Osmotic shock stimulates de novo synthesis of two cardiolipins in an extreme halophilic archaeon

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Abstract **The present report illustrates the response to osmotic stress of an extreme halophilic archaeon, *Halorubrum* sp., isolated from the saltern ponds of Margherita di Savoia in southern Italy. The hypotonic stress induces relevant changes in the membrane lipid composition: archaeal cardiolipin content markedly increases, whereas phosphatidylglycerol (PG) decreases. Membranes isolated from this archaeon after cell disruption by osmotic shock are highly enriched in archaeal cardiolipin and reveal the presence of a novel phospholipid. Electrospray ionization mass spectrometry and NMR analyses revealed that this novel lipid has the structure of a sulfo-diglyco-diether-phosphatidic acid, i.e., a phospholipid dimer or a novel cardiolipin analogue. As NMR analyses showed that the sugars in the novel phospholipid dimer are the same and in the same order of a sulfated diglycosyl diphytanylglycerol diether (S-DGD-5) present as a major lipid component in the archaeon membranes, the novel phospholipid dimer was named S-DGD-5-PA. We conclude that osmotic shock induces a specific increase in the membrane content of the two cardiolipins and suggest that PG and S-DGD-5 are intermediates for the de novo synthesis of archaeal cardiolipin and S-DGD-5-PA, respectively.**—Lopalco, P., S. Lobasso, F. Babudri, and A. Corcelli. **Osmotic shock stimulates de novo synthesis of two cardiolipins in an extreme halophilic archaeon. *J. Lipid Res.* 2004.** 45: **194–201.**

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Cardiolipin, a dimeric phospholipid discovered in heart mitochondria, plays an important role in the function and stability of many enzymatic complexes of the respiratory chain (1). A number of substituted cardiolipins or cardiolipin analogues have been described in bacteria (2–4); the function of these cardiolipin analogues in bacteria is still unclear. Both authentic cardiolipin and cardiolipin analogues are composed of two diacylglycerol moieties and therefore possess four hydrophobic chains.

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Cardiolipins have also been found in the Archaea world (5, 6). The first evidence of the presence of archaeal cardiolipin was found in *Halobacterium salinarum*, in which a bisphosphatidylglycerol (BPG) and another phospholipid dimer composed of the glycolipid sulfated triglycosyl diphytanylglycerol diether-1 (S-TGD-1) esterified to phosphatidic acid (PA) have been described [S-TGD-1-PA, also called glycocardiolipin (GlyC)] (5).

GlyC, a major lipid component of the purple membrane (PM) of *H. salinarum*, plays an important role in the function and stability of bacteriorhodopsin (BR), the photoactivated proton pump (7), whereas BPG is only a minor lipid of PM (5, 8). Interestingly, it has been shown that the amount of GlyC in the PM of *H. salinarum* increases when the cells are exposed to hypotonic shock and that the formation of GlyC occurs at the expense of S-TGD-1 (9).

In the present report, we show that a halo-archaeal isolate collected from the salterns of Margherita di Savoia in southern Italy, which is closely related to *Halorubrum trapanicum*, actively synthesizes high amounts of the archaeal cardiolipin BPG under osmotic shock. Membranes isolated after cell disruption by osmotic shock are highly enriched in this archaeal cardiolipin and reveal the presence of a novel phospholipid. The novel phospholipid has been isolated and identified as S-DGD-5-PA, i.e., a phospholipid dimer composed of the glycolipid S-DGD-5 (a sulfo-diglycosyl-diether) esterified to PA.

Abbreviations: BPG, bisphosphatidylglycerol or cardiolipin (diphytanylglycerol ether analogue); BR, bacteriorhodopsin; ESI-MS, electrospray ionization mass spectrometry; GlyC, glycocardiolipin or 3-HSO_3-Gal*p*-β1,6-Man*p*-α1,2-Glc*p*-α1,1-[*sn*-2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol] or S-TGD-1-PA; PA, phosphatidic acid (diphytanylglycerol ether analogue); PG, phosphatidylglycerol (diphytanylglycerol ether analogue); PGP-Me, phosphatidylglycerophosphate methyl ester (diphytanylglycerol ether analogue); PGS, phosphatidylglycerosulfate (diphytanylglycerol ether analogue); PM, purple membrane; S-DGD-5, 2-HSO_3-Man*p*-α1,2-Glc*p*-α1,1-*sn*-2,3-diphytanylglycerol; S-DGD-5-PA, 2-HSO_3-Man*p*-α1,2-Glc*p*-α1,1-[*sn*-2,3-diphytanylglycerol]-6-[phospho-*sn*-2,3-diphytanylglycerol]; S-TGD-1, 3-HSO_3-Gal*p*-β1,6-Man*p*-α1,2-Glc*p*-α1,1-*sn*-2,3-diphytanylglycerol.

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We demonstrate that osmotic shock specifically induces an increase in the content of both archaeal cardiolipins and suggest that the archaeal cardiolipin and the novel cardiolipin analogue S-DGD-5-PA are synthesized at the expense of phosphatidylglycerol (PG) and the glycolipid S-DGD-5, respectively.

EXPERIMENTAL PROCEDURES

Materials

DNase I was obtained from Sigma (St. Louis, MO); all organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). Plates for TLC (Silica gel 60A; 20 × 10 cm, layer thickness 0.2 mm) were obtained from Merck.

Microorganism culture

The microorganism (MdS1 strain) used in this study was isolated from the salterns of Margherita di Savoia by enrichment culture on agar plates using a medium composed of 250 g/l NaCl, 20 g/l $MgSO_4 \cdot 7H_2O$, 3 g/l tri-sodium citrate dihydrate, 2 g/l KCl, 0.2 g/l $CaCl_2 \cdot 2H_2O$, and 10 g/l neutralized peptone (L34; Oxoid), pH 7.0, prepared as described previously (10). Routinely, the strain was grown aerobically in the light, in a gyratory shaker at 160 rpm at 37°C for ~10 days, in a liquid medium of the same composition.

Cells at the stationary growth phase were harvested by centrifugation and immediately used for lipid extraction or processed for membrane isolation.

Sequencing of 16S rRNA

For preliminary identification of the isolate, the 16S rDNA of MdS1 was amplified and sequenced as described elsewhere (11). The 513 nts determined were submitted to the Basic Local Alignment Search Tool to establish the relationships with known species of Haloarchaea.

Membrane isolation

Cells from liquid cultures were collected by centrifugation (8,000 *g* for 15 min), washed, and resuspended in 4 M NaCl. A dialysis bag (cutoff 12,000–14,000) was filled with a concentrated suspension of cells in 4 M NaCl in the presence of DNase I and left in distilled water under stirring at 4°C overnight. The lysate was centrifugated at 30,000 *g* for 40 min, and the resulting pellet containing the membranes was washed twice with buffer A (0.15 M NaCl and 0.050 M Tris/Cl, pH 7.0). Isolated membranes were resuspended in the same buffer and stored at −20°C until further use.

Time course of archaeal cardiolipin increase and PG decrease in cells diluted in low-salt medium

A suspension of cells (in 4 M NaCl) was diluted 5-fold with a hypoosmotic solution (0.1 M NaCl) and incubated at 25°C under stirring. At intervals (15, 30, 60, 90, and 180 min), equivalent aliquots of suspension were removed for the lipid extraction; the weights of the lipid extracts from these aliquots were not significantly different.

Cell disruption by French press

A suspension of cells (in 4 M NaCl) was passed three times through a French pressure (FP) cell at 2,000 pounds per square inch. After disruption, the lysate was diluted twice with 4 M NaCl, and unbroken cells and large debris were removed by centrifugation at 4,000 *g* for 20 min. Membranes were collected by centrifuging the supernatant at 30,000 *g* for 40 min.

Lipid extraction

Total lipids were extracted using the Bligh and Dyer method as modified for extreme halophiles (12); the extracts were carefully dried under N_2 before weighing. For the reextraction of whole cells, an aliquot of cell suspension (in 4 M NaCl) was extracted by standard procedure; then, organic solvents [chloroform-methanol-H_2O (1:1:0.9; v/v/v)] were again added to the denatured cellular material and incubated at 80°C for 30 min to obtain a reextract. This procedure was repeated once more, and the two reextracts were combined.

TLC

Plates for TLC were washed twice with chloroform-methanol (1:1; v/v) and activated at 120°C before use. Total lipid extracts were analyzed by TLC in solvent A (chloroform-methanol-90% acetic acid (65:4:35; v/v/v). All lipids were detected by spraying with 5% sulfuric acid, followed by charring at 120°C.

Isolation and purification of individual archaeal lipids

The isolation of individual lipid components of the total extracts was performed by scraping the silica gel in each band from the TLC plate and extracting each lipid band from the silica as described previously (5). If necessary, each component was further purified by rechromatography in neutral solvent B [chloroform-methanol-water (65:25:4; v/v/v)].

Quantitation of individual lipids of the total extracts

The determination of the archaeal cardiolipin and PG content of the lipid extracts was performed by densitometric analyses. For quantitative determination, peak data of the unknowns are correlated with data from calibration standards chromatographed on the same plate. In particular, the staining intensity was evaluated by video densitometry using ImageJ software (http://rsb.info.nih.gov/ij). The standard curves of authentic standards for BPG and PG were linear in the concentration range 1–10 μg.

Mass spectrometry

For negative ion electrospray ionization mass spectrometry (ESI-MS) analyses, dried samples of lipid extracts were dissolved in chloroform-methanol (1:1; v/v). Electrospray mass spectra were obtained with an API QSTAR mass spectrometer (Applied Biosystem/MSD Sciex, Concord, ON, Canada) equipped with a Turbo ion spray interface. Tandem mass spectrometry of band 3 after ESI was performed with a collision energy of −80 V.

NMR spectroscopy

NMR spectra of isolated lipids were taken in $CDCl_3$-CD_3OD (4:3; v/v; final lipid concentration ~1 mM). All NMR analyses were performed on an AM 500 Bruker (Billerica, MA) instrument, and ^1H chemical shifts are given relative to tetramethylsilane as an internal standard.

RESULTS

In this study, we have investigated the lipid composition of an extremely halophilic archaeon with a high archaeal cardiolipin content under osmotic stress. The extremely halophilic microorganism used in the present study, MdS1, was isolated from the salterns of Margherita di Savoia in southern Italy. Analysis of the 16S rRNA gene se-

quence clearly places the isolate used in the present study within the *Halorubrum* cluster: in the 513 nts determined (positions 910–1,421, *H. halobium* numbering), only 8 nts varied from the closest match, *H. trapanicum* (98.5% similarity), indicating a close relationship consistent with two strains of the same or closely related species. Therefore, it seems appropriate to refer to the isolate as *Halorubrum* sp. (F. Rodriguez-Valera, personal communication).

In agreement with the 16S rRNA analyses, the presence of a sulfo-diglycosyl-diacylglycerol in the total cell lipid extract (see below) is consistent with the placement of the MdS1 isolate in the *Halorubrum* genus.

Initially, we analyzed by TLC (**Fig. 1**) the lipid composition of MdS1 cells suspended in 4 M NaCl (lane C) and cell lysate after dialysis against water (lane D). The individual lipid components of the extracts were identified by their retention factor (R_f) values relative to those of authentic standard markers, by their staining behavior with specific reagents (not shown), and by negative ion ESI-MS spectra of isolated individual lipids. The main lipid components present in both lipid extracts were identified (in R_f order) as monosulfated diglycosyl diphytanyl-glycerol (S-DGD), phosphatidylglycerosulfate (PGS), phosphatidylglycerophosphate methyl ester (PGP-Me), PG, and the archaeal cardiolipin BPG, plus the pigments at the solvent front. The abbreviated names of purified individual lipids are also reported in Fig. 1. As it is known that *H. trapanicum* contains a glycolipid called S-DGD-5 (2-HSO_3-Man*p*-α1,2-Glc*p*-α1,1-*sn*-2,3-diphytanylglycerol) (13), we refer to the major glycolipid of MdS1 cells as S-DGD-5.

Comparing the two lipid extracts, it is evident that the lipid extract of lysed cells contains a higher archaeal cardiolipin content and a lower PG content than does the lipid extract of whole cells. An unidentified lipid (the third band from the bottom) is present only in the lipid profile of lysed cells by dialysis.

As shown previously for the GlyC of the PM of *H. salinarum*, the data in Fig. 1 suggest that archaeal cardiolipin is formed when haloarchaeal MdS1 cells are exposed to hypotonic stress. To study in detail the response of MdS1 cells to osmotic stress, we have studied in parallel the osmotic fragility of the cells exposed to hypotonic medium and the changes in membrane lipid composition.

Figure 2 shows the time course of the development of osmotic fragility of MdS1. The cells, initially resuspended in 4 M NaCl, were diluted 5-fold or 10-fold with 0.1 M NaCl. MdS1 cells seem to adapt quite well to an external medium containing 0.8 M NaCl: they swell but do not disrupt. We have reported previously that cells of a genetically engineered strain of *H. salinarum* suspended in 0.8 M NaCl are easily disrupted (9). On other hand, MdS1 cells quickly disrupt when the extracellular medium contains only 0.4 M NaCl; it took less than 15 min for the cells to become 50% fragile and 90 min for the cells to become 80% fragile.

To follow the changes in the lipid composition of MdS1 cells undergoing osmotic shock, lipids were extracted from equivalent aliquots of cells, suspended in hypotonic medium, and taken at different times from the start of osmotic stress. The TLC results in **Fig. 3** show that changes in the lipid composition of cells occurred just after resuspension in 0.8 M NaCl. Archaeal cardiolipin increased continuously in cells resuspended in hypotonic medium; it is evident that archaeal cardiolipin increased at the expense of PG. A similar pattern of changes in lipid composition was found in disrupting cells suspended in 0.4 M NaCl or in the dialysis bag (not shown).

We have estimated by video densitometric analyses the amount of individual lipids in the TLC (Fig. 3A). The standard curves for the individual lipid components of MdS1 cells were linear in the concentration range 1–10 μg. Figure 3B shows the time course of changes in archaeal cardiolipin and the PG content of the incubation mixture during osmotic shock, relative to the experiment

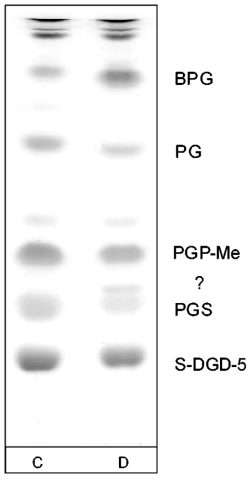

Fig. 1. TLC of lipid extracts of whole cells and cell lysate after dialysis. Lipids were extracted from MdS1 cells resuspended in 4 M NaCl (lane C) and from a concentrated suspension of cells kept in a dialysis bag (cutoff 12,000–14,000) overnight against distilled water (lane D).

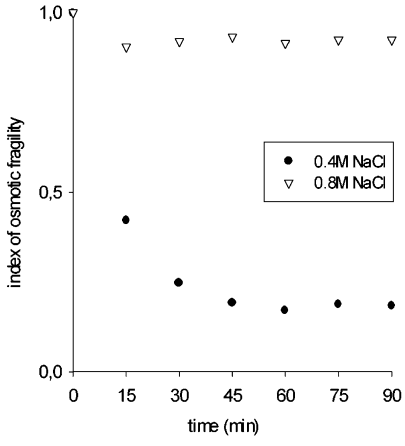

Fig. 2. Time course of the development of osmotic fragility in MdS1 cells. The cells were initially suspended in 4 M NaCl and diluted 5-fold or 10-fold with 0.1 M NaCl. Percentages of initial cell turbidity were measured at 700 nm, and the ratio was expressed as an index of osmotic fragility.

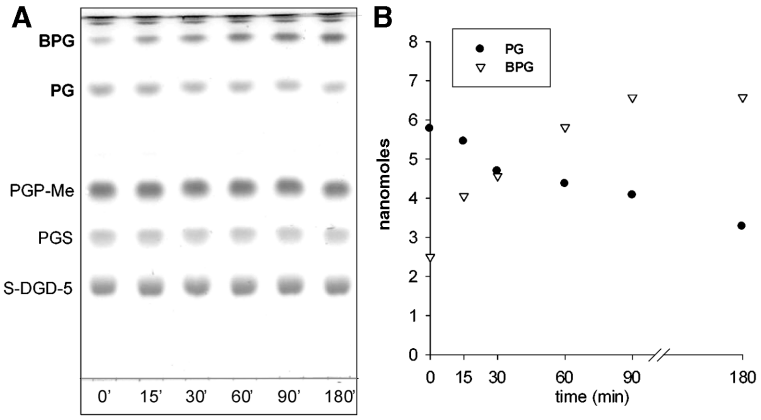

Fig. 3. Archaeal cardiolipin increase and PG decrease in cells after dilution in low-salt medium. A: TLC of lipids extracted from equivalent cell aliquots taken at different time intervals from the dilution; 40 μg of lipid extracts were loaded in each lane. B: Time course of the changes in BPG and PG contents occurring in swelling and disrupting cells after dilution. Values on the *y* axis (in nanomoles) were estimated by video densitometry, as described in Experimental Procedures.

shown in Fig. 3A. After 3 h of incubation of MdS1 cells in 0.8 M NaCl, 3 nmol of newly formed archaeal cardiolipin over 40 μg of total lipid extract were found, corresponding to the disappearance of 3 nmol of PG.

Furthermore, analyzing the plate shown in Fig. 3A, the unidentified lipid present in the extract of dialyzed cells (Fig. 1, lane D) could not be detected. This suggests that the novel lipid can be observed only when the cells disrupt by osmotic shock and not in intact swelling cells. Therefore, to identify the unidentified lipid component, we isolated a membrane fraction containing this lipid from the cell lysate.

By comparing by TLC (**Fig. 4A**) the lipid profiles of cells suspended in isotonic medium (lane C) and isolated membranes (lane M), it is evident that the presence of the unidentified lipid, named "band 3," occurs among membrane lipids only. The same lipid extracts were also analyzed by ESI-MS (Fig. 4B): the main peaks of the lipid extracts of cells (upper panel) were identified as PG at *m/z* 805.7, S-DGD-5 at *m/z* 1,055.8, and PGP-Me at *m/z* 899.6, whereas PGS at *m/z* 886 and the archaeal analogue of cardiolipin, BPG, at *m/z* 760 as a bicharged peak appear to be minor components. At *m/z* 731.7, the fragment ion diagnostic of the archaeal core is also present.

The ESI-MS spectrum of the lipid extracts of isolated membranes (Fig. 4B, lower panel) confirms that archaeal cardiolipin (at *m/z* 760.1) is highly enriched in the lipid extract of membranes, compared with that of whole cells, whereas PG (at *m/z* 806.1) is significantly reduced, in agreement with TLC data. An unidentified peak (at *m/z* 885.1) can be observed in the lipid profile of the membranes.

All of the individual lipids present in the lipid extract of archaeal cardiolipin-rich membranes have been isolated, purified, and analyzed by TLC and ESI-MS (**Table 1**).

TLC results from isolated and purified band 3 are shown in **Fig. 5A**. This lipid gave a positive test on TLC

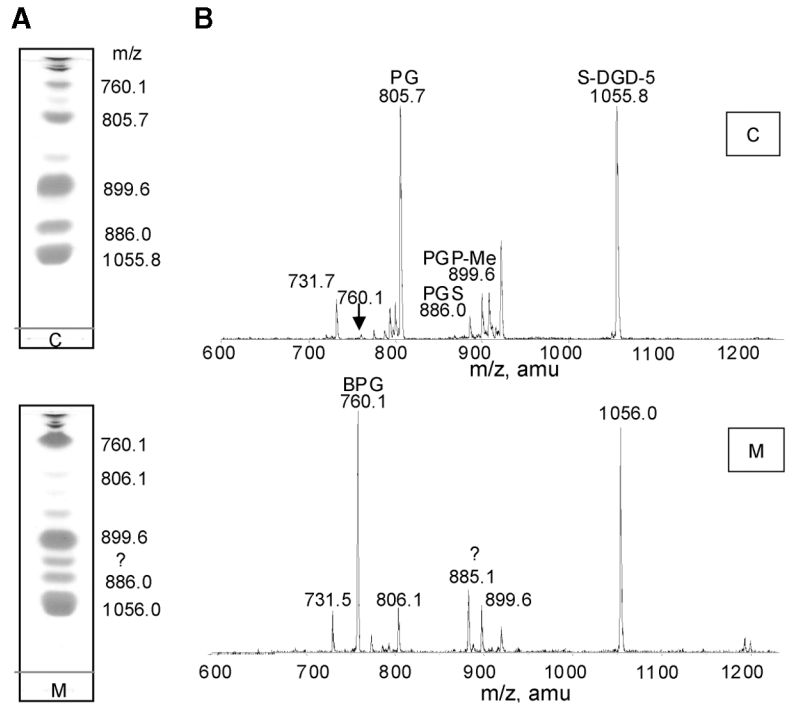

Fig. 4. Analyses of lipid extracts of whole cells and isolated membranes. TLC (A) and electrospray ionization mass spectrometry (ESI-MS) spectra (B) of lipid extracts of cells resuspended in 4 M NaCl (lane C) and of membranes isolated after disrupting the cells by dialysis against water (lane M). Eighty micrograms of lipid extracts were loaded on the plate. The *m/z* values (negative ions) are shown.

TABLE 1. TLC retention factor in solvent A, calculated mass, and electrospray ionization mass spectrometry (negative mode) ion peaks of various lipid components of MdS1 cells and membranes

Lipid	TLC Retention Factor	Calculated Mass	Mass Spectrometry Ion Peaks	
			$[M-H]^-$	$[M-2H]^{2-}$
		Da	*m/z, amu*	
S-DGD-5	0.31	1,057.5	1,055.8	
PGS	0.40	886.6	886.0	
Band 3[a]	0.46	1,772.6	1,770.7	885.1
PGP-Me	0.54	900.7	899.6	
PG	0.78	806.7	805.7	
BPG	0.89	1,522.3	1,521.3	760.0
Neutral lipids plus pigments	0.99–1.0			

amu, atomic mass unit; BPG, bisphosphatidylglycerol; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; PGS, phosphatidylglycerosulfate; S-DGD-5, sulfated diglycosyl diphytanylglycerol diether.

[a] Lipid band 3 was detected only in lipid extracts of BPG-enriched membranes.

plates for sugar, sulfate, and phosphate, indicating that it is a sulfo-glyco-phospholipid. ESI-MS (negative mode) analysis (shown in Fig. 5B) of the purified glycolipid showed $[M-H]^-$ and $[M-2H]^{2-}$ parent ions as well as some fragmentation ions. The main ion peak is the bischarged molecular ion $[M-2H]^{2-}$ at *m/z* 884.4, and the monocharged molecular ion is present at *m/z* 1770.7 along with $[M-2H-Na]^-$ at *m/z* 1792.7 as a minor peak. Fragmentation ions present at *m/z* 79.0 and 96.8 are diagnostic of a phosphate group ($[PO_3^-/H_2PO_4^-]$ ion pair); the less-intense ion peak at *m/z* 1690.5 is diagnostic for the loss of a labile sulfate group ($[M-SO_3]^-$ = 1770.7-80 = 1690.7). Also, the fragmentation ions at *m/z* 731, 1056, and 241 correspond to the archaeal analogue of PA, to S-DGD-5, and to a sugar phosphate or sulfate group, respectively.

The fragmentation pattern of the novel sulfo-glyco-phospholipid appears to be very similar to that of S-TGD-1-PA or GlyC, previously characterized in the PM isolated from *H. salinarum* (5), suggesting that band 3 consists of a sulfodiglycosyl-diphytanylglycerol esterified to the phosphate group of PA, i.e., an S-DGD-5-PA.

MS-MS analysis of purified band 3 (Fig. 5C) confirmed the proposed structure. To compare the sugars present in the novel lipid with those present in the major glycolipid of MdS1, we have analyzed isolated from MdS1 membranes NMR in parallel the sulfodiglycosyl-diphytanylglycerol by proton and band 3. The NMR spectrum of band 3 (**Fig. 6**, upper panel) in the region characteristic of glycosyl protons (3.2–5.1 ppm) was identical to that of S-DGD-5 (Fig. 6, lower panel). In particular, the two anomeric proton signals (doublets) present in the spectrum of band 3 had the same chemical shifts as those in the spectrum of S-DGD-5; these doublets, centered at ~4.95 and 4.68 ppm in both spectra, could be assigned to the anomeric protons of α-glucopyranosyl and α-mannopyranosyl residues. These data confirmed that the two glycolipids have the same sugars in the same sequence in the polar head, giving further support to the structure reported in **Fig. 7**. We conclude that the lipid called band 3 is a novel phospholipid dimer composed of S-DGD-5 and PA, S-DGD-5-PA.

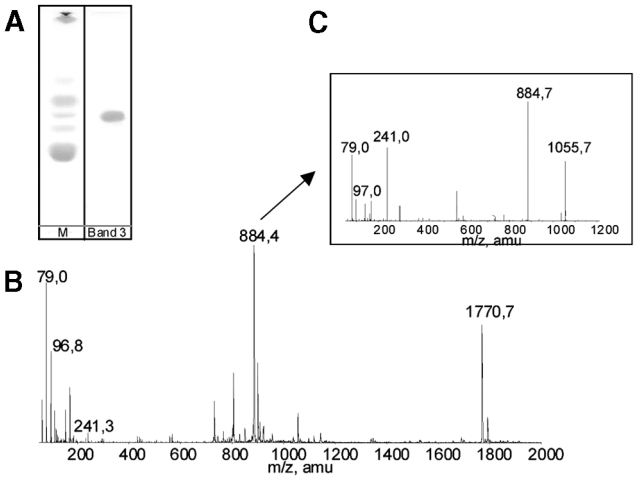

Fig. 5. TLC (A), negative ion ESI-MS (B), and MS-MS (C) spectra of isolated and purified band 3.

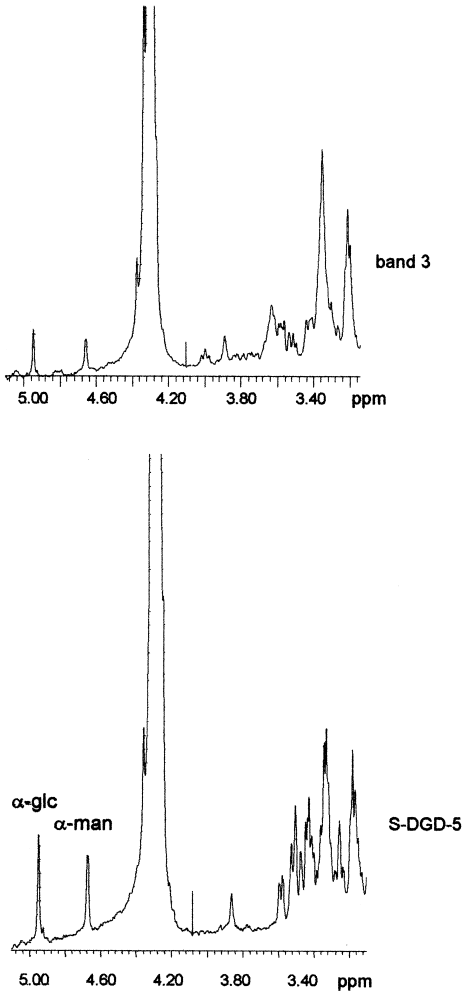

Fig. 6. ^1H NMR spectra of band 3 (upper panel) and S-DGD-5 (lower panel).

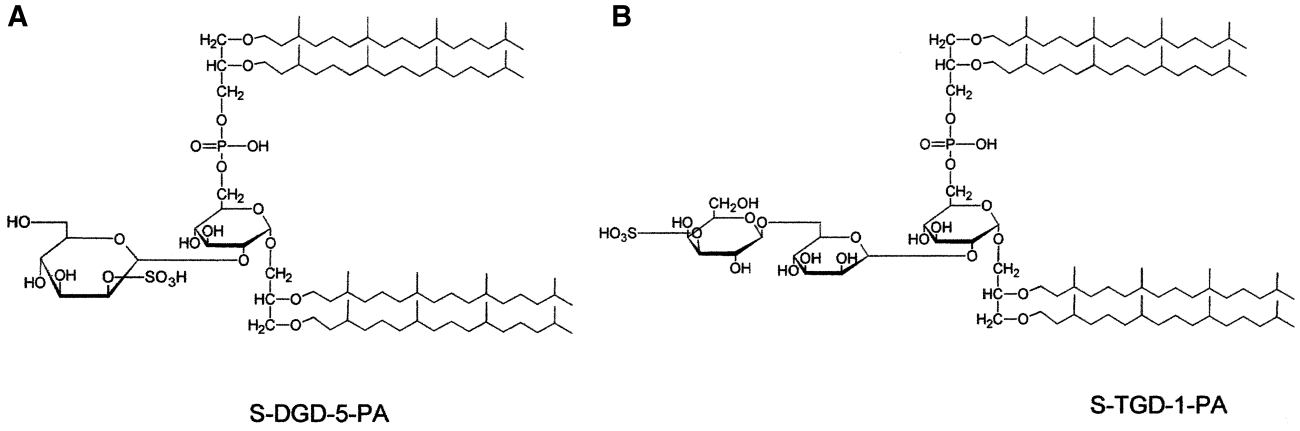

Fig. 7. Structures of S-DGD-5-phosphatidic acid (S-DGD-5-PA) (A) and sulfated triglycosyl diphytanylglycerol diether-1-PA (S-TGD-1-PA) of purple membrane of *H. salinarum* (B).

Together, these data reveal that the lipid composition of disrupted cells after dialysis and of membrane fractions isolated from these dialyzed cells are different from those of whole cells in isotonic medium because of the increased archaeal cardiolipin content and the appearance of the novel phospholipid dimer S-DGD-5-PA.

Finally, to test our hypothesis that it is the osmotic shock that induces de novo synthesis of the two cardiolipins in MdS1 cells, we analyzed total lipids extracted from cells mechanically disrupted by French press and from membranes isolated from these disrupted cells. The TLC results shown in **Fig. 8** demonstrate that the lipid profiles of cells disrupted by French press (lane C FP) and isolated membrane (lane M FP) were very similar to that of whole cells (lane C). In particular, the archaeal cardiolipin did not significantly increase in either the disrupted cell profile or the membrane profile, and no band 3 was present in the membrane isolated under these experimental conditions.

DISCUSSION

Osmotic stress is a common challenge encountered by many life forms. The behavior of the cell envelopes under different osmotic conditions has not been extensively explored. Nevertheless, several lines of evidence suggest that cell envelope composition plays an important role (14, 15).

Previous results demonstrated that in some microorganisms, the removal of the cell wall affects the composition of the plasma membrane (16). It also has been reported that important variations in membrane lipid composition occur in *Bacillus subtilis* during osmoadaptation; when the bacteria were grown in hypertonic medium, a decrease in PG and an increase in cardiolipin content were observed (17). Similar changes have been seen in other bacteria, such as *Escherichia coli* (18) and *Staphylococcus aureus* (14) cultured in hypersaline medium. Furthermore, an increase in the amount of glycolipids was observed in *B. subtilis* (17) and in *Saccharomyces cerevisiae* (15) growing in hypertonic NaCl medium.

Changes in lipid composition also occur when microorganisms living in high-salt medium are subjected to hypotonic stress. The archaeal GlyC is normally absent in *H. salinarum*, but relevant amounts of it were found in cells lysed by osmotic shock (9). Archaeal cardiolipin cell content also increased in the same cells upon osmotic shock, but this phenomenon could not be examined in detail because of the low archaeal cardiolipin cell content (9).

As archaeal cardiolipin-enriched membranes were found in an extremely halophilic microorganism of the genus *Halorubrum* (19), we thought that this microorganism could represent a good cellular system in which to investigate archaeal cardiolipin formation by osmotic shock.

In this study, we found that upon osmotic downshift, the cells of the halophilic microorganism *Halorubrum* sp. isolated from salterns of Margherita di Savoia (Italy) synthesize archaeal cardiolipin. We were able to quantitate the amount of synthesized archaeal cardiolipin by TLC video densitometry, and by comparing the quantities of various lipid components in the lipid extracts of swelling cells, we found that a decrease of PG occurs that corresponds to the archaeal cardiolipin increase.

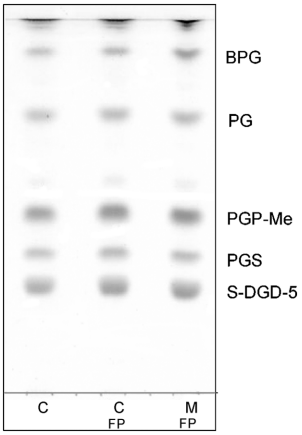

Fig. 8. TLC of lipid extracts from whole cells (lane C) disrupted by French press (lane C FP) and membranes isolated after French press disruption (lane M FP).

The fact that the archaeal cardiolipin increase did not occur in the lipid extracts of cells disrupted by French press and of membranes isolated from this cell lysate suggests that the formation of cardiolipin during dialysis is attributable specifically to osmotic shock.

The present report also demonstrates the presence of a novel phospholipid dimer in *Halorubrum* sp. and shows that a membrane fraction isolated from this archaeon after cell disruption by osmotic shock is highly enriched in both archaeal cardiolipin and the novel phospholipid.

ESI-MS and NMR analyses revealed that the novel phospholipid has the structure of a sulfo-glyco-diether-PA, namely S-DGD-5-PA. In fact, NMR analyses showed that the sugars in the novel phospholipid are the same and in the same order as those of the glycolipid S-DGD-5 isolated from the *Halorubrum* membranes, in which the sulfated mannose is linked to glucose.

We could not study the time course of S-DGD-5-PA synthesis because of its low content in MdS1 cells; on the other hand, the relatively high amount of S-DGD-5 does not easily allow the recording of small decreases.

We suggest that the change in phospholipid composition induced by osmotic shock is attributable to de novo synthesis of the two cardiolipins, BPG and S-DGD-5-PA, at the expense of PG and S-DGD-5, respectively.

Furthermore, S-DGD-5-PA formation can be detected only after cell disruption by osmotic shock, whereas the archaeal cardiolipin increase can be observed in intact swelling cells as well. Therefore, the two synthesis pathways of *Halorubrum* could be influenced and regulated by osmotic stimuli of different intensities, and the rate of synthesis of the two cardiolipins could be different.

The changes in the lipid composition of the representative of the genus *Halorubrum* described in the present study are analogous to those recently observed in other halophiles of the genus *Halobacterium* (9). Previously, we demonstrated that the phospholipid dimer S-TGD-1-PA is present in very low amounts in the cells of *H. salinarum* under physiological conditions and is synthesized de novo during cell lysis by osmotic shock at the expense of the glycolipid S-TGD-1.

Whether the cardiolipin analogue syntheses require specific enzymes it still not clear. Is a cardiolipin synthase active in the archaeal microorganisms? Are there other enzymes required to synthesize S-DGD-5-PA in *Halorubrum* and S-TGD-1-PA in *Halobacterium*? Further studies are required to address these questions.

At present, we cannot exclude the possibility that these phospholipid dimers could be chemically, rather than enzymatically, synthesized. Indeed, in our previous study on the S-TGD-1-PA of the PM of *H. salinarum* (9), we noted that in the PM, in which S-TGD-1-PA is abundant after osmotic shock, a specific enzyme to synthesize this lipid does not exist. It is well known that the only protein of the PM is BR and that no other proteins are present.

The *Halorubrum* cells used in the present study adapt to osmotic downshift better than a genetically engineered strain of *H. salinarum* cells characterized previously (9). Therefore, we hypothesize that higher archaeal cardiolipin cell content could protect the cell from lysis. The archaeal cardiolipin increase that occurs in cells undergoing osmotic shock may represent the physiological response of the microorganisms to low external osmolarity. The modifications in lipid composition could affect the physicochemical properties of the membrane such as bilayer thickness, membrane fluidity, and transport properties.

Recently, some types of mechanically gated (MG) channels have been identified in Archaea. These channels are implicated in myriad physiological processes and, like bacterial MG channels, are activated by mechanical force transmitted via the lipid bilayer (20).

Hoch (21) argues that the conversion of two PG molecules to cardiolipin plus glycerol increases the order on the membrane surface and that cardiolipin may be engaged in the regulation of ionic lateral conduction by the plasma membrane, because it is well known that the glycerol moiety of the cardiolipin molecule takes part in the lateral conduction of protons through H-bonded networks. The increase in cardiolipin concentration might serve as a barrier against the high ionic level or contribute to the active transport (14). In addition, the free glycerol, a well-known osmoprotectant, could contribute to the resistance of the cell to osmotic stress.

On the other hand, recent work on the ABC transport system of *Lactococcus lactis* showed that this system senses osmotic stress through alterations in membrane physicochemical properties. The electrostatic interactions between phospholipids and the transporter, in particular the polar lipid fraction, are essential to the osmosensing mechanism (22).

In summary, this work indicates that during the adaptation of cells to hypoosmolarity, the membrane undergoes significant biochemical and structural changes in terms of increased cardiolipin content. We are directing our research toward further clarification of the role of the two archaeal phospholipid dimers in the studied microorganism. In a preliminary study, we have shown that archaeal cardiolipin is tightly bound to a cytochrome c oxidase (19), whereas the function of S-DGD-5-PA is still unknown.

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