerophosphate ($C_3H_6O_5$); Gro, glyceryl ($C_3H_7O_2$); Phy, phytanol ($C_{20}H_{41}O$); DPG, diphytanylglyceryl ($C_{43}H_{87}O_3$).

TABLE IV

Hydrolysis products of major lipids of Halococcus saccharolyticus

The TLC pure lipid components were methanolysed in 2 M methanolic HCl and hydrophobic products were extracted with petroleum ether (see Materials and Methods section). All products were identified by TLC relative to authentic standards.

Lipid	Species	Hydrophobic products R_F in solvents			Water-soluble products R_F in solvents		
		D	E	identity [a]	G	H	identity [a]
PGP	C_{20}-C_{20}	0.41	0.42	$DPG_{20\text{-}20}$	0.12	–	GBP
	C_{20}-C_{25}	0.44	0.45	$DPG_{20\text{-}25}$	0.19	–	GP (minor)
PG	C_{20}-C_{20}	0.41	0.42	$DPG_{20\text{-}20}$	0.19	–	GP
	C_{20}-C_{25}	0.43	0.45	$DPG_{20\text{-}25}$	0.19	–	GP
PGX	C_{20}-C_{20}	–	0.04	MPG			
		–	0.42	DPG (trace)			
		–	0.81	methoxyphytol	0.12	–	GBP
		–	0.87	methoxyisophytol	0.19	–	GP (minor)
		–	0.92	isoprene hydrocarbons			
S-DGD (GL-4)	C_{20}-C_{20}	0.41	0.42	$DPG_{20\text{-}20}$	–	0.22	Glucose
						0.27	Mannose
P-TGD (Gl-2)	C_{20}-C_{20}	0.03	0.04	MPG	–	0.19	Galactose
		0.41	0.42	DPG (trace)		0.22	Glucose
		0.66	0.81	isoprenyl products		0.27	Mannose
		0.75	0.87				
			0.92				

[a] Abbreviations: DPG, diphytanylglycerol (molecular species indicated by subscripts); MPG, monophytanylglycerol; GBP, glycerolbiphosphate; GP, glycerophosphate.

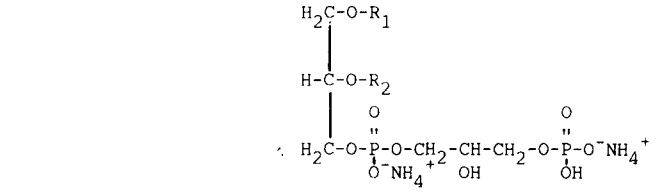
that the major lipids, PGP (total) and GL-4 accounted for 52% and 22%, respectively, of the total polar lipids by weight, or 57% and 20%, respectively, on a mol% basis. PG and PA accounted for about 14% and 3% on a mol% basis, respectively. The quantitative phospholipid composition (mol%) in *H. saccharolyticus* thus shows a lower content of PGP and a much higher content of PG than in *H. cutirubrum* [4]. Other differences are the presence of the unidentified phospholipid PGX and the unidentified phosphoglycolipid in the halococci but not in halobacteria. However, it is of interest that the mol% of the glycolipid GL-4 (S-DGD) in *H. saccharolyticus* is very similar to that of the sulfated triglycosyldiether (S-TGD) in *H. cutirubrum* [4].

Identification of polar lipid components

Phosphatidylglycerophosphate (PGP). This component could be resolved by TLC in solvent A into two P-positive molecular species (Table I): the C_{20}-C_{20} species (slower-moving), and the C_{20}-C_{25} species (faster-moving), in approximately equal proportion. These species were separated and purified by preparative TLC as described in Material and Methods section and identified by ^{1}H-NMR and negative FAB-MS analyses.

Both the C_{20}-C_{20} and the C_{20}-C_{25} molecular species showed ^{1}H-NMR signals (in $CDCl_3$) expected for saturated isopranoid ether-linked chains and glycerophosphate groups: 0.82 ppm (isopranoid (CH_3), 1.0–1.1 ppm ($(CH_3)_2C$-), 1.2–1.3 ppm (CH_2), 1.48–1.50 ppm (-CH), 3.42–3.44 ppm (RCH_2-O-), 3.56–3.58 ppm ($-CH_2$-O-R, -CH-O-R), 3.9–4.0 ppm ($-CH_2$-O-P).

Negative FAB-MS showed molecular ion peaks ([$M-1$]$^-$ and/or [M–18]$^-$ and [M – 35]$^-$) and fragmentation peaks ([M–189]$^-$ expected for the two molecular species of PGP diammonium salt (Table III). For further identification, the C_{20}-C_{20} species was converted to its tri- and tetramethyl derivatives; the ^{1}H-NMR spectra of both derivatives showed a doublet at 3.72–3.76

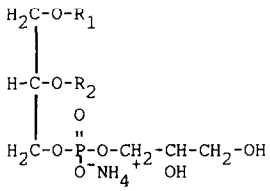


PGP(NH_4)$_2$

C_{20}-C_{20} species: R_1=R_2=phytanyl
C_{20}-C_{25} species: R_1=phytanyl
R_2=sesterterpanyl

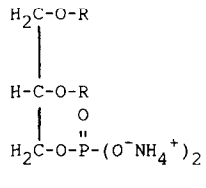
PGX(NH_4)$_2$

C_{20}-C_{20} species:
R_1=phytanyl;R_2=phytenyl



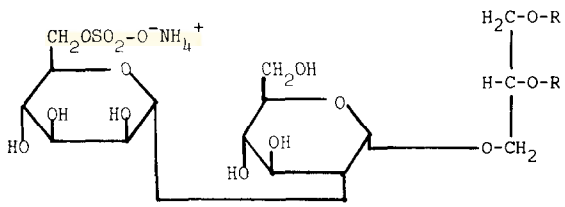
PG(NH_4)

C_{20}-C_{20} species: R_1=R_2=phytanyl
C_{20}-C_{25} species: R_1=phytanyl
R_2=sesterterpanyl



PA(NH_4)$_2$

C_{20}-C_{20} species: R=phytanyl



S-DGD(NH_4)

C_{20}-C_{20} species: R=phytanyl

Fig. 1. Structures of lipid components in *H. saccharolyticus*.

ppm characteristic of P-OCH_3 groups. The negative FAB-MS spectra (Table III) showed a parent ion at m/z 913 $[M-15]^-$ for the trimethyl derivative (calcd. M, 928), and at m/z 927 $[M-15]^-$ for the tetramethyl derivative; both spectra showed ion fragments at m/z 745 and 731, corresponding to monomethyl $[PA]^-$ and $[PA]^-$ ions, respectively.

After methanolic-HCl hydrolysis (see Material and Methods section), the water-soluble products from both species were identified by TLC as glycerolbiphosphate (Table IV). The hydrophobic products of each of the above species showed, on TLC in solvent system E [12], a major spot corresponding to the C_{20}-C_{20} or the C_{20}-C_{25} species of diphytanyl glycerol, respectively (Table IV). The latter were identified by CI-MS: C_{20}-C_{20} species (calcd. M, 652), m/z 653 $[M+1]^+$; C_{20}-C_{25} (calcd. M, 722), m/z 723 $[M+1]^+$.

These results show that the major phospholipid in *H. saccharolyticus* is PGP (diether analogue), which occurs in two molecular species with C_{20}-C_{20} and C_{20}-C_{25} glycerol diether lipid cores (Fig. 1).

Phosphatidylglycerol (PG). As described for PGP, the PG component could also be resolved,by TLC in solvent A (Table I), into C_{20}-C_{20} and C_{20}-C_{25} molecular species, in about equal proportion. The separated and purified species were identified as follows: Negative FAB-MS showed the expected molecular ion peaks ($[M-18]^-$) and fragmentation peaks ($[M-92]^-$ expected for the two molecular species of PG ammonium salt (Table III). After methanolic-HCl hydrolysis, the water-soluble products of both species contained only glycerophosphate, and the hydrophobic products contained the C_{20}-C_{20} and the C_{20}-C_{25} molecular species of diphytanylglycerol (DPG) (Table IV). These results show that the second most abundant phospholipid is phosphatidylglycerol (PG, diether analogue), which occurs in C_{20}-C_{20} and C_{20}-C_{25} molecular species (Fig. 1).

Phosphatidic acid (PA). This minor component had mobilities in acidic and basic solvents (solvents A and C, respectively) identical to those of standard synthetic PA (Table I), and appeared to be largely the isopranyl C_{20}-C_{20} species. This was confirmed by negative FAB-MS (Table III) which showed a major parent ion at m/z 731 $[M-35]^-$ (calcd. M for C_{20}-C_{20}-species of $PA(NH_4)_2$, 766); the presence of traces of the isopranyl C_{20}-C_{25} species was indicated by a weak ion peak at m/z 801 $[M-35]^-$ (calcd. M for C_{20}-C_{25} species, 836). The structure of the major species of PA is shown in Fig. 1. Isoprenyl PA has been detected as a biosynthetic intermediate in *H. cutirubrum* [9] and isopranyl PA has been found in relatively high proportion in a halophilic archaebacterium from a U.K. salt mine [19].

Unidentified phospholipid (PGX). This component had TLC mobilities somewhat higher than those for the C_{20}-C_{25} species of PGP in solvent systems A and B (Table I), suggesting that PGX may be a higher molecular species of PGP, such as C_{25}-C_{25} (mol. wt., 1026), or a cyclyzed form of PGP (mol. wt, 868 or 938 for C_{90}-C_{20} or C_{20}-C_{25}, respectively), as was found in *Natronococcus occultus* [20]. However, none of these possibilities proved to be correct, since negative FAB-MS showed a major parent ion at m/z 899, and a minor ion peak at m/z 969. These ion peaks were two mass units lower than those for the C_{20}-C_{20} and C_{20}-C_{25} species of PGP $[M-NH_4]^-$ ion peaks, respectively (Table III). However, the presence of traces of saturated PGP is indicated by the fragmentation ion peak at m/z 731, corresponding to saturated $[PA]^-$ ion (Table III).

These results suggest that PGX may be an unsaturated form of PGP similar to the isoprenoid intermediates of PGP biosynthesis detected in *H. cutirubrum* [9]. In support of this proposal, the ^{1}H-NMR spectrum of PGX (in $CDCl_3$) showed signals at 1.60–1.65 ppm for allylic CH_3 groups, at 2.04 ppm for allylic CH_2 groups, and at 5.08 ppm (doublet) for olefinic isoprenoid protons. Furthermore, the methanolic-HCl hydrolysis products of PGX consisted of isoprenyl methanolysis products (methoxyphytol, methoxyisophytol and isoprene hydrocarbons) and only traces of diphytanylglycerol; glycerolbiphosphate was the major water-soluble product detected (Table IV). Finally, it should be noted that the unsaturated isoprenyl species of PGP, detected as biosynthetic intermediates, had TLC mobilities greater than those of the fully saturated PGP species [9].

PGX thus appears to be largely the isoprenyl derivative of PGP, the major species being C_{20}-C_{20}, as shown in Fig. 1.

Sulfated diglycosyldiphytanylglycerol (S-DGD) (GL-4). This major glycolipid component had TLC mobilities (Table I) identical to those of authentic S-DGD-1 (2,3-diphytanyl-1-[6-HSO_3-α-mannopyranosyl-1-2-α-glucopyranosyl]-*sn*-glycerol) isolated from *H. mediterranei* [8]. Elemental analysis of GL-4 ammonium salt [Found: C(%), 56.3; H(%), 9.41; N(%) 0.59; S(%), 2.65; Calcd. for $C_{55}H_{111}O_{16}NS \cdot 4H_2O$, mol. wt. 1146.6, C(%), 57.61; H(%), 9.93; N(%), 1.22; S(%), 2.80] was in agreement with that for a sulfated diglycosyldiphytanylglycerol structure. Negative FAB-MS of the ammonium salt of GL-4 showed a strong parent ion with m/z 1055 $[M-NH_4]^-$ and a fragmentation ion with m/z 757 $[M-NH_4-\text{phytanol}]^-$ (Table III), consistent with the C_{20}-C_{20} molecular species of S-DGD (calcd. M, 1073 for the NH_4 salt) as the only molecular species. The ^{1}H-NMR spectrum of GL-4 in perdeuterated dimethylsulfoxide was identical with that of S-DGD-1 from *H. mediterranei*, the major diagnostic signals being: 0.8 ppm (isopranoid CH_3), 1.0–1.1 ppm ($(CH_3)_2C$-), 1.2–1.3 ppm (CH_2), 1.46–1.50 (-CH), 4.90–4.91 (doublet; anomeric H of α-glucoside), 4.96–4.98 (doublet, anomeric H of α-mannoside).

Methanolysis of GL-4 gave diphytanylglycerol as the

only hydrophobic product and glucose and mannose as water-soluble products (Table IV). Permethylation of GL-4 yielded a heptamethyl derivative (calcd. *M*, 1154) which gave $[M - CH_3]^-$ and $[M - 2CH_3]^-$ ion peaks on negative FAB-MS with *m/z* 1140 and 1127, respectively (Table II). Methanolysis of the permethylated GL-4, yielded diphytanylglycerol and partially methylated sugars which were converted to alditol acetates and identified by GC-MS as being derived from 3,4,6-trimethylglucose and 2,3,4-trimethylmannose (retentions relative to 2,3,4,6-tetramethylglucose, 1.37 and 1.48, respectively). These results thus establish the identity of GL-4 with the S-DGD-1 of *H. mediterranei* [8], having the structure shown in Fig. 1.

Phosphoglycolipid (GL-2). This minor component had low R_F values on TLC in solvents B and C (Table I), and stained positively with both the sugar and phosphate stains, indicating that it is a phosphoglycolipid. Methanolic-HCl hydrolysis of GL-2 yielded glucose, mannose and galactose as water-soluble products and a mixture of hydrophobic products containing monophytanylglycerol (MPG) and isoprenyl products, plus traces of diphytanylglycerol (DPG) (Table IV). The identity of the hydrophobic products was confirmed by CI-MS which showed parent ion peaks for monophytanylglycerol (*m/z* 373 $[M + 1]^+$, calcd. *M*, 372), phytol (*m/z* 297 $[M + 1]^+$, calcd. *M*, 296), methoxyphytol and methoxyisophytol (*m/z* 311 $[M + 1]^+$, calcd. *M*, 310) and phytene (*m/z* 279 $(M + 1]^+$, calcd. *M*, 278).

These results indicate that GL-2 is a phosphorylated triglycosylglyceroldiether (P-TGD) with one unsaturated isoprenyl chain. The presence of an isoprenyl chain in GL-2 was supported by its ^{1}H-NMR spectrum (in $CDCl_3$), which showed signals at 1.60–1.65 ppm for allylic CH_3 groups, at 2.0 ppm for allylic CH_2 groups, and at 5.1–5.2 ppm for olefinic isoprenoid protons. Furthermore, negative-ion FAB-MS of the P-TGD-diammonium salt (calcd. *M*, 1250) did not show the expected parent ion $[M - 18]^-$ at *m/z* 1232, but only fragmentation ions with *m/z* 619/617, 601 and 583 which could represent ions $[M - 633$ (phytanylphytenylglyceryl)], $[M - 633\text{-}NH_3]^-$ and $[M - 633\text{-}2NH_3]^-$, respectively. Thus, the phosphoglycolipid (GL-2) appears to have the structure: Phospho-(Gal-Man-Glc)-phytanylphytenylglycerol. Further studies are necessary to establish unambiguously the overall structure of P-TGD, as well as the sugar sequence and linkage positions unambiguously.

The findings presented here show that the species of *Halococcus* from salt flats in Spain which we examined have a characteristic lipid composition clearly distinguished from those of *Halobacterium, Haloferax* and *Haloarcula* species, as well as the haloalkaliphiles (see reviews in Refs. 3,4 and Table I). For example, the presence of C_{20}-C_{20} and C_{20}-C_{25} molecular species of PGP and PG and the absence of PGS in the halococci shows a similarity with the haloalkaliphiles, but the halococci are distinguished by the presence of glycolipids which are absent in haloalkaliphiles [7,12]. It is of interest that the same molecular species of PGP and PG, as well as a glycolipid, have also been found in a non-alkaliphilic, non-pigmented extremely halophilic bacterium (strain No. 172) isolated from solar evaporated sea sand [21]; the glycolipid in this bacterium is more polar than S-DGD, and may be a novel sulfated triglycosyl diether (Kamekura, M., Matsubara, T., Moldoveanu, N. and Kates, M., unpublished data).

On the other hand, the presence of S-DGD in *halococcus* species is reminiscent of *Haloferax* species, but the latter lack the phosphoglycolipid found in the *Halococcus* species. The halococci examined here are further distinguished from halobacteria by their lack of any sulfated triglycosyldiethers, although these appear to be present in at least two species of *Sarcina (H. morrhuae)* [1,2]. Further studies of a wider range of halococci species are necessary to verify the unique lipid pattern of this group of extreme halophiles.

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