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Salt Tolerance of Archaeal Extremely Halophilic Lipid Membranes*

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The membranes of extremely halophilic Archaea are characterized by the abundance of a diacidic phospholipid, archaetidylglycerol methylphosphate (PGP-Me), which accounts for 50–80 mol% of the polar lipids, and by the absence of phospholipids with choline, ethanolamine, inositol, and serine head groups. These membranes are stable in concentrated 3–5 M NaCl solutions, whereas membranes of non-halophilic Archaea, which do not contain PGP-Me, are unstable and leaky under such conditions. By x-ray diffraction and vesicle permeability measurements, we demonstrate that PGP-Me contributes in an essential way to membrane stability in hypersaline environments. Large unilamellar vesicles (LUV) prepared from the polar lipids of extreme halophiles, *Halobacterium halobium* and *Halobacterium salinarum*, retain entrapped carboxyfluorescein and resist aggregation in the whole range 0–4 M NaCl, similarly to LUV prepared from purified PGP-Me. By contrast, LUV made of polar lipid extracts from moderately halophilic and non-halophilic Archaea (*Methanococcus jannaschii*, *Methanosarcina mazei*, *Methanobrevibacter smithii*) are leaky and aggregate at high salt concentrations. However, adding PGP-Me to *M. mazei* lipids results in gradual enhancement of LUV stability, correlating with the PGP-Me content. The LUV data are substantiated by the x-ray results, which show that *H. halobium* and *M. mazei* lipids have dissimilar phase behavior and form different structures at high NaCl concentrations. *H. halobium* lipids maintain an expanded lamellar structure with spacing of 8.5–9 nm, which is stable up to at least 100 °C in 2 M NaCl and up to ~60 °C in 4 M NaCl. However, *M. mazei* lipids form non-lamellar structures, represented by the Pn3m cubic phase and the inverted hexagonal H_{II} phase. From these data, the forces preventing membrane aggregation in halophilic Archaea appear to be steric repulsion, because of the large head group of PGP-Me, or possibly out-of-plane bilayer undulations, rather than electrostatic repulsion attributed to the doubly charged PGP-Me head group.

Microbial growth occurs over a wide range of salt concentrations spanning the whole range from fresh water environments to NaCl concentrations of 3–5 M (1–4). To survive under conditions of high salinity, halophilic and halotolerant microorganisms have developed specific cell and cell membrane adaptations (5). Eubacteria and other halotolerant organisms use organic solutes from the surroundings or synthesize compatible osmolytes to balance the high external osmotic pressure (1, 6). Extremely halophilic Archaea maintain osmotic balance by accumulating salts through osmoregulation (4) and have adapted their membranes to have low proton and sodium permeability at high salt concentrations (7). The membrane destabilizing effects caused by NaCl may be compensated for by a shift in lipid composition; for example, in *Vibrio costicola*, by a decrease in non-bilayer-forming phosphatidylethanolamine and a corresponding increase in phosphatidylglycerol (8).

Halophilic and halotolerant microorganisms exist in all three domains of life: *Archaea*, *Bacteria*, and *Eukarya* (1, 4, 9). However, the membrane lipids of the organisms in the different domains differ significantly in structure and composition. Archaeal lipids are characterized by ether linkages and isoprenoid chains, mainly phytanyl and bisphytanediyl, in contrast to the ester linkages and straight fatty acyl chains of non-Archaea (10–13). Noteworthy, archaeal lipids have an *sn*-2,3 enantiomeric configuration, opposite to the *sn*-1,2 configuration of the glycerophospholipids from the other domains (10). With respect to their composition, the membranes of extremely halophilic Archaea have several unique characteristics that vary little within specific genera (14). Phospholipids with ethanolamine, inositol, and serine head groups are generally absent and specific phosphatidylglycerol phospholipids, sometimes including several sulfated glycolipids, predominate. Most notably, extreme halophiles contain a major, ubiquitous phospholipid, archaetidylglycerol[3] methylphosphate (PGP-Me),[4] which is an archaeal analogue of phosphatidylglycerol methylphosphate (Ref. 15 and Fig. 1). PGP-Me accounts for 50–80% of the polar membrane lipids of extreme halophiles but is absent and replaced by other lipids in moderate halophiles and non-halophiles (Table 1). For example, in the moderate halophile *Methanosarcina mazei*, which contains no PGP-Me, the phosphoinositol phospholipids are most abundant (16). The extreme halophiles also contain archaetidylglycerol (PG), and some strains have minor amounts of sulfated PG (10).

Because of its abundance in extreme archaeal halophiles, the diacidic phospholipid PGP-Me can be expected to play a major role in determining the properties of their membranes. It is believed that it contributes to membrane stability in hypersaline environments (10); however, it has neither been tested nor is its mechanism understood. To elucidate the role of PGP-Me, we performed a comparative study on the stability of large unilamellar vesicles (LUV) made of polar lipid extracts from several archaeal strains including extreme halophiles, moderate halophiles, and non-halophiles, which strongly differ by PGP-Me content (Table 1). Both measures used (leakage of entrapped carboxyfluorescein (CF) and LUV aggregation) demonstrated that the LUV stability at high salt con-

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[3] Archaetidylglycerol denotes archaeal phosphatidylglycerol with ether-linked isoprenoid chains (Fig. 1).
[4] The abbreviations used are: PGP-Me, archaetidylglycerol methylphosphate; PG, phosphatidylglycerol or archaetidylglycerol; PGP, archaetidylglycerol phosphate; PC, phosphatidylcholine; LUV, large unilamellar vesicles; CF, 5(6)-carboxyfluorescein; OG, *n*-octyl β-D-glucopyranoside; FAB-MS, fast atom bombardment mass spectrometry.

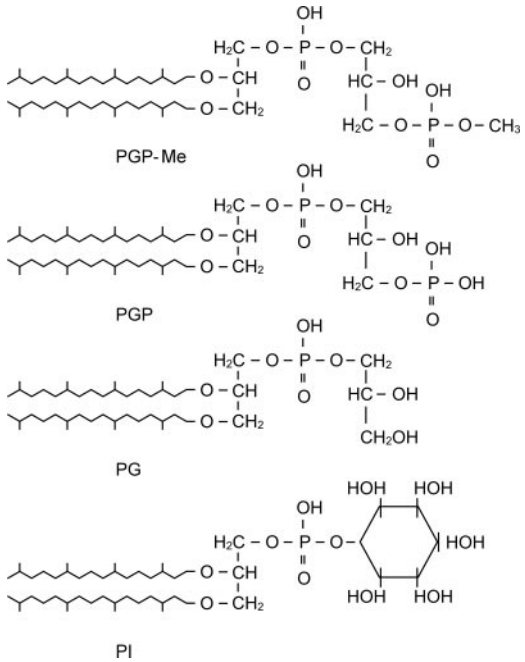

FIGURE 1. **Structure of PGP-Me, PGP, PG, and PI (15, 32, 33).**

TABLE 1
Content of the phospholipids PGP-Me and PG in Archaea used in this study

Organism	Halophilicity	PGP-Me	PG
		% w/w[a]	
H. halobium	Extreme	78 ± 3	9 ± 4
H. salinarum	Extreme	62 ± 5	23 ± 4
N. magadii	Extreme	36 ± 14	32 ± 13
		21 ± 4 (20,25)[b]	11 ± 6 (20,25)[b]
H. volcanii	Extreme	43 ± 2; 44 ± 3[c]	35 ± 2[c]
H. minutum	Extreme	61 ± 3	8 ± 2
		14 ± 5[b]	3 ± 2[b]
Halococcus morrhuae 14039	Extreme	50 ± 2	15 ± 1
M. mazei	Moderate	0	19 ± 10[d]
M. jannaschii	Moderate	0	0
M. smithii	Slight	0	0
Thermoplasma acidophilum	Slight	0	Minor

[a] % w/w of total polar lipids.
[b] PGP-Me and PG with C20-C25 alkyl chains, instead of the usual C20-C20 chains.
[c] Ref. 18.
[d] Ref. 34.

centrations correlates with the PGP-Me content of the membranes. These data were substantiated by low angle x-ray diffraction measurements, which showed that the polar lipids from *Halobacterium halobium* (extreme halophile) and *M. mazei* (moderate halophile) have dissimilar phase behavior and form different structures at high salt concentrations. *H. halobium* lipids, typified by a very high PGP-Me content of ~78% (Table 1), maintained an expanded lamellar structure stable up to least 4 M NaCl in broad temperature ranges about the physiological temperatures, whereas *M. mazei* lipids, which have no PGP-Me, formed non-lamellar structures, represented by the Pn3m cubic phase and the inverted hexagonal H_{II} phase, at high NaCl concentrations. In this way, both the LUV and the x-ray diffraction data sets consistently demonstrate a critical role of PGP-Me for the bilayer stabilization in hypersaline environments.

EXPERIMENTAL PROCEDURES

Lipid Isolation and Composition—The Archaea used in this study are shown in Table 1. The source of cultures and growth conditions were as reported previously (17–19). Lipids were extracted from freeze-thaw cell pastes by the Bligh and Dyer method using methanol/chloroform/water (2:1:0.8, v/v), and total polar lipids were purified from neutral lipids by acetone precipitation and washing (20). The total polar lipid extracts were then analyzed by negative ion fast atom bombardment mass spectrometry (FAB-MS), and the analysis of ions obtained was consistent with that reported earlier (17, 18). The m/z values from negative ion FAB-MS spectra were compared from batch to batch to confirm that similar proportions of core lipids were present in each lipid mixture extracted from fresh biomass (12). The amounts of PGP-Me and PG in the different organisms are given in Table 1. PGP-Me and PG were estimated from relative signal heights of lipid signals in negative ion FAB-MS spectra of total polar lipids ($n = 3$). PGP-Me was taken as the composite of signals of m/z 899, 899+sodium and 885. The relatively small 885 signal represents the composite of PGP fragment (loss of Me) of PGP-Me and PG-sulfate present as a minor lipid in *Halobacterium* species (10). Branched chain 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine and 1,2-diphytanoyl-*sn*-glycero-3-phosphoglycerol, were purchased from Avanti Polar Lipids, Inc.

Purification of PGP-Me and PG—Total polar lipids of *Haloferax volcanii* were separated by thin layer chromatography on Silica gel 60 glass plates using chloroform/methanol/acetic acid/water (85:22.5:10:4, v/v). PGP-Me and PG lipid bands were detected and recovered as described (18). Negative ion FAB-MS established a purity of >98% for both PGP-Me (m/z 899.5) and PG (m/z 805.3).

Preparation of LUV—LUV were prepared by extrusion. Lipids dissolved in chloroform/methanol (2:1) were dried under a stream of nitrogen, and placed under vacuum for 1 h. 4 mg of lipid were hydrated in 20 mM HEPES, pH 7.4, containing 20 mM 5,6-CF and 500 mM NaCl. They were probe-sonicated using Virsonic 60 sonicator for 15 s at 4-watt RMS and frozen at −20 °C overnight. After thawing to room temperature, the suspensions were probe-sonicated for 30 s in two 15-s bursts (4-watt RMS) and extruded through a 0.2-μm polycarbonate filter (20 passes) using an Avanti mini-extruder assembly (Avanti Polar Lipids Inc). LUV were sized by quasi-elastic light scattering using a DynaPro LSR particle size analyzer. Non-entrapped CF was removed by passing the dispersions through Sephadex PD-10 columns (21).

LUV Permeability—Leakage of CF from LUV was used as a measure of the bilayer integrity. Because entrapped CF is self-quenched, its leakage results in a fluorescence increase recorded using an SLM-Aminco 500C spectrofluorometer. In a typical experiment, 25–40-μl volumes of LUV containing 20 mM CF were dispensed into 2.5 ml of hypertonic solutions of 4 M NaCl. Leakage measurements were also made with LUV dispensed into 4 M KCl, 4 M sorbitol, and 4 M glycerol solutions. The LUV leakage was measured as the CF fluorescence increase over a period of 10 min divided by the maximum possible CF fluorescence. To obtain the maximum CF release, LUV were lysed after the initial 10 min by addition of 0.5% *n*-octyl β-D-glucopyranoside (OG) or by sonication of the sample (Fig. 2).

LUV Aggregation—For salt-induced aggregation studies, LUV were prepared as described above in phosphate-buffered saline (10 mM sodium phosphate, pH 7.1, 0.16 M NaCl). Aliquots of LUV diluted with phosphate-buffered saline containing various NaCl concentrations were examined by phase contrast microscopy to confirm aggregation. Average diameters were determined with a particle sizer (Nicomp 270) following 30-min incubations at 25 °C after dilution (Table 2).

X-Ray Sample Preparation and Measurements—Polar lipid extracts from *H. halobium* and *M. mazei* dissolved in chloroform were used. Chloroform was removed with a stream of argon, and the lipids were dried under high vacuum for 24 h. Lipid dispersions with 10% (w/v) lipid

content were prepared by hydration and vortexing at room temperature, followed by 5 freeze-thaw cycles between dry ice and room temperature, accompanied by vortexing during the thawing steps. The dispersion media used were 20 mM HEPES, pH 7.2, containing 0, 2, or 4 M NaCl, respectively. The dispersions were stored at 4 °C for ~1 day and equilibrated at room temperature for several hours prior to the x-ray measurements. The samples were vortexed again and loaded into x-ray capillaries immediately before their measurement. *H. halobium* samples at all NaCl concentrations and also *M. mazei* samples at 0 M NaCl remained with time visually completely homogeneous. In contrast, *M. mazei* samples at 2 and 4 M NaCl tended to segregate spontaneously into lipid-dense portions and lipid-free solution. X-ray patterns were recorded from the lipid-dense portions in the latter two dispersions. Use of a "cold" freeze-thaw procedure for preparation of the dispersions eliminated effects of higher temperatures on their initial structure. In this way, we were able to distinguish between "initial" states of the dispersions that were not heated above room temperature, and their states assumed after heating-cooling temperature scans. Because of beam time limitations, such scans were performed at a relatively fast rate of 10 °C/min. Thus, the phase transition temperatures referred to further in the text may be higher than the equilibrium transition midpoints, which can only be obtained using very low, quasistatic scan rates. This circumstance, however, does not affect in any substantial way the conclusions derived from the x-ray data.

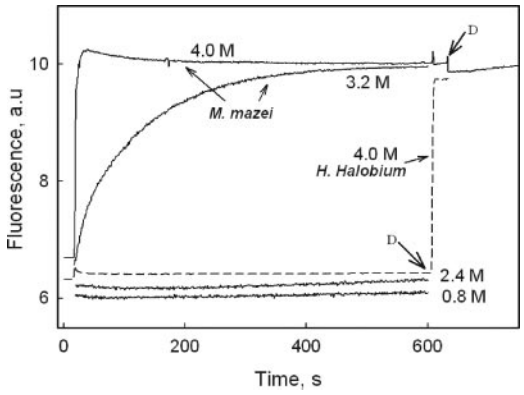

FIGURE 2. **Salt-induced leakage of LUV.** Time course of CF leakage from LUV at different NaCl concentrations for the extreme halophile *H. halobium* (*dashed line*) and for the moderate halophile *M. mazei* (*solid lines*, 0.8, 2.4, 3.2, 4.0 M NaCl). The *arrows* with *D* indicate addition of detergent (0.5% OG) or sonication of the samples applied to achieve maximum fluorescence levels.

Low angle x-ray patterns were recorded at stations 18D, BioCAT and 5IDD, DND-CAT, APS, Argonne, using two-dimensional 2048 × 2048 MAR detectors at a sample-to-detector distance of ~200 cm. Spacings were determined from axially integrated two-dimensional images using the FIT2D program and silver behenate as calibration standard. A temperature-controlled capillary sample holder was used. All measurements were started at 20 °C, and temperature scans in the range 0–100 °C were performed at a rate of 10 °C/min. The sample holder was mounted on a motorized stage, and by moving it with respect to the incident beam it was possible to ensure that the patterns recorded were representative of the whole sample volumes.

RESULTS

LUV Properties at High NaCl Concentrations—LUV represent a convenient experimental model frequently used to test the effect of various factors on the membrane stability and integrity. Because our aim was to elucidate the effect of PGP-Me on the membrane stability, we compared the properties of LUV made of polar lipid extracts from extremely halophilic, moderately halophilic, and non-halophilic Archaea, which strongly differ by PGP-Me content. As an important reference point, we also studied the properties of LUV prepared from purified PGP-Me.

The procedure applied for LUV preparation resulted in dispersions of stable LUV, which did not release entrapped CF, regardless of their lipid composition. Leakage of CF was observed, however, upon dilution of these dispersions with concentrated NaCl solutions. In a typical experiment, a 25–40-μl volume of the initial LUV dispersion was dispensed into 2.5 ml of concentrated NaCl solution, and the course of CF leakage was monitored by recording the fluorescence increase with time. These measurements revealed large differences among LUV prepared from the polar lipids of different archaeal strains. For example, LUV from the total polar lipids of the extreme halophile *H. halobium* remain stable and show minimal CF leakage even at the highest 4 M NaCl concentration. In contrast, LUV prepared from the polar lipids of the moderate halophile *M. mazei* are stable at low NaCl concentrations only and show increasing rates of CF release with increases in the NaCl concentration (Fig. 2). Results of CF release measurements in 4 M NaCl for several archaeal strains are summarized in Fig. 3. Highest LUV stability (lowest CF release) was exhibited by lipids from the extreme halophiles *H. halobium* and *Halobacterium salinarum*, which also have the highest PGP-Me content. Polar lipids from moderately halophilic and non-halophilic strains, which have no PGP-Me, display lowest stability (highest CF release). The extreme halophiles *H. volcanii* and *Halobacterium minutum* are typified by intermediate CF leakage values, with

TABLE 2

LUV size (nm) and stability with increase of the external NaCl concentration

LUV were prepared in 0.16 M NaCl, and their mean diameters ± S.D. (nm) were measured after 30 min of incubation following dilution into higher NaCl concentrations.

Lipid kind	0.16 M NaCl	2 M NaCl	3 M NaCl	4 M NaCl
H. halobium[a]	148 ± 107	125 ± 101	141 ± 106	166 ± 114
H. salinarum	125 ± 58	159 ± 58	134 ± 66	251 ± 106
N. magadii	68 ± 39	117 ± 62	98 ± 56	174 ± 81
H. volcanii	78 ± 55	87 ± 64	130 ± 68	160 ± 88
H. minutum	77 ± 39	96 ± 49	96 ± 51	82 ± 51
H. morrhuae	116 ± 55	129 ± 67	171 ± 73	132 ± 75
M. mazei	122 ± 60	—[b]	—[b]	—[b]
M. jannaschii	184 ± 150	—[b]	—[b]	—[b]
M. smithii	234 ± 121	—[b]	—[b]	—[b]
T. acidophilum	149 ± 113	—[b]	—[b]	—[b]
Archaeal PGP-Me	80 ± 62	126 ± 97	ND[c]	116 ± 92
Archaeal PG	55 ± 34	273 ± 195	—[b]	—[b]
Diphytanoyl PG	158 ± 96	682 ± 501	—[b]	—[b]
Diphytanoyl PC	250 ± 185	205 ± 149	194 ± 132	288 ± 185

[a] Average diameter of 188 ± 89 nm following dilution in 5 M NaCl.
[b] Vesicle aggregation.
[c] ND, not determined.

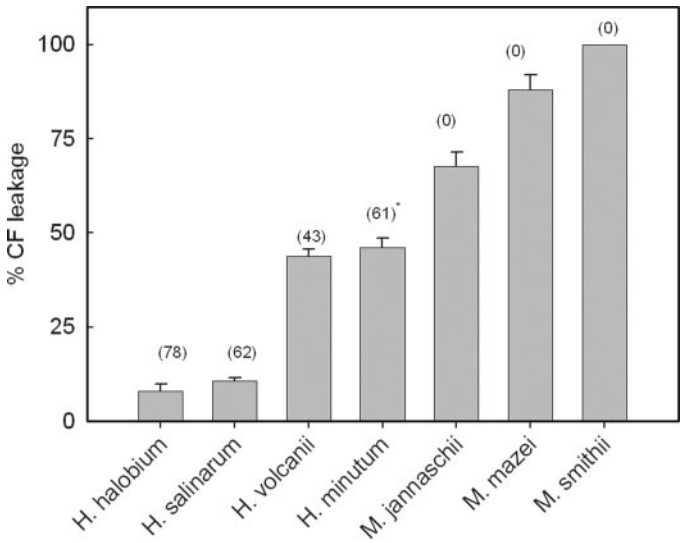

FIGURE 3. **Stability of different Archaea in hypersaline solution.** CF leakage from LUV prepared from the polar lipids of different Archaea after 10 min of incubation in 4 M NaCl solutions. The *numbers* in *parentheses* show the PGP-Me content as shown in Table 1. *Error bars* were calculated from 4–10 experiments for each column.

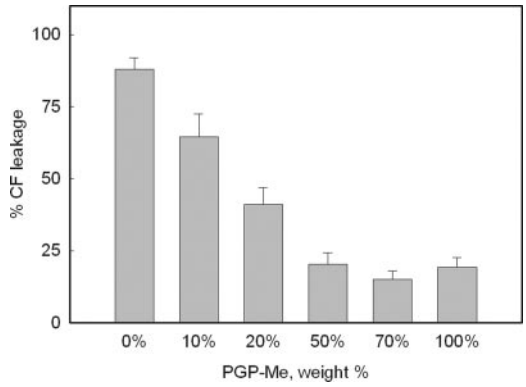

FIGURE 4. **PGP-Me content and LUV stability in hypersaline solution.** LUV was prepared by mixing specific percentages of purified PGP-Me and *M. mazei* polar lipids. CF leakage from LUV was measured after 10 min of incubation in 4 M NaCl solutions. The PGP-Me amounts in the mixtures are given as weight %. *Error bars* were calculated from 4–10 experiments for each column.

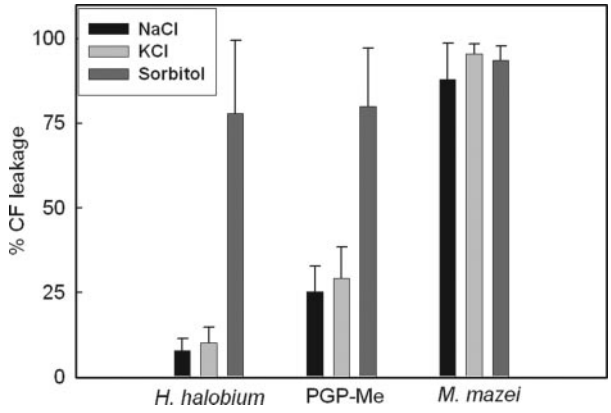

FIGURE 5. **CF leakage from LUV in electrolyte and nonelectrolyte solutions.** CF leakage after 10 min of incubation in 4 M solutions of NaCl, KCl, and sorbitol. Comparison between *H. halobium* total polar lipids (*left*), purified PGP-Me (*middle*), and *M. mazei* total polar lipids (*right*). *Error bars* were calculated from 4–10 experiments for each column.

H. minutum LUV being seemingly more leaky than might be expected based solely on their PGP-Me content. This indicates that other lipids might also contribute to the membrane stabilization/destabilization. Similar to *Natronobacterium magadii*, *H. minutum* contains a proportion of PGP-Me with C20-C25 isoprenoid chains in addition to the usual C20-C20 chains. Such asymmetric chains may be expected to impart instability to the bilayers, as *N. magadii* vesicles of similar phospholipid content have been shown to leak entrapped CF even in isotonic saline (*22*).

LUV aggregation was also monitored as another indicator for the salt tolerance of LUV with different lipid compositions (Table 2). In keeping with the CF leakage data, the LUV from extreme halophile lipids exhibited marked resistance to aggregation in the whole NaCl range up to 4 M NaCl, although an increase in their average diameter was seen in most cases. Aggregation was also monitored by phase contrast microscopy, and the increase in LUV size was always associated with aggregation of LUV. We did not attempt to investigate if fusion of LUV was involved. By contrast, LUV from lipids isolated from moderately halophilic and non-halophilic strains were found to aggregate at all tested NaCl concentrations.

In view of the observed correlations of the LUV stability with their PGP-Me content, it was of importance to investigate the properties of LUV prepared from purified PGP-Me. These experiments showed that PGP-Me vesicles do not aggregate in concentrated NaCl solutions (Table 2) and also exhibit a relatively low CF leakage of ~20% (Figs. 4 and 5), comparable to that of LUV made of extreme halophile lipids. Noteworthy, LUV made of archaeal PG were found to aggregate in 2 M NaCl (Table 2), thus suggesting that PGP-Me and PG have completely different effects with regard to their contribution to the membrane stability.

In a further test of the ability of PGP-Me to impart stability to membranes in hypersaline environments, LUV were prepared from mixtures of *M. mazei* polar lipids (which are unstable in high salt) with purified PGP-Me. Fig. 4 shows that increasing the proportion of PGP-Me in these mixtures leads to gradual reduction in CF leakage to levels typical for the most stable extreme halophiles *H. halobium* and *H. salinarum*. These results clearly demonstrate the ability of PGP-Me to stabilize the lipid membranes in solutions with high NaCl concentrations.

To compare the NaCl effect with that of other solutes, measurements of CF leakage from LUV were also made in 4 M solutions of KCl, sorbitol, and glycerol. These data show that NaCl and KCl have similar effects on LUV stability, dependent on the PGP-Me content, whereas sorbitol (Fig. 5) and glycerol (data not shown) cause high CF leakage regardless of the LUV lipid composition.

All the above described measurements were performed with LUV in strongly asymmetric environments, up to 4 M external solute concentrations *versus* internal NaCl concentrations of 0.5 M in the CF leakage and 0.16 M NaCl in the LUV aggregation experiments. To test if these large osmotic gradients caused LUV instabilities, we attempted to prepare vesicles with high internal NaCl concentrations. Whereas *H. halobium* lipids were found to form stable vesicles at higher salt concentrations, attempts to prepare vesicle dispersions from *M. mazei* lipids at 1.5, 2, and 4 M NaCl concentrations were not successful and resulted in formation of lipid aggregates. For example, the yield of liposomes from *M. mazei* lipids at 1 M NaCl was less than 5%. These results support the notion that the presence of PGP-Me is of critical importance for both the membrane formation and its integrity in high salt solutions.

Low Angle X-Ray Diffraction—To gain further insight into the effect of PGP-Me, we compared the phase behavior of polar lipid extracts from the extreme halophile *H. halobium* and the moderate halophile *M. mazei* as a function of NaCl concentration and temperature. Relatively low lipid contents of 10% (w/v) were used to eliminate restricted volume effects and to ensure sufficiently large aqueous spaces in the

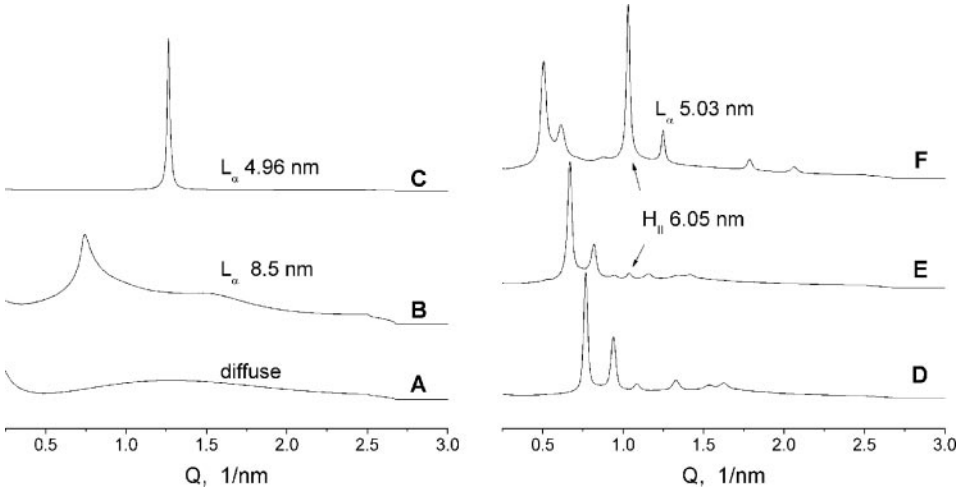

FIGURE 6. **Representative x-ray patterns of phases formed by *H. halobium* and *M. mazei* polar lipid extracts.** *A*, disorded phase typical for both extracts in the range 0–100 °C in absence of NaCl. *B*, *H. halobium* expanded lamellar Lα phase at 2 M NaCl, 20°. *C*, *M. mazei* Lα phase at 2 M NaCl, 20 °C. *D*, cubic Pn3m phase in *M. mazei* dispersions at 2 M NaCl after a heating-cooling cycle, 20 °C. *E*, initial Pn3m and H_{II} mixture in *M. mazei* dispersion at 4 M NaCl, 20 °C. *F*, mixture of Pn3m, H_{II}, and Lα phases in *M. mazei* dispersions at 4 M NaCl after a heating-cooling cycle, 18 °C.

lipid dispersions necessary for the development, in particular, of cubic lipid phases (*23*). Another reason to use low lipid contents was to ensure better compatibility of the x-ray results with the data on the stability of LUV prepared from the same lipid extracts.

In the absence of NaCl, both lipid extracts form disordered phases typified by "background" x-ray scattering in the whole temperature range 0–100 °C (Fig. 6*A*). The lack of reflections at low angles shows that both extracts form dispersions with separated, uncorrelated bilayers. These bilayers may either form closed vesicles, or assume some more complicated spatial arrangements of "sponge" type, which are not closed and would not entrap water soluble markers, such as CF. The large separation and lack of correlation between the bilayers in absence of NaCl is an indication for the existence of sufficiently large repulsive forces between them. Such forces might arise from electrostatic repulsion of electrically charged bilayer surfaces. Another possible origin of a repulsive force is the out-of-plane undulations of flexible, liquid crystalline lipid bilayer (*24*). Further, steric repulsion between bilayers comprising lipids with large, polymeric head groups, *e.g.* containing polyethylene glycol conjugates, may also result in disordered lamellar phases (*25*). Whereas the electrostatic repulsion would be screened and suppressed with increase of the NaCl concentration, the latter two kinds of repulsive interactions would not be substantially affected by the NaCl concentration, but may be expected to increase with the temperature. It was thus of importance to test the effects of both NaCl and temperature on the phase behavior of the lipid extracts.

At 2 M NaCl, both lipid extracts initially form lamellar (Lα) phases at 20 °C. The lamellar phase of *H. halobium* with spacing of 8.5 nm is characterized by broad peaks revealing relatively poor correlation between the bilayers (Fig. 6*B*). It is stable in the whole range up to 100 °C, although it markedly, but reversibly, disorders at high temperatures (Fig. 7*A*). In comparison to *H. halobium*, the initial lamellar phase of *M. mazei* lipids at 2 M NaCl is much more compact, better correlated, and typified by sharp reflections with lamellar spacing of 4.96 nm (Fig. 6*C*). However, it is not stable at higher temperatures and converts into a mixture of an inverted hexagonal phase, H_{II}, and a cubic Pn3m phase, with transition onset at ~45 °C (Fig. 8*A*). The latter transformation is not reversible, and the Lα phase of *M. mazei* does not reform upon cooling. It is replaced by a well ordered Pn3m cubic phase with temperature-independent lattice constant of 11.6 nm, originating from the high temperature Pn3m phase, and with no detectable residues of the H_{II} and Lα phases. The final Pn3m phase is typified by 6 prominent reflexes indexing on a Pn3m lattice in the ratios $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}:\sqrt{8}:\sqrt{9}$ (Fig. 6*D*). A number of smaller reflections at higher angles that are visible at higher magnification (not illustrated) are also consistent with a Pn3m assignment.

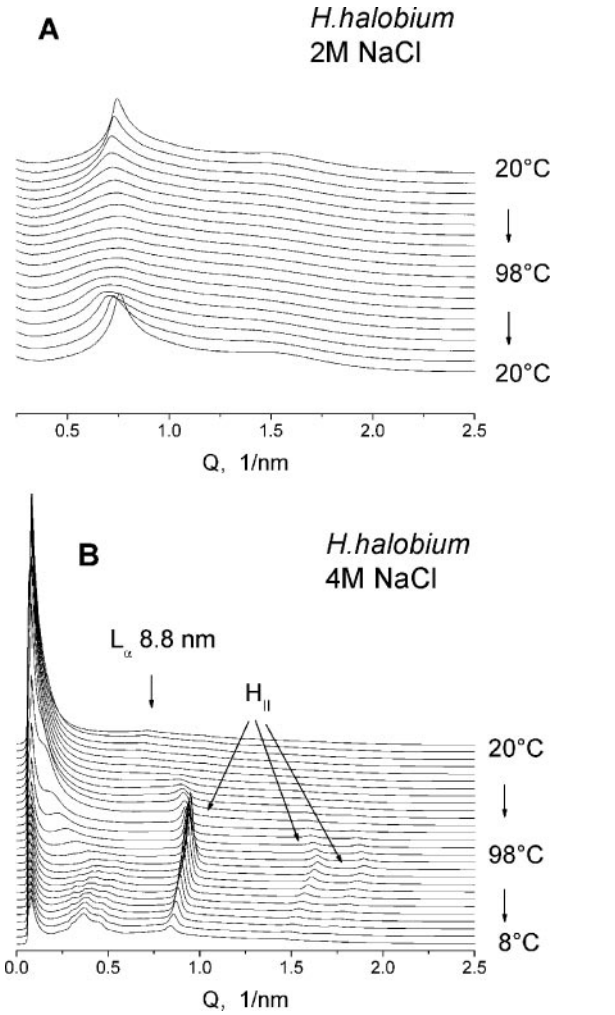

FIGURE 7. **Overview of the effect of temperature on *H. halobium* lipid dispersions.** Diffraction patterns recorded every minute are shown. *A*, 2 M NaCl; *B*, 4 M NaCl.

At the higher concentration of 4 M NaCl, the initial lamellar phase of *H. halobium* lipids at 20 °C appears little changed with respect to the phase at 2 M NaCl. It remains poorly correlated and centered around a broad lamellar spacing of 8.8 nm (Fig. 7*B*). However, upon heating it retains its

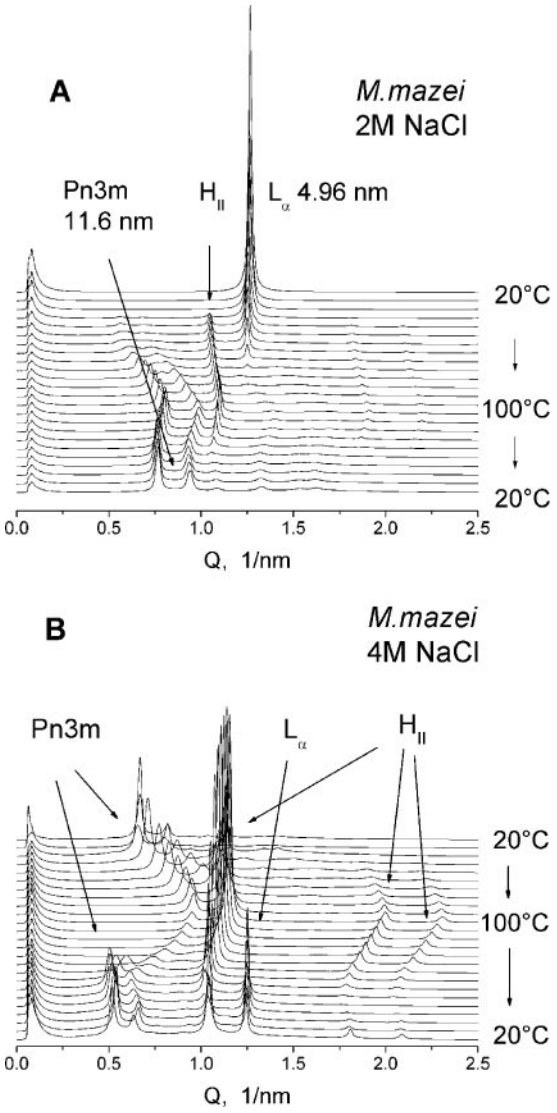

FIGURE 8. **Overview of the effect of temperature on *M. mazei* lipid dispersions.** Diffraction patterns recorded every minute are shown. *A*, 2 M NaCl; *B*, 4 M NaCl.

stability up to ~60 °C and converts into inverted hexagonal phase, H_{II}, followed by the gradual appearance of several reflections at scattering angles typical for the lipid cubic phases. These peaks are broad, poorly resolved, and cannot be unambiguously assigned. They may arise from a poorly ordered cubic phase, or from a mixture of different cubic phases. The evolution of these peaks with temperature shows that they originate from extremely low angles, corresponding to spacings above ~50 nm, which are under the direct beam stop and cannot be recorded. Upon cooling, the initial lamellar ordering does not recover but is replaced by the poorly resolved phase mixture formed at high temperatures. By contrast with *H. halobium*, the initial state of *M. mazei* lipid dispersions at 4 M NaCl is not lamellar. It is represented by a mixture of a Pn3m cubic phase (lattice constant 13.6 nm) with a small amount of H_{II} phase with 6.05 nm spacing (Fig. 6*E*). However, the state of the dispersion after a heating-cooling cycle may be represented by a mixture of all three phases Pn3m, H_{II}, and Lα as shown in Figs. 6*F* and 8*B*, although it should be noted that the lamellar phase was found to only form at temperatures below ~20 °C.

DISCUSSION

The data collected in this work strongly suggest that PGP-Me, a major phospholipid, which accounts for a large proportion of the polar lipids in extreme archaeal halophiles, plays an essential role in the membrane stabilization at high NaCl concentrations. This conclusion is based on several lines of evidence: 1) The extent of release of the water soluble fluorescent marker CF from LUV strongly correlates with the membrane PGP-Me content (Figs. 3–5). The CF leakage is minimal for LUV prepared from the polar lipids of the extreme halophiles *H. halobium* and *H. salinarum*, which have highest PGP-Me content, as well as for LUV prepared from purified PGP-Me. On the other hand, CF leakage is maximal for LUV prepared from the polar lipids of moderately halophilic and non-halophilic strains, such as *M. mazei*, *Methanococcus jannaschii*, *Methanobrevibacter smithii*, which contain no PGP-Me. 2) LUV prepared from the polar lipids of *M. mazei* gradually increase in salt stability in proportion to the percentage of PGP-Me incorporated, gradually approaching the stability of LUV from the extreme halophiles *H. halobium* and *H. salinarum* (Fig. 4). 3) LUV prepared from lipid extracts with high PGP-Me content and from purified PGP-Me remain stable and resist aggregation up to at least 4 M NaCl. In contrast, LUV prepared from the polar lipids of moderately halophilic and non-halophilic archaeal strains are unstable and readily aggregate at increased NaCl concentrations (Table 2). 4) *H. halobium* polar lipids were found to form stable vesicles at NaCl concentrations up to 4 M NaCl, in concurrence with prior evidence that stable LUV could be made in high NaCl concentrations from the polar lipids of the Archaea *H. salinarum* and *H. vacuolatum* (7). By contrast, attempts to prepare vesicle dispersions from *M. mazei* polar lipids at higher NaCl concentrations such as 1.5, 2, and 4 M NaCl were not successful and resulted in formation mostly of lipid aggregates. For example, the yield of liposomes from *M. mazei* lipids at 1 M NaCl was less than 5%. 5) According to the x-ray data, *H. halobium* and *M. mazei* polar lipids have dissimilar phase behavior and form different structures at high NaCl concentrations. *H. halobium* lipids maintain an expanded lamellar structure with spacing of 8.5–9 nm, which is stable up to at least 100 °C in 2 M NaCl and up to ~60 °C in 4 M NaCl (Fig. 7). Such behavior is fully consistent with the observed property of these lipids to form stable, non-leaky vesicles under the same conditions. In contrast, *M. mazei* lipids readily form non-lamellar structures, represented by the Pn3m cubic phase and the inverted hexagonal H_{II} phase, at high NaCl concentrations (Fig. 8). Such behavior indicates that *M. mazei* lipids are unable to form vesicles under these conditions, in agreement with our observations on the *M. mazei* LUV properties.

In general, the present results show that the membrane stability of extremely archaeal halophiles in hypersaline environment is basically determined by PGP-Me. However, there are also indications that other lipids may also contribute to the membrane stabilization/destabilization. For example, LUV stability for the extreme halophiles *H. halobium* and *H. salinarum* appears to be somewhat higher than that of LUV made of purified PGP-Me (Figs. 3–5). Another example is represented by the LUV of *H. minutum* lipids, which appear to be more leaky than can be expected on the basis of their PGP-Me content. As already mentioned under "Results," the lesser membrane stability in the latter case could be attributed to the presence of PGP-Me with asymmetric C25-C20 chains (Table 1).

Our x-ray results show that the expanded lamellar phase of *H. halobium* lipids is rather stable in salt solutions. It can only be converted to non-lamellar state by a combination of 4 M NaCl concentration with heating to temperatures above 60 °C (Fig. 7*B*). This transformation results in formation of poorly ordered, unresolved phases with spacings in the range typical for the lipid-inverted cubic phases. Similarly, a previous freeze-fracture study has shown that PGP-Me has a tendency to form an inverse cubic phase in hypersaline conditions of 5 M NaCl (26).

At ambient temperatures, LUV with high PGP-Me contents remain stable in 4 M NaCl or KCl solutions but not in 4 M sorbitol and glycerol solutions (Fig. 5). It is known that sugar alcohols and polyols have a dehydrating effect on the lipid polar head groups tending to reduce the head group surface areas of contact with the aqueous medium. As a consequence of that, these substances have been shown to suppress the lamellar liquid crystalline phase Lα and to favor formation of lamellar gel and inverted hexagonal phases at the expense of the Lα phase (27–29). It is therefore not surprising that high sorbitol and glycerol concentrations have the ability to destabilize PGP-Me membranes thus causing high CF leakage from the PGP-Me LUV.

It has been suggested in earlier studies using synthetic diphytanoyl PC that the salt stability of halophilic Archaea is caused by the presence of isoprenoid chains instead of straight fatty acyl chains (30). Because the isoprenoid chains are characteristic of all Archaea and are not specific to extreme halophiles, it appears that the presence of such chains alone cannot significantly modulate salt tolerance. This conclusion is consistent with our data on LUV aggregation. It can be seen from Table 2 that both PGP-Me and diphytanoyl PC form LUV, which have relatively constant sizes and do not aggregate up to at least 4 M NaCl. In contrast, archaeal PG and diphytanoyl PG form LUV, which increase significantly in size at 2 M NaCl and aggregate at concentrations of 3 and 4 M NaCl. It is clear on the basis of these data that the contribution of head group to the LUV stability is much more significant than that of the isoprenoid chains.

The much higher stability of LUV made of PGP-Me in comparison to LUV made of PG indicates that the stabilizing effect of PGP-Me is actually because of the methylphosphate group in the polar head of PGP-Me, as this group represents the only structural difference between PGP-Me and PG (Fig. 1). The notion that the large head group of PGP-Me is responsible for the membrane stabilization imparted by this lipid is strongly supported by our x-ray study, which disclosed profound differences in the phase behavior of *H. halobium* and *M. mazei* polar lipid extracts at high NaCl concentrations. For both extracts, addition of 2 M NaCl results in conversion of their disordered dispersions into lamellar phases (Fig. 6, *A–C*). However, these two lamellar phases have completely different characteristics. *M. mazei* lipids undergo a transition from uncorrelated bilayers into a compact, well correlated lamellar phase with short lamellar spacing of 4.96 nm at 20 °C (Fig. 6*C*). We therefore assume that the dominant repulsive force disordering the *M. mazei* lamellar phase in absence of NaCl is electrostatic repulsion and that the latter repulsion is completely screened at 2 M NaCl.

Changing the NaCl concentration from 0 to 2 M NaCl also results in appearance of correlation between the bilayers in *H. halobium* lipid dispersions. Whereas this observation indicates that electrostatic forces play a role in this case too, it is noteworthy that the lamellar phase still remains poorly correlated and with a relatively large spacing for lipid lamellar phases of 8.5 nm (Fig. 6*B*). Because its ordering is not affected by a further increase of NaCl from 2 M to 4 M, it is clear that another repulsive interaction of non-electrostatic origin, which cannot be screened by NaCl, is also present in this system. As noted under "Results," such repulsive interaction might arise, in principle, as a result of out-of-plane fluctuations of the lipid bilayers or because of steric repulsion between opposing bilayers caused by the presence of lipids with large head groups. It is remarkable in this connection that PGP-Me, a lipid with large head group, represents ~78% of the *H. halobium* polar lipids and is thus a natural candidate for the role of the lipid giving rise to steric repulsion between neighboring lipid bilayers. Also, PGP-Me is completely absent from *H. mazei* lipid extracts, which were found to form a compact lamellar phase with short spacing of 4.96 nm at 2 M NaCl concentration.

It is noteworthy also that the lamellar phase of *H. halobium* strongly disorders with increase of temperature (Fig. 7*A*). This effect is reversible and provides another indication for poorly correlated, weakly bound lipid bilayers, which easily disorder with increase of temperature. *H. halobium* bilayers remain poorly correlated at high NaCl concentrations suggesting that *H. halobium* polar lipid extracts should be able to form unilamellar vesicles, which would remain stable and would not aggregate in the range 0–4 M NaCl. This is in full agreement with our observations on the properties of LUV dispersions. In contrast, the phase state of *M. mazei* polar lipid extracts at 4 M NaCl is represented by mixtures of non-lamellar phases including the inverted hexagonal H_{II} and cubic Pn3m phases, and no Lα phase at temperatures above ~20 °C. Obviously, no stable LUV could be expected to form from *M. mazei* lipids at higher NaCl concentrations, again in agreement with our experiments on LUV formation.

In summary, the x-ray and LUV data consistently substantiate the conclusion that the remarkable salt tolerance of *H. halobium* is caused by the ability of *H. halobium* polar lipids to form stable, poorly interacting lipid bilayers in a broad range of NaCl concentrations and temperatures. The phospholipid PGP-Me plays a principal role in the bilayer stabilization. No lipids similar to PGP-Me are present in moderately halophilic and non-halophilic Archaea tested here, which, respectively, are typified by a much lower salt tolerance. The mechanism of the PGP-Me stabilizing effect at high NaCl concentrations apparently involves steric repulsion that prevents close approach and aggregation of the lipid bilayers. We attribute the latter effect to the large polar head group of PGP-Me and intend to undertake a detailed structural characterization of the lipid phases formed by pure PGP-Me, once sufficient amounts of this lipid become available for x-ray studies. It can be also noted that salt-tolerant liposomes may find various applications as salt may be used as a preservative to prevent microbial contamination, or for delivery of oral vaccines to marine species, thus combining to advantage the known adjuvant activity of archaeal liposomes (31) and their salt stability.

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Salt Tolerance of Archaeal Extremely Halophilic Lipid Membranes

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