Structural elucidation of a novel phosphoglycolipid isolated from six species of *Halomonas*

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Abstract The structure of a new phosphoglycolipid from the halophilic Gram-negative bacteria *Halomonas elongata* ATCC 33173[T], *Halomonas eurihalina* ATCC 49336[T], *Halomonas almeriensis* CECT 7050[T], strain Sharm (AM238662), *Halomonas halophila* DSM 4770[T], and *Halomonas salina* ATCC 49509[T] was elucidated by NMR and mass spectroscopy studies. In all of the species examined, the polar lipid composition consisted of 1,2-diacylglycero-3-phosphorylethanolamine, 1,2-diacylglycero-3-phosphoryl-glycerol, bisphosphatidyl glycerol, and the new phosphoglycolipid PGL1. The structure of PGL1 was established to be (2-(α-D-glucopyranosyloxy)-3-hydroxy-propyl)-phosphatidyl diacylglycerol. C16:0;C18:1 and C16:0;C19:cyclopropane are the most abundant acyl chains linked to the phosphatidylglycerol moiety of each isolated PGL1. All of the species presenting the lipid PGL1 belong to *Halomonas* rRNA group 1, suggesting that the new phosphoglycolipid could be a chemotaxonomic marker of this phylogenetic group.—Giordano, A., F. M. Vella, I. Romano, and A. Gambacorta. **Structural elucidation of a novel phosphoglycolipid isolated from six species of *Halomonas*. *J. Lipid Res.* 2007. 48: 1825–1831.**

Supplementary key words *Halomonas elongata* ATCC 33173[T] • *Halomonas eurihalina* ATCC 49336[T] • *Halomonas almeriensis* CECT 7050[T] • strain Sharm (AM238662) • *Halomonas halophila* DSM 4770[T] • *Halomonas salina* ATCC 49509[T] • nuclear magnetic resonance spectroscopy • tandem mass spectrometry

Halophile organisms are able to withstand fluctuations of extracellular salinity using two strategies that can operate simultaneously. The first one involves the cytoplasmic accumulation of small organic compounds, called osmolites or compatible solutes, which enable the cell to reduce the water loss and to maintain the cell pressure by reducing the osmotic potential between the cell and the cytoplasm (1, 2). The second one consists of modifying the membrane structure, generally affecting phospholipids and their fatty acid composition. In halophilic Gram-negative bacteria, the increase in salt concentration usually corresponds to a decrease of the zwitterionic phospholipid phosphatidylethanolamine (PEA) and an increase of the anionic lipids phosphatidylglycerol (PG) and/or bisphos-

phatidyl glycerol (DPG) as well as the incorporation of cyclopropanic rings and double bonds in fatty acid chains (3, 4). Regulation of the phospholipid composition of the cell membrane is also used by the microorganisms to adjust the proton permeability of the membrane to the growth temperature of the cell (5, 6).

Phosphoglycolipids have a relatively limited distribution among Gram-negative bacteria compared with Gram-positive bacteria. Among euryhaline microorganisms, *Halomonas* species are widely distributed throughout saline environments and constitute a remarkably high percentage of the total microbial community in hydrothermal vent habitats. The polar lipid composition shows a general pattern, because PEA, PG, and DPG are the major lipid components. The presence of unknown lipids was first described in 1990 by Franzmann and Tindall (7), who reported the occurrence of unidentified periodate-Schiff-positive phospholipids in some species of the *Halomonas* genus. In 1998, Yagi and Maruyama (8) described in a patent the presence of new glucosyl phosphatidyl glycerol derivatives in *Halomonas marina*. In this study, the structure of a new phosphoglycolipid present in six strains belonging to the genus *Halomonas* was elucidated by NMR and mass spectroscopy studies.

MATERIALS AND METHODS

Isolation and culture conditions

During the summer of 2005, samples of water with mat were collected from the salt lake inside Ras Muhammad Park in Egypt; pH was 8.5 and temperature was 30°C.

Isolation of Sharm strain was carried out on a saline medium (medium 1) containing the following components: 2.0 g/l KCl, 20 g/l MgSO$_4$·7H$_2$O, 150 g/l NaCl, 3.0 g/l sodium citrate, 5.0 g/l yeast extract, 5.0 g/l casamino acids, 0.36 μg/l MnCl$_2$·4H$_2$O, and 50 μg/l FeSO$_4$. The strain was isolated by dilution-plating tech-

Abbreviations: CD, circular dichroism; DPG, bisphosphatidyl glycerol; DQF-COSY, double quantum-filtered correlation spectroscopy; FAME, fatty acid methyl ester; HMBC, heteronuclear multiple bond coherence; HSQC, heteronuclear single quantum coherence; PEA, phosphatidylethanolamine; PG, phosphatidylglycerol.

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nique on solid medium 1. Solid medium for purification and maintenance of the strain was prepared by the addition of 1.8% agar to medium 1. Routinely, the strain was grown on liquid and/ or solid medium 1. In liquid medium, growth was followed by measuring the absorbance at 540 nm. *Halomonas elongata* ATCC 33173[T] (9), *Halomonas eurihalina* ATCC 49336[T] (10), *Halomonas salina* (11), and *Halomonas halophila* DSM 4770[T] (12) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Brunschweig, Germany). *Halomonas almeriensis* CECT 7050[T] was kindly provided by Martinèz-Checa et al. (13). All of the strains were grown in the media at their optimal growth conditions, as suggested in the literature.

Lipid extraction and characterization

Complex lipid analyses were performed using freshly harvested cells of the strains (5 g) that were lyophilized and extracted. Cells in the stationary phase were collected by centrifugation and lyophilized. Dry cells were extracted with chloroform-methanol-water (65:25:4, v/v) and evaporated under reduced pressure. The lipid extract was analyzed by two-dimensional TLC on silica gel plates cut to 10 cm × 10 cm. The TLC products were developed in chloroform-methanol-water (65:25:4; first dimension) and chloroform-methanol-acetic acid-water (80:12:15:4; second dimension). The lipid extract was then fractionated by flash chromatography on silica gel with methanol-chloroform (1:9, v/v), methanol-chloroform (2:8), methanol-chloroform (3:7), and methanol-chloroform-water (25:75:4). The fraction eluted with chloroform-methanol-water (25:75:4) was purified by preparative TLC eluting with chloroform-methanol-water (25:75:4; retention factor of phosphoglycolipid PGL1, 0.25). Polar lipids were quantified by the weight of the isolated fractions.

^{13}C NMR spectra were acquired on a Bruker DPX-300 operating at 300 MHz using a dual probe. [1]H, [1]H-^{1}H double quantum-filtered correlation spectroscopy (DQF-COSY), heteronuclear multiple bond coherence (HMBC), and heteronuclear single quantum coherence (HSQC) experiments were recorded on a 600 MHz Bruker instrument. Chemical shifts were given in ppm (δ) scale; the methanol signal was used as the internal standard (δ 3.34 ^{1}H; δ 49.0 ^{13}C). Mass spectra of the unknown phosphoglycolipid were recorded on a QTof-/micro/mass spectrometer (Waters) equipped with an electrospray ionization source in negative ion mode.

Fatty acid methyl esters (FAMEs) were obtained from complex lipids by acid methanolysis incubating phosphoglycolipids with 0.5 M methanol-HCl at 80°C for 1 h. The solvent was removed under vacuum, and the residue was mixed with chloroform and water. FAMEs partitioned into the organic phase. GC-MS analysis of FAMEs was performed with an HP5890 series II plus-5989B equipped with an HP-V column with a flux of 45 ml/min. The analysis program was 60°C for 1 min, 60–140°C at 25°C/min, 140–200°C at 5°C/min, and 200–300°C at 10°C/min.

PGL1 (2 mg) was incubated in 1 M methanol-HCl (1 ml) at 60°C for 12 h. The reaction mixture was dried and partitioned between chloroform and water-methanol (8:2). The aqueous layer containing the sugar was concentrated, filtered on a Sep-Pak cartridge, and dried. Benzoyl chloride (0.5 ml) and dry pyridine (0.5 ml) were added to the methanolysis products. After 12 h at room temperature, the mixture was dried and purified on silica gel (hexane/diethyl ether gradient) to give 0.7 mg and 0.4 mg of pure α- and β-tetra-benzoate. The absolute stereochemistry of the sugar was determined by comparison of the circular dichroism (CD) spectra of the benzoate derivatives with samples prepared from commercial α- and β-methyl-D-glucopyranose,
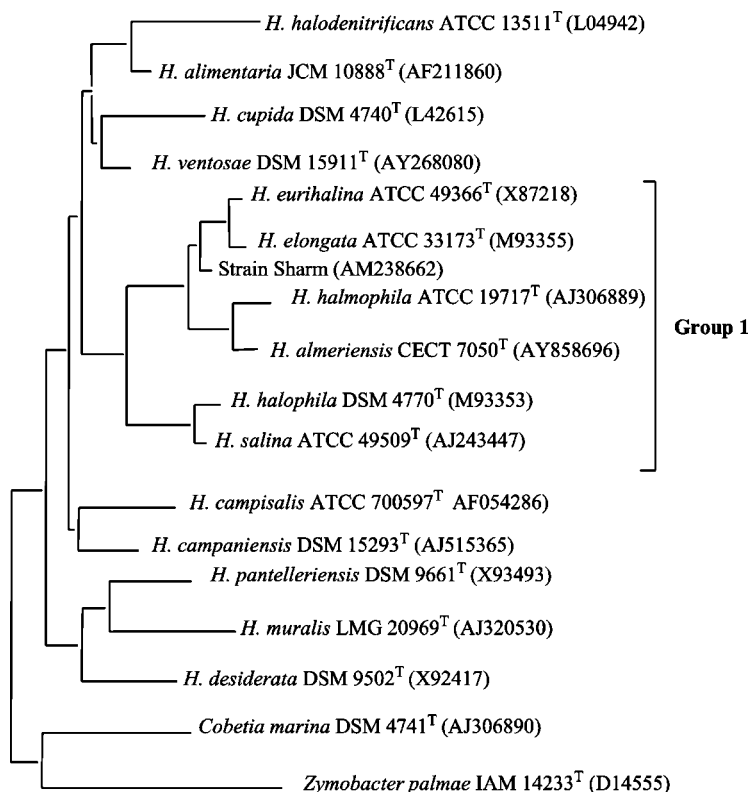

Fig. 1. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain Sharm and other *Halomonas* species. The bar indicates 0.01 substitutions per nucleotide position. Strains belonging to rRNA group 1 of *Halomonas* are marked with a bracket.

TABLE 1. Polar lipid pattern and FAME composition of PGL1 in the examined *Halomonas* species

Variable	Sharm Strain	*H. elongata*	*H. eurihalina*	*H. salina*	*H. halophila*	*H almeriensis*
Lipids (%)						
Phosphatidylethanolamine	13	65	61	50	75	49
Phosphatidylglycerol	51.3	22	25	16	4	2
Bisphosphatidyl glycerol	0.7	12	12	30	19	38
Lipid 1	35	1	2	4	2	11
FAME (% in PGL1)						
$C_{16:0};C_{16:1}$				7	16	
$C_{16:0};C_{18:1}$	10	60	77	46	70	67
$C_{16:0};C_{19cyc}$	81	40		38	30	
$C_{16:1};C_{20:0}$			16			33
$C_{19cyc};C_{19cyc}$	9					

FAME, fatty acid methyl ester.

respectively. For CD (*n*-hexane), $[\theta]_{248}$ 1640 for α derivative and $[\theta]_{235}$ 613 for the β derivative.

RESULTS

The strain Sharm was isolated from samples of water with mat collected from a saline lake inside Ras Muhammad Park in the promontory of Sinai Peninsula in Egypt. The Gram-negative microorganism grew aerobically in enrichment medium containing up to 30% NaCl, with an optimum at 5–15% NaCl. The 16S rRNA gene sequence (EMBL database accession number AM238662) was determined (data not shown) and compared with all sequences currently available for members of the genus *Halomonas* and related taxa. The results are presented as a phylogenetic dendrogram (**Fig. 1**) showing that strain Sharm is a member of the genus *Halomonas*. The strain clustered together with the species *H. elongata* and *H. eurihalina* and other species of the rRNA group 1, according to the phylogenetic classification of *Halomonas* (14).

The total lipid compositions of strain Sharm and the five related species, *H. elongata, H. eurihalina, H. salina, H. almeriensis,* and *H. halophila,* are reported in **Table 1**. The isolate Sharm possessed three phospholipids, PEA (13%), PG (51.4%), and DPG (0.7%), and the new phosphoglycolipid PGL1 (33.0%), identified by NMR studies. Polar lipid analysis of the other *Halomonas* strains showed that these species contain the same lipid pattern but with different distribution (Table 1).

The structure of phosphoglycolipid PGL1 and NMR assignments are reported in **Fig. 2** and **Table 2**. The presence of spin systems corresponding to one sugar unit (H1-H6), two glycerols (H1′-H3′ and H1″-H3″), and fatty acids was suggested from ^1H, ^{13}C, and ^1H-^1H DQF-COSY spectra. The HSQC spectrum gave the assignment of carbons directly bonded to protons. The ^1H and ^{13}C NMR spectra contained methyls (δ_H 0.88), methylene chains (δ_H 1.25–2.42), double bonds (δ_H 5.33, δ_C 130.0) in methylene chains, and carbonyl carbons (δ_C 173.2, 173.4), suggesting the presence of fatty acid moieties. Moreover, the presence of ^1H signals at ~-0.4 ppm indicated the presence of a cyclopropanic ring.

The HSQC spectrum (**Fig. 3**) also revealed one anomeric methine (δ_H 4.99, δ_C 98.8), suggesting the presence of one sugar unit with α-configuration (J_{1-2} = 3.6). Starting from the anomeric proton H1, interpretation of COSY correlations (**Fig. 4**) allowed us to assign the δ_H of the protons of the sugar ring (Fig. 4). The high values of the coupling constants J_{2-3} (9.90 Hz), J_{3-4} (9.0 Hz), and J_{4-5} (9.2 Hz) indicated the axial orientation of H2, H3, H4, and H5; thus, the sugar was assigned as a glucopyranose ring in the $^4C_1$ conformation.

Four oxymethylenes and two oxymethines were assigned as two glycerol units on the basis of the spin system from ^1H-^1H COSY and their directly bonded carbons (Fig. 4). Coupling constants of the carbon signals indicated the presence of a phosphate unit linking the two glycerols. In particular, the small values of $J_{C3′-P}$ and $J_{C1″-P}$ are diagnostic for the presence of a phosphate group directly linked to
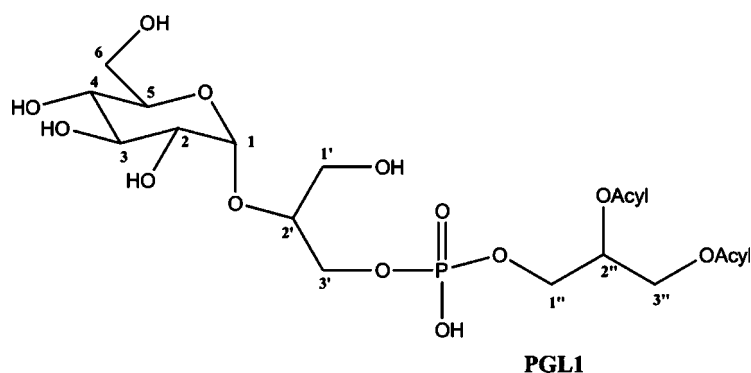

Fig. 2. Structure of phosphoglycolipid PGL1 [(2-(α-D-gluco-pyranosyloxy)-3-hydroxy-propyl)-phosphatidyl diacylglycerol].

TABLE 2. NMR data of PGL1 in CDCl₃/CD₃OD

Variable	δ	Signal Multiplicity[a] (J)	δ
	ppm	Hz	ppm
Glu			
1	4.99	d (3.6)	98.8
2	3.42	dd (9.9, 4.1)	72.4
3	3.70	m	74.3
4	3.25	dd (9.9, 9.2)	71.8
5	3.90	m	72.4
6	3.89	m	62.1
gly1			
1′	3.67–3.76	m	61.0
2′	3.90	m	78.3 ($J_{2'\text{-}P}$ = 7.7 Hz)
3′	4.03	m	65.9 ($J_{3'\text{-}P}$ = 5.5 Hz)
gly2			
1″	4.02	m	64.3 ($J_{1''\text{-}P}$ = 3.7 Hz)
2″	5.25	m	71.4 ($J_{2''\text{-}P}$ = 8.6 Hz)
3″	4.42	dd (12.2, 3.3); dd (12.2, 6.9)	62.6
	4.19		

[a] d, doublet; dd, double doublet; m, multiplet.

these carbons. HMBC cross-peak H2′/C1 (3.90/98.8) showed that the glucose molecule is attached to the C2 of the first glycerol moiety (**Fig. 5**). Furthermore, long-range correlations of the H2″ and H3″ with the carbonyl carbons demonstrated that the two acyl groups were located on C-2″ and C-3″ oxydryls (5.25 and 4.42–4.19/169.2).

The stereochemistry of the glucose unit was determined by comparison of the CD spectra of the purified benzoylated α- and β-methyl glucopyranoses. The CD curves of the benzoyl derivatives were identical to those of the corresponding compounds obtained from benzoylation of commercial α- and β-methyl-D-glucopyranoses, indicating the D absolute stereochemistry of the sugar unit.

The fatty acid moieties were determined by tandem MS spectra and GC-MS analysis (Table 1). The mass spectra

contained several molecular ion peaks indicating differences in the type and distribution of the acyl chains on the PG backbone. For Sharm strain, the most abundant were determined to be C16:0 and C19:cyclopropane, corresponding to the highest peak of m/z 923 in the MS spectrum (**Fig. 6A**). In tandem MS spectra using negative ion electrospray ionization (Fig. 6B), fragmentation of the molecular ion m/z 923 (M-H) led to fragment ions 295 (C19:cyc-H) and 255 (C16-H). Mass spectrometry analysis of phosphoglycolipids isolated from the other *Halomonas* species allowed us to establish the identity of acyl chains linked to the glyceryl moiety. It was shown that all lipids carry two acyl chains, the most abundant being C16:0;C18:1. The fatty acid composition was also confirmed by GC-MS analysis of methyl esters obtained by acid methanolysis of the phosphoglycolipids. The structure of the phosphoglycolipid present in Sharm strain, *H. elongata*, *H. eurihalina*, *H. salina*, *H. almeriensis*, and *H. halophila* was established to be 2-(α-D-glucopyranosyloxy)-3-hydroxy-propyl)-phosphatidyl diacylglycerol.

DISCUSSION

Polar lipids and their fatty acids can be used as biochemical markers because many of them have unique structures. Lipid structures related to that described for Sharm strain were found in microorganisms classified in genera not related to *Halomonas*, such as *Mycoplasma*, *Acholeplasma*, and *Streptococcus* (15–19). The presence of phosphoglycolipids in several strains belonging to the *Halomonas* genus was detected by Franzmann and Tindall (7), who analyzed the general composition of polar lipids of many members of the family Halomonadaceae. In 1998, Yagi and
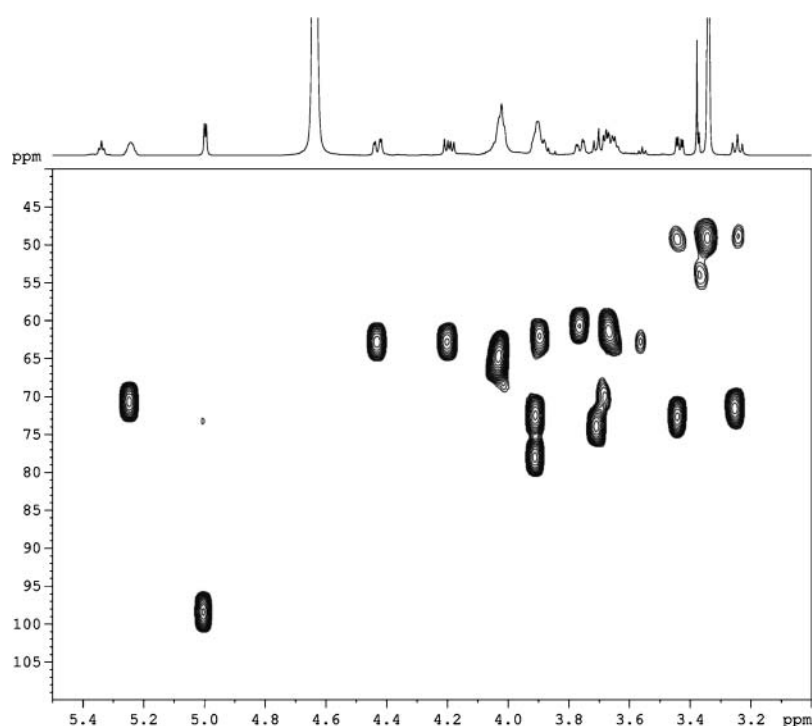

Fig. 3. Sections of heteronuclear single quantum coherence and ^{1}H NMR spectra of PGL1. ^{1}H-^{13}C correlations relative to glucose and the phosphatidylglycerol (PG) moiety are visible.
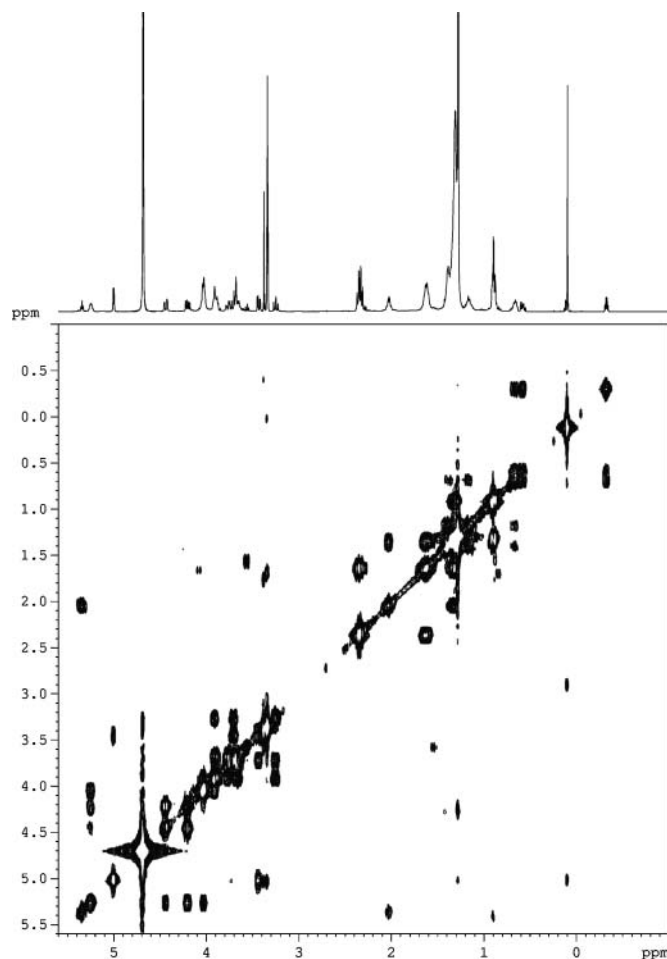
Fig. 4. Double quantum-filtered correlation spectroscopy spectrum of PGL1. ^{1}H-^{1}H correlations of the glucose ring and the PG moiety are visible.

Fig. 5. ^{1}H-^{13}C heteronuclear multiple bond coherence correlations between H2″ and H3″ and the carbonyl carbons of acyl chains (4.42–4.19/169.2) and between the secondary oxymethine proton of PG and the anomeric carbon (3.90/98.8).

Maruyama (8) described in a patent new glucosyl phosphatidyl glycerol derivatives in *H. marina*.

In connection with our ongoing search for new halophilic and alkalohalophilic phenotypes on different continents (20–23), we isolated a new halophilic strain from a salt lake inside Ras Muhammad Park in Egypt. Phylogenetic analysis of Sharm strain indicated that the strain clustered together with the species *H. elongata* and *H. eurihalina*, suggesting that it could belong to the rRNA group 1, according to the Arahal et al. (14) classification of *Halomonas*. Chemotaxonomic characterization of this strain showed the presence of a large amount of an unidentified compound in the polar lipid extract, positive to sugar and phosphate reaction on TLC.

Interestingly, lipid analysis of the species most related to Sharm strain shows that this new lipid is present in five other members of *Halomonas* rRNA group 1, confirming a relationship between these species. *H. elongata*, *H. eurihalina*, *H. salina*, *H. almeriensis*, and *H. halophila*, grown at their optimal growth conditions, possess the same polar lipids of the Egyptian isolate, although in different proportions. PEA is the major lipid in all of the species with the exception of the Egyptian isolate. Phosphoglycolipid
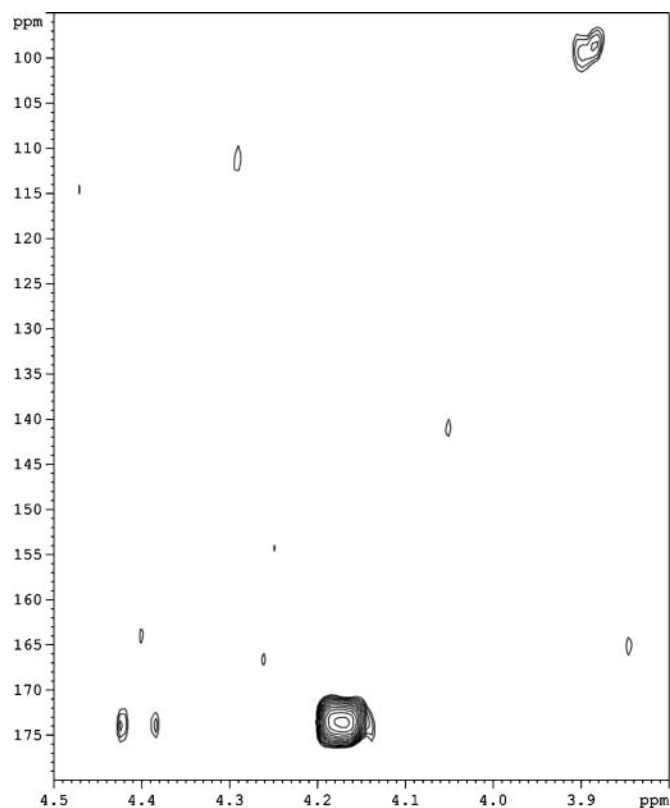
PGL1 accounts for 35% of the total lipid composition of Sharm strain but is present in lower amounts (between 2% and 11%) in the other *Halomonas* species.

NMR and mass spectroscopic analyses allowed us to establish the structure of PGL1. The chemical shifts and the coupling constants of ^{1}H NMR and ^{13}C spectra indicate the presence of one residue of glucopyranose in α configuration. The anomeric carbon is directly linked to a diacylated PG molecule. An interesting feature of PGL1 is its unusual linkage of the sugar to the secondary oxydryl group of PG, a structure resembling that of glycosylated teichoic acids of Gram-positive bacteria. *Halomonas* species are usually rich in C18:1 and C16:0 acyl chains, whereas an increment of cyclopropanic rings is a common response to higher salinity. The FAME composition of each purified phosphoglycolipid agrees with this general rule and is quite superimposable on that of the total lipid fraction. In all of the species, C16:0;C18:1 and C16:0;C19:cyclopropane are the most abundant acyl chains linked to the PG moiety.

To survive in harsh environments, microorganisms modify their membrane structures to modulate the chemical composition of lipids. One of the possible biological roles of phosphoglycolipids in halophilic bacteria is to ensure the osmotic stability of the cellular membrane. Bulky head groups would enhance steric protection through hydrogen bonding via glycosyl head groups (24). This study elucidates the structure of a new phosphoglycolipid that is
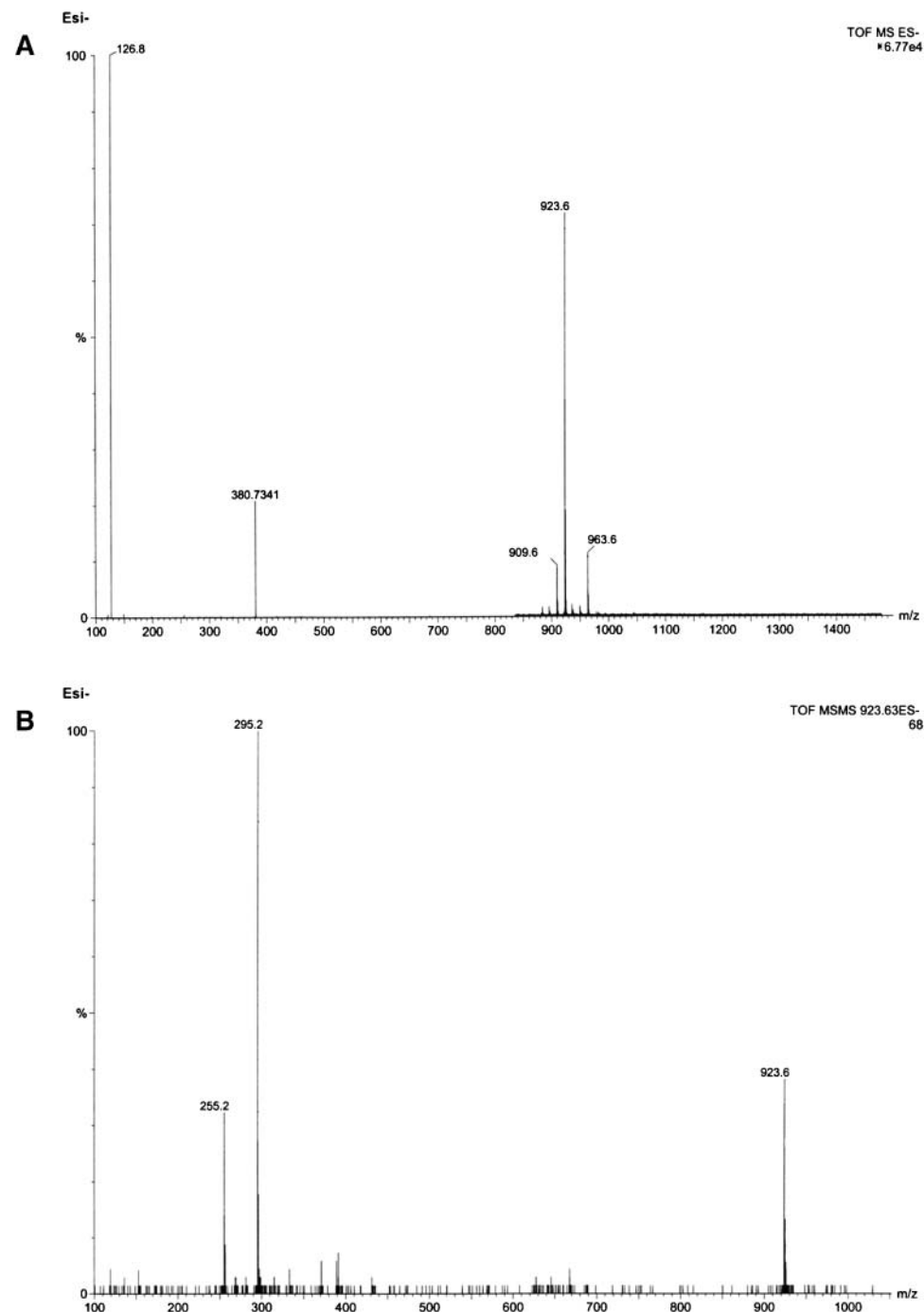
Fig. 6. Negative-ion electrospray ionization-MS spectra of PGL1 from Sharm strain. A: Ion peaks of m/z 909, 923, and 963 correspond to a different distribution of acyl chains ($C_{16:0};C_{18:1}$, $C_{16:0};C_{19cyc}$, and $C_{19cyc};C_{19cyc}$, respectively). B: Tandem MS-QTof spectrum of precursor ion m/z 923.

present in all of the species of *Halomonas* rRNA group 1. It would be interesting to extend detailed lipid characterization to all *Halomonas* species to determine whether the new phosphoglycolipid is a chemotaxonomic marker of group 1 or whether it is a common feature of *Halomonas*.

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