Osmotic shock induces the presence of glycocardiolipin in the purple membrane of *Halobacterium salinarum*

Simona Lobasso, Patrizia Lopalco, Veronica M. T. Lattanzio, and Angela Corcelli[1]

Dipartimento di Fisiologia Generale ed Ambientale and Dipartimento di Chimica, Università di Bari, via Amendola 165/a, 70126 Bari, Italy

Abstract In the purple membrane (PM) of *Halobacterium salinarum* is present a phospholipid dimer consisting of sulfo-triglycosyl-diether (S-TGD-1) esterified to the phosphate group of phosphatidic acid (PA), i.e., S-TGD-1-PA, called glycocardiolipin (GlyC) (Corcelli, A., M. Colella, G. Mascolo, F. P. Fanizzi, and M. Kates. A novel glycolipid and phospholipid in the purple membrane. 2000. *Biochemistry.* 39: 3318–3326). The GlyC content of whole cells, PM, and other cell fractions of *H. salinarum* have been analyzed. GlyC is a nonabundant phospholipid in *H. salinarum* cells, and it represents one of the major phospholipids of isolated PM. In this report, we show that *a*) GlyC is formed during the isolation of PM, *b*) GlyC increase in *H. salinarum* cells is specifically induced by osmotic shock, and *c*) in correspondence with GlyC increase, a decrease of S-TGD-1 levels occurs. The changes in membrane lipid composition observed during the isolation of PM are due to de novo synthesis of GlyC from S-TGD-1.—Lobasso S., P. Lopalco, V. M. T. Lattanzio, and A. Corcelli. **Osmotic shock induces the presence of glycocardiolipin in the purple membrane of *Halobacterium salinarum*. *J. Lipid. Res.* 2003.** 44: **2120–2126.**

Supplementary key words bacteriorhodopsin • cardiolipin • archaeal lipids

The finding of cardiolipin analogs in the extreme halophilic Archaea arises from an investigation into the characteristics of delipidated bacteriorhodopsin (BR), the photoactivated proton pump in the purple membrane (PM) of *Halobacterium salinarum* (1).

By analyzing lipids of delipidated BR fractions by electrospray ionization-mass spectrometry (ESI-MS), it was found that the composition of the residual lipids in delipidated BR is different from that of the PM in that the delipidated fractions have much higher proportions of two bicharged peaks at 760 *m/z* and 966 *m/z*, corresponding to two novel membrane lipids. This suggested that these novel lipids are more resistant to detergents than the other lipid components of PM, or, alternatively, that the novel lipids are more strongly bound to BR and may be involved in stabilizing the BR trimer structure.

The two novel PM lipids were isolated, purified by thin-layer chromatography (TLC), and analyzed by conventional analytical and spectroscopic methods to determine their chemical structure. Combining ESI-MS data and proton and phosphorus nuclear magnetic resonance (NMR) data of the purified novel lipids, together with the identification of their acid degradation products, allowed the elucidation of the chemical structures of the novel PM phospholipids (2). Their structures are, respectively, of a phosphosulfoglycolipid, 3-HSO_3-Gal*p*β1-6Man*p*α1-2Glc*p*α-1-1-[*sn*-2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol] (called glycocardiolipin, or GlyC), and a glyceroldiether analog of bisphosphatidylglycerol, *sn*-2,3-di-*O*-phytanyl-1-phosphoglycerol-3-phospho-*sn*-2,3-di-*O*-phytanylglycerol (archaeal BPG) (**Fig. 1**). Both novel lipids have two diphytanylglycerol moieties in their molecule, making their dimeric structures analogous to that of eukaryal cardiolipin.

Interestingly, GlyC consists of a sulfotriglycosyldiphytanylglycerol esterified to the phosphate group of phosphatidic acid (PA); in addition, its polar head group is composed of the same sugars in the same sequence and anomeric configuration as in sulfo-triglycosyl-diether-1 (S-TGD-1), the major membrane glycolipid of *Halobacterium*, namely βGal*p*→αMan→αGlc. Also, the sulfate group is located on C-3 of the galactose residue, as in S-TGD-1 (3). We will refer to it as S-TGD-1-PA (see structure in Fig. 1A) or GlyC, adopting a previously introduced abbreviation (4). The

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Abbreviations: BPG, archaeal analog of bisphosphatidylglycerol, or cardiolipin (diphytanylglycerol analog); 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; PGP-Me, phosphatidylglycerophosphate methyl ester (diphytanylglycerol ether analog); PM, purple membrane; S-TeGD-1, 3-HSO_3-Gal*p*-β1,6-Man*p*-α3,1-galactofuranose-α1,2-Glc*p*-α-*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.

[1] To whom correspondence should be addressed.
e-mail: a.corcelli@biologia.uniba.it

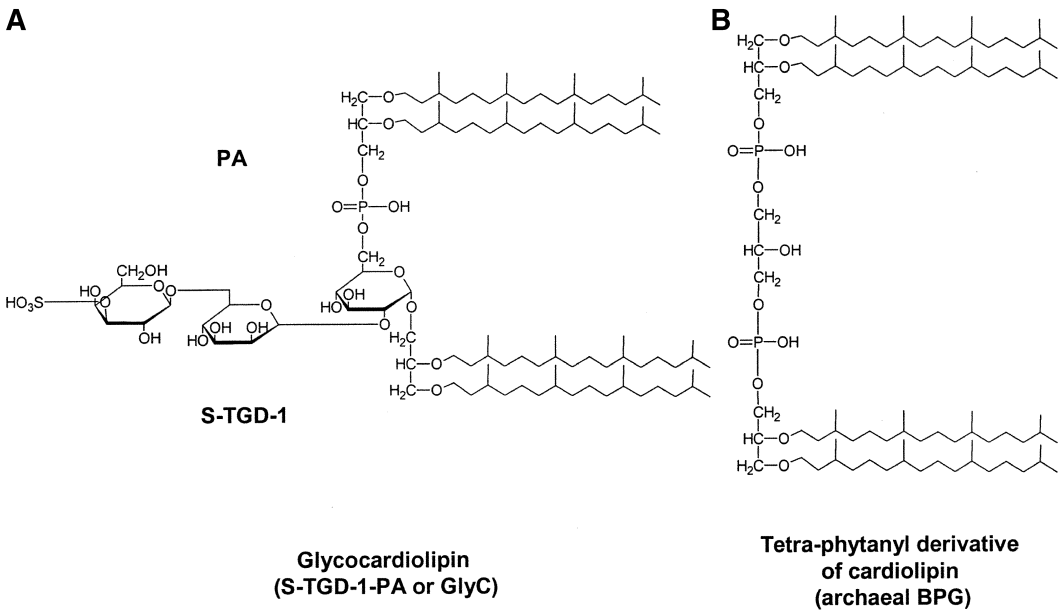

Fig. 1. Lipid structures of archaeal glycocardiolipin (A) and bisphosphatidylglycerol (B). PA, phosphatidic acid; S-TGD-1, sulfo-triglycosyl-diether-1.

cardiolipin/BR stoichiometries in the PM have also been estimated; one GlyC per BR is present in the PM, while BPG is only a minor component (4).

Here, by analyzing the distribution of GlyC in the various fractions obtained in the course of PM isolation, we show that GlyC is present in very low amounts in the cells of *H. salinarum* under physiological conditions, and that it is de novo synthesized and accumulated mainly in the PM during cell lysis by osmotic shock.

MATERIALS AND METHODS

Materials

DNase I was obtained from Sigma. All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). TLC plates (silica gel 60A), obtained from Merck, were washed twice with chloroform-methanol (1:1; v/v) and activated at 120°C before use.

Microorganism cultures

The engineered high-producing BR strain of *H. salinarum* used in this study was kindly provided by Richard Needleman (5); the *H. salinarum* NRC-1 strain was kindly provided by Aharon Oren. The *H. salinarum* cells were grown in light at 37°C in liquid growth medium containing neutralized peptone (L34, Oxoid) prepared as previously described (6).

PM isolation

PMs were isolated from the high-producing BR strain as previously described (6). Briefly, a dialysis bag (cutoff 12,000–14,000) was filled with a concentrated suspension of halobacterial cells in 4 M NaCl in the presence of DNase I and left in water under stirring at 4°C overnight; the purple lysate was centrifuged at 28,000 *g* for 40 min, and the colorless supernatant decanted. The purple pellet was resuspended in 0.1 M NaCl and again centrifuged; this was repeated two more times, and the last pellet (resuspended in distilled water) was layered over a step gradient (60%, 35%, and 15% sucrose) and then centrifuged at 100,000 *g* for 18 h at 10°C. The purple band was collected, and sucrose was removed by dialysis. Finally, the collected PMs were suspended in water and frozen (−20°C). During PM isolation, samples of various cellular fractions were stored to be extracted (whole cells, lysed cells after dialysis, supernatant after the first centrifugation, and PM).

Cell disruption by French press

A suspension of cells (in 4 M NaCl) was passed through a French pressure cell at 2,000 psi three times and left overnight at 4°C. To remove salt from cells broken by French press, an aliquot of a previous sample was dialyzed in the presence of DNase I against water at 4°C overnight.

Time course of GlyC increase during the dialysis

Cells suspended in 4 M NaCl were dialyzed against water at 4°C for different incubation times (15, 30, and 60 min and 12 h). At intervals, lipids were extracted from equivalent aliquots of the lysate by the standard procedure (see below). The weights of the lipid extracts from these aliquots were not significantly different.

Time course of GlyC increase in halobacterial cells diluted in low-salt medium

A suspension of high-producing BR or NRC-1 cells in 4 M NaCl was diluted 5-fold with a hypo-osmotic solution (0.1 M NaCl) and incubated at 25°C under stirring. At intervals, equivalent aliquots of suspension were removed for determination of osmotic fragility ($Abs_{700\ nm}$) and the lipid extraction.

Lipid extraction

Total lipids were extracted using the Bligh and Dyer method, as modified for extreme halophiles (7); the extracts were carefully dried under N_2 before weighing. Typically, before lipid extraction, whole cells were suspended in 4 M NaCl, while PMs were in water. In the case of the lipid extraction from PM high salt, 8 mg of PM in water was centrifuged (28,000 *g* for 40 min) and resuspended in 4 M NaCl (5 ml) before the extraction. For the experiment of lipid re-extract of cells, an aliquot of cell suspension (in 4 M NaCl) was extracted by the standard procedure; then, organic solvents (chloroform-methanol;Δ:3) were

again added to the denatured cellular material and incubated at 80°C for 30 min to obtain a re-extract; this procedure was repeated once more, and the two re-extracts were combined.

Lipid analyses

Total lipid extracts were analyzed by TLC on silica gel (20 cm × 10 cm, layer thickness 0.2 mm). Lipids were eluted with Solvent A (chloroform-methanol-90% acetic acid; 65:4:35) and unless otherwise specified, detected by spraying with sulfuric acid in water, followed by charring at 120°C for 45 min.

The quantitative analyses of GlyC content were performed by video densitometry, using the software ImageJ (http://rsb.info.nih.gov/ij). The lipid standard curves were linear in the concentration range of 1–10 μg. For ESI-MS analyses, dried samples of lipid extract were dissolved in chloroform-methanol (1:1). Electrospray mass spectra 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 a 7125 Rheodyne valve, 5 μl of sample into a 25 μl/min flow of chloroform-methanol delivered by a Harvard model 11 syringe pump (South Natick, MA). 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 amu; mass step, 0.1 amu; dwell time, 0.2 ms. With the orifice voltage used, CID-MS spectra were obtained showing [M-H]- and $[M\text{-}2H]^2$-parent ions as well as some fragmentation ions.

RESULTS

GlyC in whole cells and PM of *H. salinarum*

To investigate the location and distribution of GlyC in the *H. salinarum* cells, we studied the distribution of total cell lipids and GlyC in the course of cell fractionation. **Table 1** reports the total lipid content and the lipid/BR ratio of the various fractions obtained in the course of PM isolation by following the fractionation protocol of the *H. salinarum* cells, originally developed by Oesterhelt and Stoeckenius (6). We found that *a*) ~25% of lipid material was lost during the dialysis, *b*) 40% of lipids remained in the clear colorless supernatant of the first centrifugation, and *c*) PM lipids represented only ~30% of total cell lipids; at the same time, 70% of starting BR was typically recovered in PM after sucrose gradient.

Figure 2A shows the TLC of the lipid extracts of the whole cells of *H. salinarum*, of lysed cells after dialysis, of PM, and of other membrane small fragments present in the supernatant. It can be seen that GlyC is almost absent among lipids extracted from the whole cells, while it is present in lysed cells after dialysis against water, in PM, as well as in the cell material left in the first supernatant.

TABLE 1. Lipid recovery and lipid/BR ratio during PM isolation

	Lipid	Lipid/BR
	%	*w/w*
Cells	100	1
Lysed cells	75	0.8
Supernatant	40	nd
PM	30	0.4

BR, bacteriorhodopsin; nd, not determined; PM, purple membrane. As BR is absent in the supernatant, the lipid/BR ratio was nd.

Data illustrated in Fig. 2B show the negative-mode ESI-MS analyses of the lipid extract of whole cells and PM; in agreement with data in Fig. 2A, it can be seen that the diagnostic peak for GlyC at 966 *m/z* is absent in the ESI-MS spectrum of the whole-cell lipids, while it is much higher in the ESI-MS spectrum of PM lipids. The discrepancy in the cell GlyC content before and after the osmotic shock could depend on the fact that GlyC is not readily extractable from the whole cells without a prior treatment to disintegrate the cells.

Does efficiency of cardiolipin extraction depend on experimental conditions?

It has been previously reported that the efficiency of cardiolipin extraction from microorganisms may be significantly affected by the experimental conditions (8–10).

As cells of *H. salinarum* were usually suspended in an isotonic 4 M NaCl solution, while lysed cells after dialysis or PM were in medium containing very low or no salt, we examined the possibility that the low content of GlyC in the cell lipid extract could depend on a low efficiency of cardiolipin extraction from the cell membranes in the presence of high salt. Therefore, we have *a*) re-extracted and analyzed lipids from the denatured proteins remaining after the first lipid extraction of the cells resuspended in isotonic medium to check for the presence of GlyC among residual lipids, and *b*) checked if the lipid extraction of PM resuspended in 4 M NaCl yields a lipid extract having a lower amount of cardiolipins or is lacking them.

TLC analyses in **Fig. 3** show that GlyC is absent in the re-extract (Fig. 3A) and present in the extract from PM in high salt (Fig. 3B); the methanol-chloroform phase resulting after the cell re-extraction was dried, and lipids were dissolved in chloroform and analyzed by TLC; no GlyC was found in this last residual lipid fraction (data not shown).

Furthermore, the mechanical disintegration of the cells before lipid extraction, independent of the method used, could improve the recovery of GlyC in the lipid extract. Therefore, we have analyzed total lipids extracted from *H. salinarum* cells disrupted by French press. TLC in Fig. 3C shows that GlyC levels in the total lipid extract of cells disrupted by French press are very low and comparable with those in the extracts of the whole cells. Furthermore, disrupted cells after French press were dialyzed to low osmolarity, and lipids were extracted after complete salt removal. Figure 3D shows that GlyC did not significantly increase in these experimental conditions.

GlyC is formed during the isolation of PM

Data in Fig. 2 indicate that GlyC is a nonabundant phospholipid in the whole cells and is formed during the dialysis, when the cells are disrupted by osmotic shock. To study the time course of the GlyC increase during the dialysis, we have analyzed by TLC lipids of equivalent cell aliquots taken at different times from the starting of dialysis and estimated the amount of newly formed GlyC by video densitometry (**Fig. 4**). As the standard curves for the de-

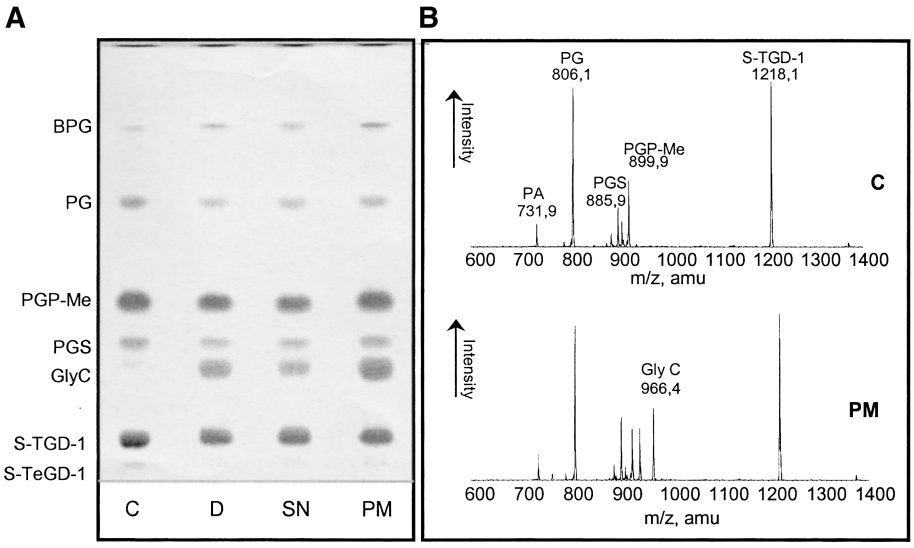

Fig. 2. Lipid analyses of different fractions collected in the course of purple membrane (PM) isolation. A: Thin-layer chromatography (TLC) of the lipid extracts of whole cells (C), lysed cells after dialysis (D), supernatant (SN) and PM; 60 μg of lipid extracts have been loaded onto each lane. The abbreviated names of individual lipid components in the extract have been reported. B: Electrospray ionization-mass spectrometry (−) spectra of the lipid extracts of whole cells (C) and PM.

termination by video densitometry of the amounts of individual phospholipids and glycolipids of PM are linear in the 1–10 μg range, to perform a quantitative analysis of the other major lipids present in the extracts before and after the dialysis, from 10 to 40 μg of lipid extracts were analyzed. By comparing the cell lipid profiles before and after the dialysis, it could be seen that in conjunction with the GlyC increase, S-TGD-1 and phosphatidylglycerophosphate methyl ester (PGP-Me) slightly decreased; data in **Fig. 5** illustrate the S-TGD-1 and PGP-Me decrease at the end of dialysis in five different experiments. At the end of the dialysis, the S-TGD-1/GlyC molar ratio was found to be 3 and, therefore, identical to that in isolated PM determined by NMR analyses (4). Finally, video densitometric analysis showed that at the end of dialysis, the minor PM lipid component BPG also increases (Fig. 4A).

Osmotic fragility and GlyC synthesis

To shed light on the changes in lipid composition during the dialysis and to rule out the possibility that they could be the consequence of some loss of lipids through the dialysis membrane, in further experiments we have avoided the use of the dialysis bag and studied the effect of osmotic shock on the lipid composition of *H. salinarum* by directly diluting cells in low-salt medium. Two different *H. salinarum* strains, high-producing BR and NRC-1, were used in the following experiments.

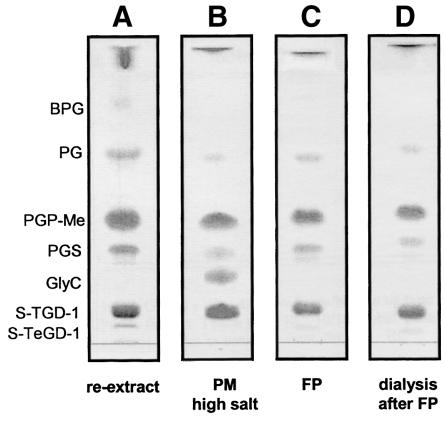

Fig. 3. A: TLC of lipid re-extract of cells in 4 M NaCl. B: Lipid extract of PM suspended in 4 M NaCl (PM high salt); lipids from cells disrupted by French press before (C) and after dialysis (D). Sixty micrograms of lipid extracts have been loaded onto each lane (details in Materials and Methods section).

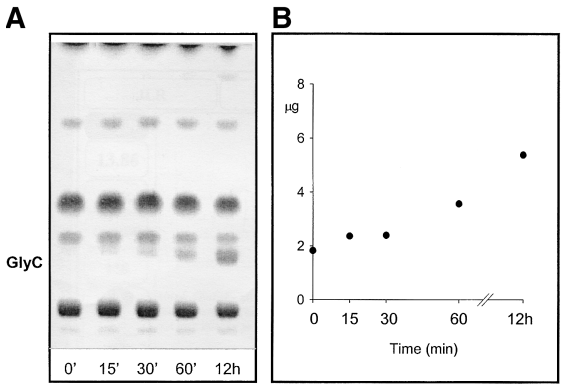

Fig. 4. GlyC increase during dialysis of high-producing bacteriorhodopsin (BR) *Halobacterium salinarum* cells. A: TLC of lipids extracted from equivalent aliquots of cells undergoing dialysis at different incubation times. Eighty micrograms of lipid extracts have been loaded onto each lane. B: Time course of the rise in GlyC content during the dialysis. The GlyC content at the different incubation times has been estimated by video densitometry (details in Materials and Methods section).

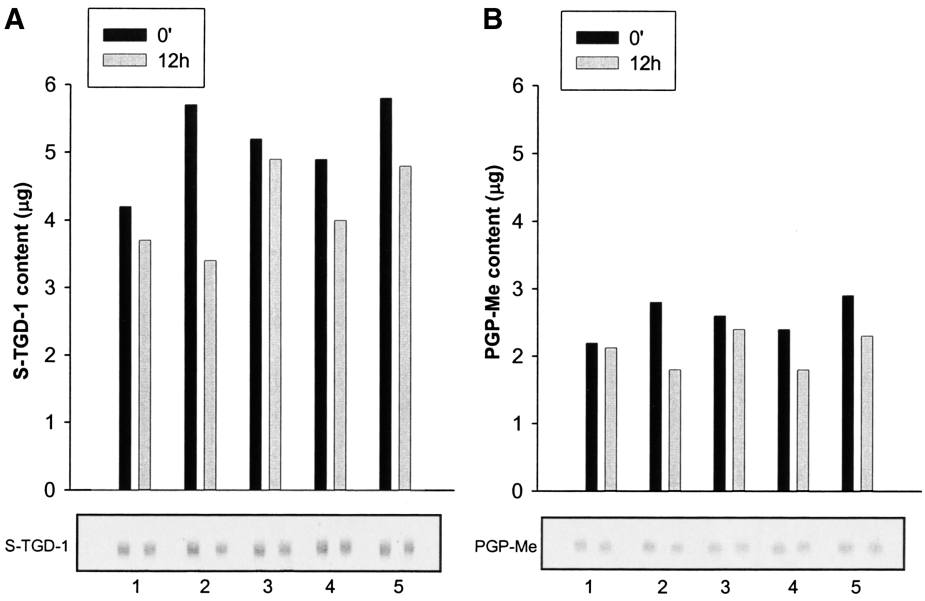

Fig. 5. S-TGD-1 and phosphatidylglycerophosphate methyl ester (PGP-Me) content in high-producing BR *H. salinarum* cells before and after the dialysis. Fifteen micrograms of lipid extracts of cells before and after dialysis have been loaded on the plate (TLC). The bar graphs illustrate the results of video densitometric analyses of S-TGD-1 and PGP-Me content before (0 minutes) and after (12 h) the dialysis in five experiments (1–5).

In order to follow and quantitate the changes in GlyC and possibly other membrane lipids in samples of swelling and disrupting cells, lipids had been extracted from equivalent aliquots of cells taken at different time intervals from the start of the hypotonic shock. During the osmotic shock, the total cell lipid content and the lipid/BR ratio did not significantly change. **Figure 6A** shows the time course of GlyC increase during the osmotic shock constructed by video densitometry, together with the plot of osmotic fragility of high-producing BR halobacterial cells. Three hours after the osmotic shock, ~2 μg (i.e., 1 nmol) of GlyC over 40 μg of total lipids were newly formed in the cells (Fig. 6A), and the S-TGD-1/GlyC molar ratio was equal to 6 (data not shown).

The lipid profiles of the extracts of NRC-1 cell aliquots taken at different time intervals after dilution in low-salt medium showed not only an increase in the GlyC content during the osmotic shock but also a clear increase of BPG (data not shown). The increase of BPG could not be easily detected in high-producing BR cells because of the very low content of BPG.

After 3 h of cell incubation in hypotonic medium, 3.8 μg of GlyC over 20 μg of total lipid extract were formed in NRC-1 cells; the final S-TGD-1/GlyC molar ratio was found to be ~3. The amount of newly formed GlyC is, therefore, higher in NRC-1 cells than in high-producing BR cells under the same experimental conditions.

Finally, by reducing the amount of extracts loaded on the plate and the time of charring, we were able to find the experimental conditions to show, by video densitometric analyses, that during the osmotic shock, in conjunction with GlyC increase, a decrease in S-TGD-1 content occurred in NRC-1 cells exposed to osmotic shock (Fig. 6B). The decrease in S-TGD-1 could be detected only in the NRC-1 strain, as in these cells the amount of newly formed GlyC is higher, and the final S-TGD-1/GlyC ratio is lower than in the high-producing BR halobacterial cells. In addition, as previously found in the experiment shown in Fig. 5, video densitometric analysis revealed that PGP-Me also decreases during the osmotic shock (data not shown).

DISCUSSION

Cardiolipin is well known as a characteristic phospholipid of the inner mitochondrial membrane of eukaryotic cells. From a structural point of view, this tetra-acyl phospholipid can be considered a phospholipid dimer. The role of cardiolipin in the maintenance of optimal activity of cytochrome c oxidase, as well as other enzymes catalyzing oxidative phosphorylation, has been widely documented (11–13). Cardiolipin is also present in bacterial membranes in relation to the function of generating an electrochemical potential for substrate transport and ATP synthesis; recent crystallographic studies have shown that a cardiolipin molecule is located on the intramembrane surface of the reaction center from *Rhodobacter sphaeroides* (14). In contrast with mammals containing only "authentic" cardiolipin, bacteria contain a number of structural analogs of cardiolipin, for example, D-glucopyranosylcardiolipin in *Streptococci* sp. (15) and D-alanylcardiolipin in *Vagococci* sp. (16).

An archaeal bisphosphatidilglycerol or archaeal cardiolipin (BPG) and a glycocardiolipin (GlyC) have been found among residual lipids associated with delipidated BR fractions isolated from an engineered strain of *H. salinarum* (1) in the natural halophilic community inhabiting

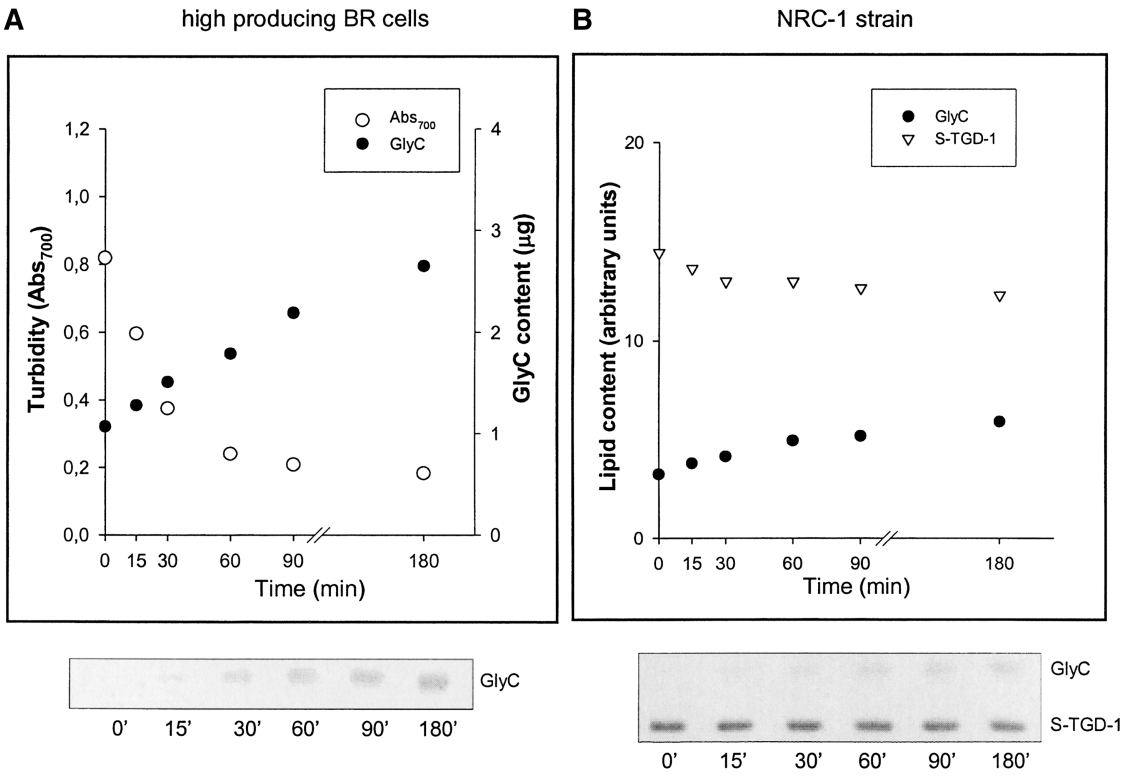

Fig. 6. Changes of lipid composition of high-producing BR (A) and NRC-1 (B) *H. salinarum* cells after dilution in low-salt medium. A: The time course of the changes in GlyC content (µg) and in turbidity ($Abs_{700\ nm}$) occurring in swelling and disrupted cells after dilution. Values of GlyC content (in µg) on the *y* axis have been estimated by video densitometry. Lipids were extracted from equivalent cell aliquots taken at different time intervals from the dilution, and 40 µg of the different extracts have been loaded on the plate. B: The time course of changes in GlyC and S-TGD-1 cellular contents after dilution, estimated by video densitometric analyses. Lipids were extracted from cells undergoing hypotonic shock at different incubation times. Twenty micrograms of different extracts have been loaded on the plate stained by spraying with H_2SO_4 followed by incubation at 120°C for 13 min.

the crystallizer brines of salterns (17), in a number of isolates from the saltern ponds of Margherita di Savoia (Italy), and in validated strains available from culture collections (17). While GlyC is only present in species producing PMs, BPG is ubiquitous in all examined members of *Halobacteriaceae*, suggesting additional roles for it in the extremely halophilic Archaea. Evidence for the association of archaeal BPG with cytochrome c oxidase in an archaeal microorganism has been recently shown (18); therefore, BPG might be only a contaminant of PM.

Very little is known about the biosynthetic pathways of cardiolipins in Archaea. Presumably, the route of archaeal cardiolipin biosynthesis passes through the common intermediates of other archaeal phospholipid pathways, while it is not known whether the final step involves a cardiolipin synthase of prokaryotic or eukaryal type. Present data show for the first time that the cardiolipin synthesis is stimulated by osmotic shock in the Archaea.

This study focuses on the GlyC of the PM of *H. salinarum*. As GlyC is highly enriched in PM compared with the whole cells, we previously suggested that GlyC is located only in the PM and not in other portions of the cellular membranes of *H. salinarum* (2). In this study, we have shown that the content of the archaeal cardiolipin GlyC increased in the extreme halophilic microorganism *H. salinarum* after osmotic shock, and that the newly formed GlyC is mainly accumulated in the PM. In other words, GlyC is normally absent in the PM patches of *H. salinarum*, and its presence in isolated PM is artificially induced by osmotic shock during the isolation procedure.

The fact that GlyC is not present in the lipid extract of cells disrupted by French press suggests that the formation of cardiolipin during the dialysis is specifically due to osmotic shock and that it is not the consequence of the cell disruption, independent of the method used to lyse the cells. Salt removal, after mechanical cell disruption, did not induce a significant increase of GlyC level, indicating that lowering salt does not per se increase GlyC levels, and that the integrity of the cell is required to efficiently synthesize GlyC. The formation of GlyC (and BPG) clearly occurs in cells undergoing osmotic shock before cell lysis and may represent the physiological response of the microorganisms to low external osmolarity. Whether the GlyC synthesis in *H. salinarum* requires a specific enzyme still is not clear; it cannot be excluded that GlyC might be chemically synthesized during PM isolation rather than by an enzymatic activity, also because it is well known that the sole protein of the large PM patches is BR, and no other enzymatic proteins are present in PM.

We have been able to quantitate the amount of newly synthesized GlyC in *H. salinarum* cells by TLC video densitometry. Furthermore, by comparing the quantities of var-

ious lipid components in the lipid extracts of swelling and disrupted cells, we have found that in conjunction with the GlyC increase, a decrease of S-TGD-1 occurs.

As the glycolipid S-TGD-1 has the same sugars in the same order as GlyC in the polar head, it can be suggested that the glycolipid S-TGD-1 is one of the precursors for the last step of the biosynthesis of GlyC. PGP-Me could represent a form of activated PA involved in the cardiolipin synthesis, but it remains to be ascertained whether its decrease during osmotic shock is correlated with GlyC synthesis.

Data reported in this study show that the other minor archaeal cardiolipin BPG also increased in *H. salinarum* cells after osmotic shock; we have not analyzed this phenomenon in detail, because the stimulation of BPG by osmotic shock is more evident and easier to study in other representatives of archaeal extreme halophiles having a higher content of BPG. The presence of BPG-enriched membranes in an extremely halophilic microorganism of the *Halorubrum* genus has been described in a preliminary report (18), and we are currently investigating the BPG formation in the presence of an osmotic shock.

The increase of cardiolipin content in microorganisms due to osmotic shock has already been reported in the literature (19, 20). In *Staphylococcus aureus* it has been demonstrated that the de novo synthesis of cardiolipin occurs during the preparation of autoplast by osmotic shock (19). It has been suggested that in *Staphylococcus*, the loss of cellular wall induced by osmotic shock could stimulate the cardiolipin synthesis to increase the buffer ability of the membrane (20). The present study shows that a specific link exists between the osmotic fragility and the de novo synthesis of cardiolipin in halobacterial cells.

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