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Presence of two novel cardiolipins in the halophilic archaeal community in the crystallizer brines from the salterns of Margherita di Savoia (Italy) and Eilat (Israel)

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Abstract Two novel cardiolipin derivatives were recently detected in *Halobacterium salinarum*, namely an archaeal analog of bisphosphatidylglycerol (BPG) and a glyco-cardiolipin (GlyC). GlyC was found to be tightly bound to bacteriorhodopsin. To obtain information on the presence and distribution of these archaeal cardiolipins, we have analyzed the lipids extracted from the crystallizer ponds of the salterns of Margherita di Savoia (Italy) and Eilat (Israel) and from cultures of representative species of the Halobacteriaceae by electrospray ionization mass spectrometry. BPG was present as a minor lipid component in the lipids extracted from the biomass of the Margherita di Savoia and the Eilat salterns, while GlyC was detected only in the extract of the biomass of Margherita di Savoia. Both compounds were enriched in the membrane fraction obtained by dialysis of the cells against distilled water. We detected BPG in all members of the Halobacteriaceae tested, but GlyC has so far been found only in the genera *Halobacterium* and *Haloarcula*. A sulfated diglycosyl diether was the major glycolipid detected in the biomass of both salterns.

Key words Cardiolipins · Glycolipids · Halophilic Archaea · Salterns

Introduction

Cardiolipin (bisphosphatidylglycerol) is well known as a minor component of the lipids of the Eucarya. It is relatively abundant in the inner mitochondrial membrane, where it is tightly bound to transmembrane enzymes associated with oxidative phosphorylation. The interaction of eucaryal cardiolipin with the intrinsic membrane proteins of mitochondria is relevant from a functional point of view. Cardiolipin has been shown to be important to achieve optimal activity of cytochrome *c* oxidase, NADH dehydrogenase, ATP synthase and other mitochondrial proteins (Kagawa et al. 1973; Serrano et al. 1976; Dale and Robinson 1988). It mediates the cooperativity between cytochrome *c* binding sites in the dimeric enzyme complex (Arnold and Kadenbach 1997). It has been proposed that cardiolipin ensures structural integrity of the environment of the proton-conducting protein and takes part directly in proton uptake of the yeast cytochrome *bc*₁ complex (Lange et al. 2001).

Cardiolipin is a major phospholipid of various Gram-positive bacteria, mycobacteria and Gram-negative bacteria (Ratledge and Wilkinson 1988; O'Leary and Wilkinson 1988; Wilkinson 1988).

Recently, crystallographic evidence has been reported for the interaction of cardiolipin with specific sites on the protein surface of the photosynthetic reaction center in the membrane of *Rhodobacter sphaeroides* (McAuley et al. 1999). However, our overall knowledge of the specific location and the functional role of bacterial cardiolipin is still quite poor.

Bisphosphatidylglycerol is not the only tetra-acylphospholipid in the prokaryotic world. Two tetra-acyl glycopospholipids, having the basic structure of phosphatidyl- α -kojibiosyl-diacylglycerol and a D-alanine derivative of cardiolipin, have been isolated from streptococci and characterized (Fischer and Landgraf 1975; Fischer and Arneith-Seifert 1998). Furthermore two novel glucosyl and lysyl derivatives of cardiolipin have been found in Gram-positive bacteria (Gutberlet et al. 2000).

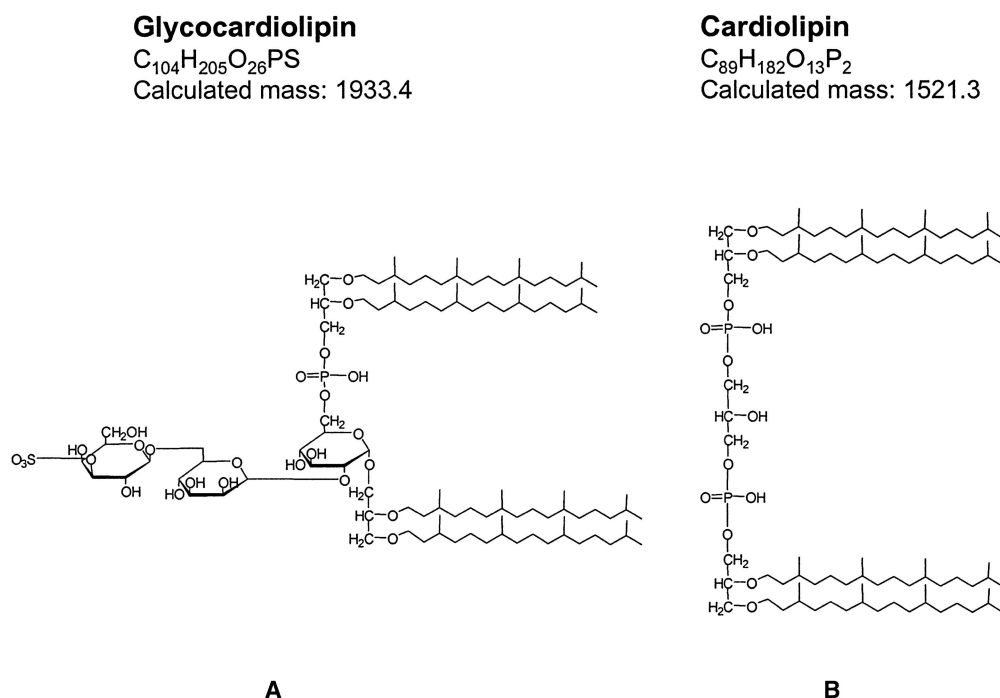
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Fig. 1. Structures of the novel cardiolipin analogs in the purple membrane of *Halobacterium salinarum*: **A** glycocardiophilin, **B** cardiolipin



In the archaeal domain, cardiolipins have been detected only recently. The first evidence that cardiolipins are present in the Archaea came from our studies of the purple membrane of *Halobacterium salinarum* (Corcelli et al. 2000, 2002). Two structural analogs of eucaryal cardiolipin – a phospholipid and a glycolipid – were detected in the purple membrane of an engineered strain of *Hbt. salinarum*. Their structures are, respectively, *sn*-2,3-di-*O*-phytanyl-1-phosphoglycerol-3-phospho-*sn*-2,3-di-*O*-phytanylglycerol (bisphosphatidylglycerol, BPG) and 3-HSO₃-Galpβ1-6Manpα1-2Glcα1-1-[*sn*-2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol] (glycocardiophilin, GlyC). The molecular structures of the two archaeal cardiolipins are shown in Fig. 1. These novel cardiolipins were found to be tightly bound to bacteriorhodopsin, the light-driven proton pump of *Halobacterium*, and appear to have a key role in its stability and function (Lopez et al. 1999; Corcelli et al. 2000). GlyC and bacteriorhodopsin are present in the purple membrane in equimolar amounts, while BPG appears to be a minor component (Corcelli et al. 2002).

To obtain information on the presence and distribution of cardiolipins among the halophilic Archaea, both in their natural habitat and in culture, we used electrospray ionization mass spectrometry (ESI-MS) to analyze the lipids extracted from the crystallizer ponds of the salterns of Margherita di Savoia (southern Italy) and Eilat (Israel) and from cultures of representative species of the Halobacteriaceae. ESI-MS analysis is a highly sensitive method, enabling the analysis of complex membrane lipid mixtures and the selective detection of individual classes of phospholipids and other anionic membrane lipids in total lipid extracts. We show here that the archaeal cardiolipin BPG is present as a minor component in the lipids extracted from

the biomass of the Margherita di Savoia and the Eilat salterns, whereas GlyC was detected only in the lipid extract of the biomass of Margherita di Savoia. We detected BPG in all members of the Halobacteriaceae tested, but GlyC has so far been found only in the genera *Halobacterium* and *Haloarcula*.

Materials and methods

Sampling

Brine was collected from the crystallizer ponds of the salterns of Margherita di Savoia on 7 February, 2001 and of the Israel Salt Company at Eilat on 3 October, 2001. Bacteria were collected by centrifugation (30 min, 7,000 g), resuspended in 4 M NaCl or brine and kept at 4°C until use.

Archaeal strains and culture conditions

The following halophilic Archaea were used as reference strains: *Haloferax volcanii* ATCC 29605^T, *Haloferax gibbonsii* ATCC 33959^T, *Haloferax denitrificans* ATCC 35960^T, *Haloferax mediterranei* ATCC 33500^T, *Halorubrum saccharovororum* ATCC 29252^T, *Haloarcula vallismortis* ATCC 29715^T, *Haloarcula marismortui* ATCC 43049^T, *Halogeometricum borinquense* ATCC 700274^T, *Halobacterium salinarum* NRC 1 ATCC 700922, and *Halobacterium salinarum* NRC 817.

The Archaea were grown in the light at 37°C in liquid medium containing (all concentrations in g l⁻¹): NaCl, 250;

MgSO₄·7H₂O, 20; trisodium citrate dihydrate, 3; KCl, 2; CaCl₂·2H₂O, 0.2; neutralized peptone (L34, Oxoid), 10; prepared as previously described (Lanyi and McDonald 1979). Cells were collected by centrifugation and resuspended in 4 M NaCl before lipid extraction.

Isolation of membranes from biomass by dialysis

Cell pellets were collected by centrifugation, resuspended in 4 M NaCl and dialyzed against distilled water. The disrupted cells were centrifuged at 40,000 *g* for 40 min to collect membranes. Isolated membranes were resuspended in distilled water and immediately used for lipid extraction.

Purple membrane isolation

Purple membrane was isolated and purified on a sucrose density gradient, as previously described (Oesterhelt and Stoekenius 1974), from cultures of an engineered BR high-producing strain (L33) of *Hbt. salinarum*, the kind gift of Richard Needleman (Ni et al. 1990).

Lipid extraction and analysis

All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). Plates for thin-layer chromatography (TLC) (Silica gel 60A), obtained from Merck, were washed twice with chloroform:methanol (1 : 1, v/v) and activated at 120°C before use. The novel cardiolipin standards were isolated and purified as previously described (Corcelli et al. 2000).

Lipids were extracted using the Bligh and Dyer method as modified for extreme halophiles (Kates 1986).

Total lipid extracts were analyzed by thin-layer chromatography on silica gel 60A plates (Merck, 20 × 10 cm, layer thickness 0.2 mm). Lipids were eluted with chloroform:methanol:90% acetic acid (65:4:35, v/v), and detected by spraying with 0.5% sulfuric acid in ethanol, followed by charring at 120°C.

For ESI-mass spectrometry analyses, dried samples of lipid extracts were dissolved in chloroform:methanol (1 : 1, v/v). Electrospray mass spectra (ESI-MS) were obtained with an API 165 mass spectrometer (Applied Biosystems/MSD Sciex, Concord, Ontario, Canada) equipped with a turboion-spray interface. The samples were analyzed by loop injection introducing, by means of a 7125 Rheodyne valve, 5 µl of sample into a 25 µl/min flow of chloroform:methanol (1 : 1, v/v) delivered by a Harvard model 11 syringe pump (South Natick, MA, USA). The instrumental conditions were as follows: nebulizer gas flow (air), 1.2 l/min; curtain gas flow (nitrogen), 1.2 l/min; needle voltage, 5,600 V; interface temperature, ambient; orifice voltage, -150 V; ring voltage, -200 V; mass range, 50–2,000 a.m.u.; mass step, 0.1 a.m.u.; dwell time, 0.2 ms. With the orifice voltage used, CID-MS spectra were obtained showing [M-H]⁻ and [M-2H]²⁻ parent ions as well as some fragmentation ions.

Results

Microscopic examination of the pellets obtained by centrifuging brines from the Margherita di Savoia and the Eilat saltern crystallizer ponds showed a dominance of flat, square-to-rectangular cells, similar to those reported earlier from the Eilat salterns and similar systems worldwide (Benloch et al. 1995, 1996; Oren et al. 1996). No *Dunaliella* cells were observed in the pellets upon microscopic examination. *Dunaliella* cells heavily loaded with β-carotene granules, such as occur in the salterns, are lighter than the brine and they float during centrifugation. We recovered yields of 9 mg and 1 mg lipids per liter from the Margherita di Savoia and Eilat brines, respectively.

ESI-MS analyses of the total unprocessed lipid extracts of the biomass of Margherita di Savoia and Eilat are shown in Fig. 2A, B. Figure 2C shows the TLC of the both lipid extracts. The TLC lipid profiles of both extracts are quite complex. Identification of the two novel archaeal cardiolipins, expected to be present as minor lipid components in such halophilic microorganisms that may be present in natural brines, is therefore difficult on the basis of TLC data.

An additional problem in the identification of the archaeal cardiolipins in the TLC lipid profile of Fig. 2C arises from the fact that the *R_f* values of glycardiolipin and cardiolipin are very close to those of the major sulfoglycolipid S-DGD-1 and the abundant reddish pigments, respectively.

The ESI-MS spectra, as presented in Fig. 2A, B are far more informative on the biomass composition. The main peaks present in Fig. 2A, B correspond to the molecular ions of the different kinds of polar lipids (phospholipids and glycolipids) present in the membranes of various halophilic microorganisms of the biomass. As glycolipids of extremely halophilic Archaea might serve as chemotaxonomic markers for classification of these organisms, the presence of certain glycolipid peaks in the total lipid extract of the biomass can be considered indicative of the presence of various genera of the Halobacteriaceae family (Oren and Gurevich 1993; Oren 2001).

Table 1 lists the main peaks observed in the ESI-MS lipid profiles of various halophilic microorganisms cultured in our laboratory. It should be noted that unsulfated diglycosyl, triglycosyl, and tetraglycosyl lipids, when present, appear as minor peaks in the ESI-MS spectra only, as they are difficult to ionize. On the basis of these data it was possible to assign various ESI-MS peaks detected in the brine extracts to known polar lipids of archaeal halophiles (Table 2). The major peak at *m/z* 1,056 found in both salterns shows that monosulfated diglycosyl diether lipids, such as occur in the genera *Haloferax*, *Halobaculum*, and *Halorubrum*, are far more abundant in the saltern biomass than, for example, in *Halobacterium*.

In a previous study we have shown that the two novel phospholipids of the purple membrane, GlyC and BPG, can easily be recognized within a mixture of phospholipids by a set of specific peaks present in the ESI mass spectrum; in particular we have shown that the two bicharged peaks at *m/z* 760 and 966 are diagnostic for the presence of BPG and

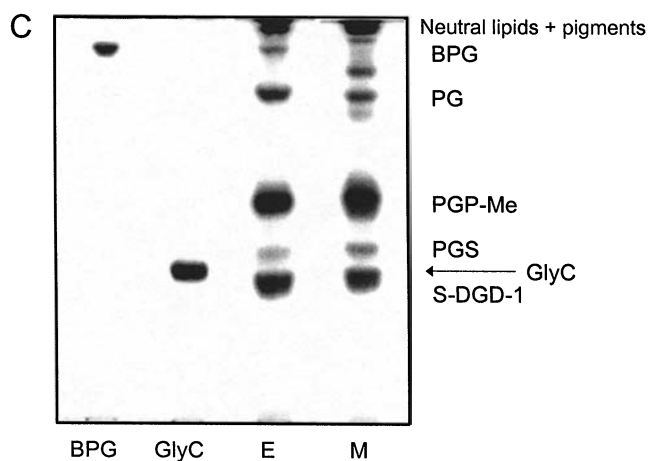
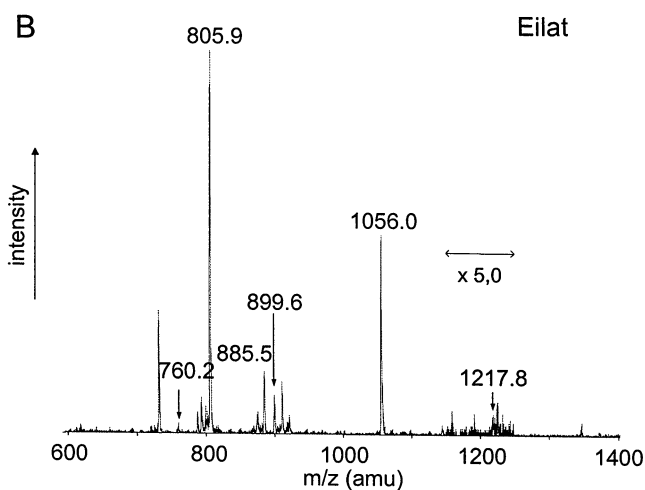
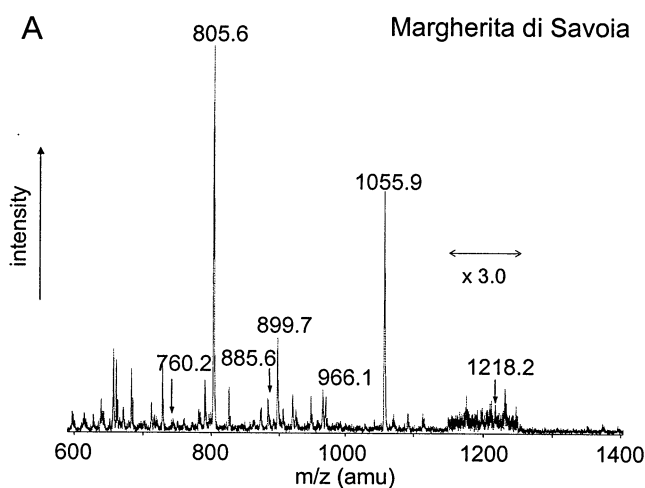


Fig. 2. ESI-MS analyses (negative ion) of the total lipid extracts of the biomass of Margherita di Savoia (**A**) and Eilat (**B**); **C** TLC profiles of lipid extracts of Margherita di Savoia (*M*) and Eilat (*E*). Standard BPG and GlyC, bisphosphatidylglycerol and glycardiolipin, respectively, were isolated and purified from the purple membrane of an engineered strain of *Hbt. salinarum* (Ni et al. 1990)

GlyC, respectively. A small peak at m/z 760 is present in both ESI-MS spectra of Fig. 2, whereas the peak at m/z 966 was detectable only in the lipids obtained from the Margherita di Savoia biomass. It is interesting to point out that the glycolipid S-TGA-1, which is part of the structure of the glycardiolipin and may be a precursor in its biosynthesis, is detectable among the lipids of Eilat biomass as well.

As the cardiolipins are lipid components specifically associated with integral membrane proteins, they appear to be enriched in isolated cellular membranes. We therefore also analyzed the lipids of membranes isolated from the saltern biomass in order to examine the presence of the glycardiolipin in an enriched membrane preparation of the biomass of Eilat. The lipid composition of membranes isolated by dialysis from the biomass of Margherita di Savoia and Eilat is shown in Fig. 3A, B, respectively. In the case of Margherita di Savoia, both the peaks at m/z 760 and m/z 966 appear to be enriched in the lipid profiles of the membranes isolated from biomass compared to the lipid profile of non-disrupted biomass cells (Fig. 2A). The lipid extract of membranes isolated from Eilat cells shows the peak at m/z 760,

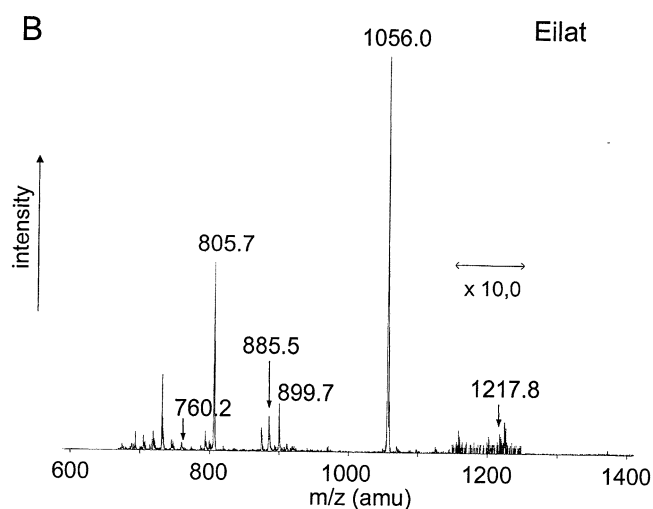
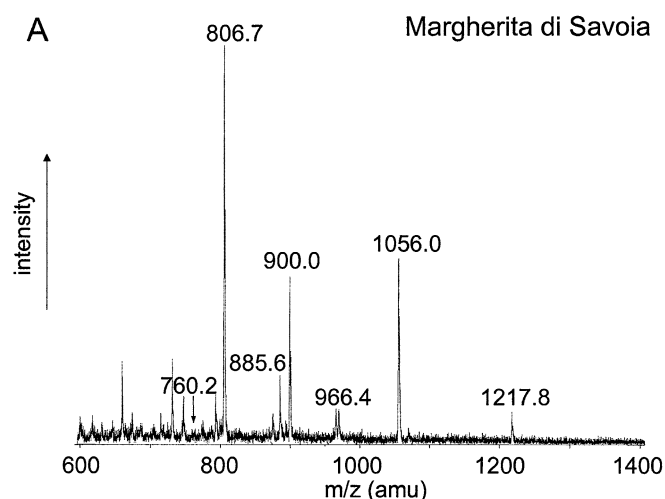


Fig. 3. ESI-MS profiles (negative ion) of total lipid extracts of membranes isolated by dialysis of the biomass of Margherita di Savoia (**A**) and Eilat (**B**)

Table 1. Observed signals in the ESI-MS (negative ion) spectra of total lipid extracts from cultures of various halophilic archaeal microorganisms

Genus, Species	Phospholipids			Glycolipids					Cardiolipins	
	806	886	900	976	1,056	1,138	1,218	1,381	760	966
<i>Hfx. gibbonsii</i> ATCC 33959	x	x	x	x	x		x ^b		x	
<i>Hfx. denitrificans</i> ATCC 35960	x	x	x		x	x ^a	x ^a		x	
<i>Hfx. volcanii</i> ATCC 29605	x	x	x		x				x	
<i>Hfx. mediterranei</i> ATCC 33500	x	x	x		x		x ^a		x	
<i>Hrr. saccharovororum</i> ATCC29252	x	x	x		x		x ^a		x	
<i>Har. vallismortis</i> ATCC 29715	x	x	x			x			x	x ^a
<i>Har. marismortui</i> ATCC 43049	x	x	x			x			x	x
<i>Hbt. salinarum</i> NRC 1	x	x	x				x	x	x	x
<i>Hbt. salinarum</i> NRC 817	x	x	x				x	x	x	x

^aPresent as a very small peak^bMinor peaks**Table 2.** Molecular ions and bicharged peaks (m/z values) of the archaeal polar lipids present in halophilic microorganisms. All the lipids listed in the table are derivatives of the C₂₀, C₂₀isopranyl glycerol diether

Lipid	m/z, amu
Bisphosphatidylglycerol (BPG)	760 ^a
Phosphatidylglycerol (PG)	806
Phosphatidylglycerosulfate (PGS)	886
Phosphatidylglycerophosphate methyl ester (PGP-Me)	900
Glycocardioliipin (GlyC)	966 ^a
Sulfated diglycosyl diphytanyl glycerol (S-DGD-1)	1,056
Diglycosyl diphytanyl glycerol (DGD-1)	976
Sulfated triglycosyl diphytanyl glycerol (S-TGD-1)	1,218
Triglycosyl diphytanyl glycerol (TGD-1)	1,138
Sulfated tetraglycosyl diphytanyl glycerol (S-TeGD-1)	1,381

^aDouble-charged peaks

but not the peak at m/z 966. The peak at m/z 1,218, representing the molecular ion of the glycolipid S-TGA-1, is clearly detectable as a minor lipid component in the mass spectrum shown in Fig. 3B.

Discussion

The differences observed in the ESI-mass spectra of the lipids extracted from the biomass of the salterns of Margherita di Savoia and Eilat show once more that in spite of the superficial resemblance of the biota found in salterns worldwide, local variations do exist. Salterns may differ in nutrient levels, and the size of the biomass, both of algae and of prokaryotes, can vary greatly (Javor 1983a, b). Qualitative differences have also been documented in studies in which the eutrophic salterns of San Francisco Bay have been compared with the oligotrophic ponds in Eilat (Litchfield et al. 2000; Litchfield and Oren 2001).

The large peak at m/z 1,056 shows that one or more monosulfated diglycosyl diethers are the dominant glycolip-

ids in the crystallizer biomass, both at Margherita di Savoia and at Eilat. Glycolipids are excellent chemotaxonomic markers for different genera within the Halobacteriaceae, and their classification is in part based on the presence of specific glycolipids (Kamekura and Kates 1999; Oren 2001). Earlier characterizations of the lipids present in the Eilat crystallizer pond biomass by thin-layer chromatography have shown a single glycolipid to be present, co-eluting with S-DGD-1 of *Haloferax* (Oren 1994; Oren et al. 1996). In the more nutrient-rich salterns in San Francisco Bay small amounts of other glycolipids may be present, but the sulfated diglycosyl diether lipids are by far the most abundant there (Litchfield et al. 2000; Litchfield and Oren 2001). Our ESI-MS data for the two salterns examined thus confirm the earlier findings.

In the present study we have shown for the first time that both novel archaeal cardiolipins, BPG and GlyC, are present among the lipids extracted from the microorganisms present in the halophilic community of the saltern of Margherita di Savoia, whereas only BPG is detectable in the total lipid extract of Eilat biomass. The first evidence that these cardiolipins are present in Archaea came from our studies of the purple membrane of an engineered strain of *Hbt. salinarum* (Corcelli et al. 2000). The purple membrane is enriched in cardiolipins, and these are tightly bound to bacteriorhodopsin. They appear to play a key role in preserving the stability of solubilized bacteriorhodopsin and in particular of the trimer aggregates (Lopez et al. 1999). The molar ratio GlyC to bacteriorhodopsin in the purple membrane has been estimated to be 1 (Corcelli et al. 2000, 2002). This molar ratio is therefore compatible with a role of GlyC as a stabilizing agent of the trimer structure and a possible location in the crevice between monomers. BPG is only a minor lipid component of the purple membrane (Corcelli et al. 2000, 2002). It has been suggested that it may be located in the inter-trimer spaces, and that it could mediate specific interactions between trimers by establishing bridging contacts between bacteriorhodopsin molecules in adjacent trimers in the purple membrane lattice (Corcelli et al. 2002).

The ubiquity of BPG in all examined members of the Halobacteriaceae, including many species that do not pro-

duce purple membrane (Table 1), suggests that this lipid may play additional roles in the extremely halophilic Archaea. It may be worthwhile to investigate whether the archaeal cardiolipins are present among the lipids tightly bound to transmembrane enzymes performing oxidative phosphorylation and in particular with archaeal cytochrome *c* oxidase.

GlyC has been found thus far only in the genus *Halobacterium* and as a very minor component in *Haloarcula* (Table 1). Bacteriorhodopsin proton pumps are also present in *Haloarcula*, although the pigment has not been documented to be organized in purple membrane (Mukohata 1994; Tatenio et al. 1994; Mukohata et al. 1999). We did not detect GlyC in representatives of the genera *Haloferax* and in *Hrr. saccharovororum*, all organisms that appear to lack bacteriorhodopsin. Our results therefore suggest that GlyC can be considered as a biomarker for the presence of bacteriorhodopsin and of the purple membrane in the biomass of the salterns.

In general, little information is available on the presence of the bacteriorhodopsin proton pump in halophile communities in salterns. In the archaeal community of an oligotrophic saltern crystallizer pond in Baja California (Mexico), 2.2 nmol l⁻¹ bacteriorhodopsin has been found, being a very high concentration relative to the biomass present (Javor 1983a). In a more eutrophic saltern in California the retinal pigment was not detected (Javor 1983b). Experiments are in progress to assess the occurrence of bacteriorhodopsin in the biomass of both salterns of Margherita di Savoia and Eilat.

Microorganism(s) producing purple membranes could easily be obtained in mixed cultures by inoculating the red brine of Margherita di Savoia in a medium containing peptone. We have found that the production of the purple membrane was greatly stimulated in nutrient-rich media. The novel cardiolipins have been found among lipids extracted from the purple membranes isolated from these microorganisms cultured from the brine of Margherita di Savoia (data not shown).

It would be interesting to ascertain the contribution of BR-producing microorganisms of *Halobacterium* and *Haloarcula* genera to the biomass of both salterns. It has been shown that, using a selective enrichment based on its ability to grow anaerobically on arginine, *Halobacterium* could be isolated from salterns worldwide (Oren and Litchfield 1999). *Halobacterium* has sulfated triglycosyl and tetraglycosyl diethers: S-TGD-1 (1-*O*-[β -D-galactose-(3'-SO₃H)-(1'→6')- α -D-mannose-(1'→2')- α -D-glucose]-2,3-di-*O*-phytanyl-sn-glycerol) (*m/z* = 1,218) and S-TeGD (1-*O*-[β -D-galactose-(3'-SO₃H)-(1'→6')- α -D-mannose-(3'←1')- α -D-galactofuranose-(1'→2')- α -D-glucose]-2,3-di-*O*-phytanyl-sn-glycerol) (*m/z* = 1,381), as well as minor amounts of the desulfated analogs TGD-1 (*m/z* = 1,138) and TeGD (*m/z* = 1,301) (Kamekura and Kates 1999; Kates and Deroo 1973; Smallbone and Kates 1981).

S-TGD-1 was detected as a minor component in both the Margherita di Savoia and the Eilat biomass, while the peak at 1,138 *m/z*, which appears only as a small peak in the ESI-MS lipid profiles of cultivated *Halobacterium* cells, could

not be detected. The glycolipid signature of *Haloarcula* was not detected in the ESI-MS spectra of the Margherita di Savoia and the Eilat crystallizer biomass. *Haloarcula* is characterized by the presence of the triglycosyl diether TGD-2 (1-*O*-[β -D-glucose-(1'→6')- α -D-mannose-(1'→2')- α -D-glucose]-2,3-di-*O*-phytanyl-sn-glycerol) (Evans et al. 1980) (expected to give a peak at *m/z* = 1,138); evidence for the presence of minor amounts of an unsulfated diglycosyl diether has also been reported (Oren 2001). Both these glycolipids are difficult to ionize and appear only as small peaks in the ESI-MS profile of the total cell lipid extract of cultivated *Haloarcula* strains.

We have recently isolated bright-red, square-shaped representatives of the genus *Haloarcula* from the biomass of Margherita di Savoia. The TLC and ESI-MS lipid profiles of these strains were identical to those of *Har. marismortui* and *Har. vallismortis*; furthermore, the 16S sequence of one of these strains showed 99% similarity to *Har. argentinensis* and *Har. marismortui* (F. Rodríguez-Valera, Universidad Miguel Hernández, Alicante, Spain, unpublished results).

The possibility exists that the presence of cardiolipins in the salterns may be attributed to an as yet uncultured type of flat, square Archaea that is abundantly present in the biomass. Microscopic examination shows that the most frequently encountered type of cell has a flat, square morphology, and often contains gas vesicles. Such square gas-vacuolated cells were first discovered in a brine pool in Sinai (Walsby 1980), and they have since been shown to be ubiquitous in hypersaline environments at or approaching halite saturation, including saltern crystallizer ponds (Guixa-Boixareu et al. 1996; Oren et al. 1996). Lipid analysis of biomass collected from the Eilat saltern dominated by these square cells showed the presence of S-DGD-1 as sole glycolipid (Oren et al. 1996). Cells with such a morphology have not yet been obtained in culture. It is clear, however, that this organism does not belong to any of the cultured genera of the Halobacteriaceae. We do know its phylogenetic position as based on 16S rRNA sequence comparisons. Amplification of archaeal 16S rRNA genes from DNA extracted from the biomass of the saltern crystallizers of Alicante (Spain) and Eilat consistently yielded a new phylotype at the highest frequency, distantly related to the genus *Haloferax* (Benlloch et al. 1995, 1996; Rodríguez-Valera et al. 1999). This phylotype belongs to the square Archaea, as shown by fluorescent oligonucleotide probes designed to specifically interact with this new phylotype (Antón et al. 1999). Electron microscopic examination of the square Archaea collected from the Sinai brine pool did not show any purple membrane to be present in thin sections or freeze-fracture and freeze-etch preparations (Stoeckenius 1981). However, flash spectroscopy of the collected biomass showed evidence for the presence of both bacteriorhodopsin and halorhodopsin activity (Stoeckenius et al. 1985). Thus, it is possible that the cardiolipins detected in the biomass of the salterns may be associated with retinal pigments in the flat square Archaea that dominate the community.

The present study shows that ESI-MS analysis of the biomass lipids is a powerful experimental approach that

provides extensive information on the types of halophilic microorganisms, that inhabit saltern brines. As ESI-MS can also give reliable quantitative results by using appropriate internal standards, we conclude suggesting that a combination of ESI-MS lipid analyses and 16S rRNA analyses may allow a full description of the distribution of different halophiles along the salinity gradient of salterns.

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