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MINI-REVIEW

Adaptive Modifications in Membranes of Halotolerant and Halophilic Microorganisms

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Abstract

Halotolerant and halophilic microorganisms can grow in (hyper)saline environments, but only halophiles specifically require salt. Genotypic and phenotypic adaptations are displayed by halophiles; the halotolerants adapt phenotypically, but it is not established whether they show genotypic adaptation. This paper reviews the various strategies of haloadaptation of membrane proteins and lipids by halotolerant and halophilic microorganisms. Moderate halophiles and halotolerants adapt their membrane lipid composition by increasing the proportion of anionic lipids, often phosphatidylglycerol and/or glycolipids, which in the moderately halophilic bacterium *Vibrio costicola* appears to be part of an osmoregulatory response to minimize membrane stress at high salinities. Extreme halophiles possess typical archaebacterial ether lipids, which are genotypically adapted by having additional substitutions with negatively-charged residues such as sulfate. In contrast to the lipids, it is less clear whether membrane proteins are haloadapted, although they may be more acidic; very few depend on salt for their activity.

Key Words: Halotolerant, microorganisms; halophile, microbial; membrane, lipids and salinity; lipid composition, microbial and salinity; salinity, effects on microbial membranes; osmotic pressure, effects on membrane lipids; bacteria, halotolerant and halophilic; phenotypic adaptation of microbial membranes to salt.

Halotolerant and Halophilic Microorganisms

Microorganisms are well known for their ability to occupy a wide variety of environmental niches, both natural and man-made, and the saline environment

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provides a well-recognized example of such an environment—often called "extreme," although this term is misleading as explained below.

A distinction must be made between "tolerance for salt" and "requirement for salt" (Larsen, 1986). Halotolerant microorganisms have no specific requirement for salt, other than the usual 100–200 mM NaCl needed by all (nonhalotolerant) organisms, but they are able to grow in up to ~1.25 M NaCl (slightly halotolerant), up to ~3.0 M NaCl (moderately halotolerant), or up to saturated (i.e., 5.2 M) NaCl (extremely halotolerant). Within the halotolerants, a distinction can be made between those for which growth rate is decreased by the addition of any salt, and those for which the growth rate reaches an optimum with the addition of some salt, but then declines at higher salinities.

Organisms that require salt for growth are called halophiles, and they are classified in a similar manner to the halotolerants into slight, moderate, and extreme halophiles (Kushner, 1978). However, there is an important distinction between the slight and moderate halophiles, on the one hand, and extreme halophiles, on the other. The slight and moderate halophiles, which include bacteria, fungi, and algae, have a requirement for 0.2–0.5 M NaCl and will grow in up to ~1.0 M and 3.5–4.0 M NaCl, respectively. In contrast, extreme halophiles include only bacteria and a few fungi, and have a much higher requirement for NaCl of 2.0–4.0 M (depending on the species), but will grow in up to saturated NaCl. Another important distinction with reference to bacteria is that slight and moderate halophiles are eubacteria and cyanobacteria, whereas extreme halophiles are archaebacteria. This has important consequences as far as adaptation to salinity (haloadaptation) is concerned, because the archaebacteria have a quite distinctive biochemistry/physiology, particularly in the composition of their membrane lipids.

Thus, as a group, the halotolerant and halophilic microorganisms cover the complete range of salinity from a few millimolar up to saturated. Indeed, it is remarkable that some individual species of algae (e.g., *Dunaliella*) and bacteria (e.g., *Micrococcus varians* ssp. *halophilus*) are capable of spanning the whole range. This is a considerable feat of adaptation not only to the direct effects of increasing NaCl concentration, but also to the inevitable osmotic stresses which that imposes. Since the cell (plasma) membrane is the semipermeable barrier between the cytoplasm and the external medium, it is perhaps not surprising that this membrane displays adaptive changes in the face of altered salinity, particularly in its lipid composition, which provides the main focus of this review.

At this point, it is useful to distinguish genotypic from phenotypic adaptation. Genotypic adaptation represents a long-term evolutionary change in the genetic makeup of the organism enabling it to grow over a

certain range of salinity. Phenotypic adaptation occurs within a population during their lifetime—i.e., it is the changes that occur within cells, and is not due to the selection of genetic variants. Both genotypic and phenotypic adaptation are displayed by halophiles; the halotolerants adapt phenotypically, often in a very similar manner to halophiles, but it is not established whether they show genotypic adaptation.

Salt-Dependent Changes in Membrane Composition

Proteins

It is well established that membrane (and cytoplasmic) proteins in extremely halophilic bacteria have a high content of acidic amino acids and generally a large excess of acidic over basic residues, compared with proteins from nonhalophiles (Lanyi, 1974; Bayley and Morton, 1978). However, the levels of acidic residues are not enough to explain why the proteins remain hydrated in $\geqslant 4$ M NaCl, nor the organisms' requirement for such high salinity (Lanyi, 1974; Werber *et al.*, 1986). In the moderately halophilic genus *Ectothiorhodospira*, those species with the highest salt requirements have membrane proteins with the greatest proportion of acidic amino acids (Imhoff *et al.*, 1983). This example of genotypic adaptation does suggest that the unusual amino acid composition of halophilic proteins is relevant to their halophilic properties.

The moderate halophile *Pseudomonas halosaccharolytica* contains proteins in its outer membrane that have an excess of acidic over basic amino acids at least as great as that in extreme halophiles, and much greater than that of slightly or nonhalophilic bacteria (Hiramatsu *et al.*, 1980). There is no consistent change in this ratio when the salinity of the growth medium is changed. Thus, it appears that halophile protein amino acid composition may well be adapted genotypically for high-salinity environments, but that phenotypic adaptation does not occur. This is very different to the situation for membrane lipids, except those of extreme halophiles (see below).

The most-studied membrane protein of extreme halophiles is bacteriorhodopsin, but ironically it does not require Na^+ for activity—i.e., it is not halophilic. Small-angle neutron and X-ray scattering, and nuclear magnetic resonance (NMR) spectroscopic studies of some cytoplasmic proteins, which were purified throughout in the presence of salt, show that simple considerations of amino acid content are inadequate to explain the proteins' special properties (Werber *et al.*, 1986; Zacchai *et al.*, 1986).

Proteins from extreme halophiles are also relatively deficient in nonpolar amino acids, compared with proteins from nonhalophiles. High

concentrations of NaCl and KCl (the latter is the main internal salt in extreme halophiles) strengthen hydrophobic bonds, so the relative deficiency of nonpolar residues will prevent halophilic proteins from adopting a conformation that is too "tight" to allow it to function (Lanyi, 1974). The relative increase in surface acidic residues will have the same effect by increasing the water-binding capacity. As an example of this phenomenon, the ferredoxin in an extremely halophilic bacterium was shown by using ^{1}H-NMR to have a loosely folded overall tertiary structure even in high salt concentrations (Gochin and Degani, 1985). Studies of purified membrane proteins have not been reported, but there is no reason to doubt that similar physicochemical effects are responsible for the stability of halophilic membrane proteins, although, of course, the added dimension of lipid–protein interactions must also be considered.

In moderate halophiles, several membrane functions, including transport (MacLeod, 1986) and respiration (Tokuda and Unemoto, 1983; Ken-Dror *et al.*, 1986), depend on Na^+ for activity. The active transport system for α-aminoisobutyric acid in *Vibrio costicola* adapts phenotypically to the NaCl concentration of the bacterial growth medium (Kushner *et al.*, 1983). Unfortunately, none of the membrane proteins responsible for these activities have been purified, so a molecular explanation for their dependence on and regulation by NaCl is not forthcoming. The membrane-bound 5′-nucleotidase in *V. costicola* has optimal activity at 2–3 M NaCl, but only in the purified state—i.e., not when in the membrane (Bengis-Garber and Kushner, 1981). The reason for this change in salt dependence is not understood. Comparative studies with the more readily purified cytoplasmic proteins provide little insight because, in moderate halophiles, they do not require salt for activity (Kamekura, 1986; Kushner, 1986). It may be more profitable to study the extracellular enzymes produced by some moderately halophilic bacteria, because several have optimal activity at high salt concentrations (Kamekura, 1986). The maximal activity of *in vitro* protein synthesis in *V. costicola* is at 0.6 M salt (Kamekura and Kushner, 1984), which is substantially lower than the optimum salt concentration of 1.0 M for growth.

Finally, another class of membrane proteins should be mentioned at this stage—namely, those enzymes of lipid synthesis that are membrane bound and respond to salt concentration changes, producing an altered lipid composition. The regulation of these enzymes by salt is considered together with the mechanism of lipid changes in a later section. Firstly the types of salt-dependent lipid changes are considered.

Lipids

The extremely halophilic bacteria (halobacteria) are all members of the archaebacteria, a separate phylogenetic group (*ur*-kingdom) (Woese *et al.*,

1978). As such they possess a distinctive cell envelope that lacks peptidoglycan, having instead a glycoprotein coat (which in halobacteria needs Na^+ for its integrity) and a membrane containing characteristic glycerol ether-linked isopranoid lipids (Woese and Wolfe, 1985). Halobacteria share these properties with other methanogenic and thermophilic archaebacteria. A special feature of halobacteria is their retinal-based pigment proteins (e.g., bacteriorhodopsin) that pump ions in a light-mediated manner (Stoeckenius and Bogomolni, 1982).

The glycerol ether lipids of halobacteria are based on so-called "core structures" comprised of phytanyl (i.e., C20 isoprenoid-derived) alkyl chains linked by ether bonds to the 2- and 3-carbon atoms of glycerol (Kates, 1978; Langworthy, 1985). These diphytanylglycerol diethers (C20, C20) are the major core structures in all halobacteria; haloalkaliphiles contain, in addition, sesterpanyl (C25) alkyl chains in C20, C25 and C25, C25 core structures (Grant and Ross, 1986). Halobacteria lack C40, C40 dibiphytanyldiglycerol tetraethers, in which pairs of phytanyl chains on two diethers are fused to give C40 alkyl chains that span the membrane. Tetraethers are the predominant lipids in thermophilic archaebacteria, and are also present in methanogenic archaebacteria (Langworthy, 1985). The absence, from halobacteria, of tetraethers that "lock" the two halves of the bilayer into what effectively becomes a monolayer might reflect the need to keep the halobacterial membrane fluid in high salinity. Although this is pure speculation on the reason for its genotypic adaptation, there is some evidence that phenotypic adaptive changes in other halophiles and halotolerants serve to raise membrane fluidity at high salt concentrations (see below).

The glycerol ether core structures are linked to a "head group" of phosphorylglycerol, to give the diphytanylglycerol diether analogs of phosphatidylglycerol, phosphatidylglycerol phosphate, and phosphatidylglycerol sulfate (Kates, 1986). The glycerol moiety in the head group may be glycosylated to give di- and trisugar glycolipids, in which the terminal sugar may also be sulfated (Kates, 1978; Smallbone and Kates, 1981).

The major phospholipid in halobacteria is phosphatidylglycerol phosphate, with smaller amounts usually of phosphatidylglycerol and, in most species, phosphatidylglycerol sulfate also (Kates, 1986; Torreblanca *et al.*, 1986). The glycolipids and glycolipid sulfates are major components in some halobacteria and there is a strong correlation between their structure and the taxonomic classification of halobacterial species (Torreblanca *et al.*, 1986; Kushwaha *et al.*, 1982). Despite these variations in phospholipid and glycolipid composition and extent of sulfation, it is significant that all halobacteria have a very high proportion of negatively charged lipids in their membranes—in the range of 1.3–1.7 negative charges per mole of polar lipid

(Kates, 1986). This parallels the relatively high proportion of acidic residues in the proteins (discussed above).

It appears, therefore, that extremely halophilic bacteria are adapted genotypically in terms of their lipid composition for life in high salinities by having a very high density of negative charges on the surface of their membrane lipid bilayer. It might be suggested that this is a necessary adaptation to counter the high cation concentration (Na^+ on the outside and K^+ on the inside of the cell membrane). As pointed out for halobacterial proteins (Lanyi, 1974), however, the amount of charge shielding provided by the lipids is quite inadequate in relation to molar concentration of cations.

The presence of ether lipids is not a halophilic adaptation, but rather a phylogenetic trait of archaebacteria. By specializing genotypically for growth at such high salt concentrations, it might be thought that halobacteria do not adapt phenotypically to altered salinity (many halobacteria live in environments where the salt concentration changes very little). One study of phenotypic adaptation has been performed on *Halobacterium cutirubrum* and *H. mediterranei*, grown between 2.6 M and saturated NaCl (Kushwaha *et al.*, 1982). *Halobacterium cutirubrum*, but not *H. mediterranei*, increases its lipid content with an increase in salt concentration; in *H. mediterranei*, there is a relative increase in sulfated diglycosyl diether and phosphatidylglycerol phosphate with a decrease in phosphatidylglycerol, but the number of negative charges per mole of polar lipid remains fairly constant at 1.3–1.4. In addition, there is a striking difference in the effect of salinity on pigmentation of the two halobacteria. In *H. cutirubrum*, the synthesis of β-carotene and bacterioruberin pigments is increased by high NaCl concentrations, whereas in *H. mediterranei* it is considerably reduced. The different phenotypic responses by these two bacterial species may reflect their different habitats and geographical origins; *H. cutirubrum* has a higher salt requirement and more complex nutritional requirements than does *H. mediterranei*. Based on these two organisms, it seems that halobacteria may adapt phenotypically, but it is impossible to interpret the meaning of the changes until more is known about the effect of salt on the conformation of the halobacterial ether lipids in membranes (see below).

Neither the slight and moderate halophiles nor the halotolerant bacteria show genotypic adaptations in lipid composition that are related specifically to their ability to grow in salt. In fact, their lipid compositions are remarkably similar to those of closely related nonhalotolerant/halophilic species. For example, in the moderate halophile *V. costicola*, phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol (cardiolipin) together account for $\sim 97\%$ of the phospholipid; this composition is essentially the same as that of nonhalophilic *Vibrio* species (Oliver and Colwell, 1973).

Nonetheless, halotolerant/halophilic bacteria do show distinctive phenotypic alterations in lipid composition that appear to be related to the salt concentration of the culture medium.

In *V. costicola*, these changes seem to be necessary for growth in high salinity because, if a culture is shifted up—i.e., the NaCl concentration of the growth medium is raised suddenly—growth stops, the phospholipid composition alters, and then growth resumes at the new rate (Kogut and Russell, 1984).

The salt-dependent changes in lipid (and fatty acid) composition are summarized in Table I. It is particularly noteworthy that, although there are differences between species, in all instances there is an increase in the anionic lipid content relative to zwitterionic lipids; this is sometimes

Table I. Summary of the Major Changes in Lipid Composition of Microorganisms in Response to an Increase in NaCl Concentration[a]

Microorganism type	Major lipid change
Gram-negative bacteria	
Halophilic	PG increase cycFA or UFA increase brFA decrease
Halotolerant	PG or DPG increase
Gram-positive bacteria	
Halophilic[b]	GL increase UFA increase brFA increase
Halotolerant	DPG and/or PG increase GL increase brFA increase
Algae	
Halophilic	None
Halotolerant	None
Yeast	
Halotolerant	Not known

[a] For bacteria and yeast, the conclusions are based on comparison of data for cultures grown at different salinities, i.e., phenotypic adaptive changes. For algae, such data are not available and, instead, comparison has been made between the lipid compositions of halophilic/halotolerant and nonhalophilic/halotolerant strains, i.e., genotypic adaptive differences.

Not all of the changes for any grouping have necessarily been observed in all members of that group. In many instances, fatty acid composition was not studied. The source references for individual species can be found in Kates (1986), Russell (1988), and Bygraves and Russell (1988).

[b] Includes *Planococcus halophilus*, which is Gram variable.

PG, phosphatidylglycerol; DPG, diphosphatidylglycerol (cardiolipin); GL, glycolipid (including phosphogylcolipid); cycFA, cyclopropane fatty acid; brFA, branched fatty acid; and UFA, unsaturated fatty acid.

accompanied by an overall decrease in membrane lipid content, although information on this parameter is less frequently reported compared with percentage compositions. The reasons why it is the anionic lipids that increase are considered in relation to lipid conformation and osmotic effects in a later section.

The interspecies differences in the identity of the anionic lipid that changes reflect not so much a different response to salinity, nor whether the bacterium is halophilic or halotolerant (or any of the subgroupings described above), but instead whether the bacterium is Gram negative or Gram positive. Gram-negative bacteria generally have a simple lipid composition, comprised mainly of phosphatidylethanolamine and phosphatidylglycerol, with smaller amounts of diphosphatidylglycerol (cardiolipin) (Harwood and Russell, 1984). The major change on raising salinity is an increase in the amount of phosphatidylglycerol relative to phosphatidylethanolamine. Sometimes, diphosphatidylglycerol also increases, but it has to be established that this is not a consequence of slower growth rates at higher NaCl concentrations, because, as growth slows, two molecules of phosphatidylglycerol are converted to one molecule of diphosphatidylglycerol (Harwood and Russell, 1984). Therefore, this change has no effect on the surface negative-charge density of the lipid bilayer (see below). Whether or not the amount of diphosphatidylglycerol changes may also depend on the composition of the growth medium; for example, in *V. costicola*, diphosphatidylglycerol levels change with salinity when cultures are grown in simple, defined media, but not in complex, rich media (M. Kogut and N. J. Russell, unpublished results).

The fatty acid composition of most Gram-negative bacteria consists of even-carbon-numbered saturated and unsaturated fatty acids; in some species, a proportion of the unsaturated fatty acids are converted to the cyclopropane derivative (Harwood and Russell, 1984). As salinity is increased, the major change in fatty acid composition is an increase in unsaturated and cyclopropane acids (Table I). This *apparently* gives an increase in membrane fluidity in that unsaturated and cyclopropane fatty acids have similar thermal properties and, at a given temperature, are more fluid compared with their saturated derivatives. However, this interpretation must be taken with some caution because, in some species, the major change is from unsaturated (already present at low salt concentration) to cyclopropane fatty acid, which would not alter membrane fluidity very much. Furthermore, in no species have direct measurements of membrane lipid fluidity at different salinities been reported. The other changes that occur in phospholipid composition, and in protein/lipid ratio in some species, will also affect membrane fluidity. Therefore, it is premature to draw conclusions about fluidity based on fatty acid compositional changes alone.

Gram-positive bacteria generally have much more complex lipid compositions, typically comprising the same phospholipids as in Gram negatives (but with more phosphatidylglycerol and diphosphatidylglycerol relative to phosphatidylethanolamine) together with a mixture of glycolipids and/or phosphoglycolipids (Harwood and Russell, 1984). The (phospho)glycolipids are anionic lipids and their amounts usually increase in high salinity, often in conjunction with increases in phosphatidylglycerol and/or diphosphatidylglycerol (Table I).

The fact that it is usually the (phospho)glycolipids that increase in Gram-positive bacteria reflects the phylogenetic differences in lipid composition between Gram-positive and Gram-negative bacteria. We have also observed differences in response to salinity between Gram-positive strains that are apparently closely related. For example, two halotolerant *Bacillus* species with very similar lipid compositions and tolerances to salt and other solutes respond differently: in one strain, it is only glycolipid content that increases, whereas, in the second strain, it is phosphatidylglycerol and diphosphatidylglycerol, together with glycolipids, that increase (Bygraves and Russell, 1988). This difference is probably not due to different conditions of culture, because both strains were grown in rich broth media.

In contrast, the different results obtained by two research groups studying strains of halotolerant *Staphylococcus aureus* are probably due to the different culture media used. Kanemasa *et al.* (1972) reported that the major anionic lipid increase was in diphosphatidylglycerol with a decrease in phosphatidylglycerol, whereas Hurst *et al.* (1984) found that phosphatidylglycerol decreased with no change in diphosphatidylglycerol. In their study, Kanemasa *et al.* (1972) used a semidefined medium, whereas Hurst *et al.* (1984) used a complex medium. In both strains, there was a decrease in lysylphosphatidylglycerol at higher salinities, a change that would increase the surface negative charge on the membrane by converting a zwitterionic lipid into an anionic lipid through removal of the lysine positive charge.

The fatty acid compositions of most Gram-positive bacteria are dominated by branched-chain fatty acids, which may increase (in halotolerants) or decrease (in halophiles) in response to higher salinity (Table I). However, this distinction between halotolerants and halophiles is tenuous, being based on data from very few species. In addition, the caveat that accompanying lipid changes will affect properties such as membrane fluidity also applies, so it is not possible at present to draw valid conclusions about the relationship of salt effects on fatty acid composition and membrane fluidity in Gram-positive bacteria.

Many fewer species of algae (mainly *Dunaliella*) have been studied, and comparative data are not available for individual species grown at different

salt concentrations. Instead, comparisons have to be made between data for halotolerant/halophilic and nonhalotolerant/halophilic strains. The lipid patterns of halophilic *Dunaliella* species are generally similar to those of halotolerant species except for the presence of up to five unidentified glycolipids in the halophiles (Evans and Kates, 1984). The lipid compositions of halotolerant/halophilic *Dunaliella* do not appear to be adapted specifically to the saline environment, but are similar to those of other photosynthetic algae particularly in that they contain the unusual zwitterionic polar lipid diacylglycerol-O-(*N*,*N*,*N*-trimethyl)homoserine (Evans *et al.*, 1982a, b). Recently, a salt-sensitive mutant of *D. tertiolecta* has been isolated (Brown *et al.*, 1987), and it would be interesting to determine its lipid composition, as this might indicate what features are related to halotolerance.

The salt dependence of lipid composition in only a single species of halotolerant yeast has been reported, namely, *Debaryomyces hansenii* (Tunblad-Johansson *et al.*, 1987). At high salinities, the overall content (on a cellular dry weight basis) of free sterol (mainly ergosterol) and phospholipid decreases, but there are only minor changes in the ratio of free sterol to phospholipid, which remains at 1.2–1.3. This value is considerably higher than that in the nonhalotolerant yeast *Saccharomyces cerevisiae*. Although this could reflect a phylogenetic difference, it could be significant for halotolerance because the overall sterol-to-phospholipid ratio is often the major determinant of membrane properties such as fluidity in eukaryotic organisms. In addition, in *D. hansenii* at high salinities the proportion of phosphatidylserine increases, while that of phosphatidylglycerol and phosphatidylcholine are unchanged. There are only minor changes in fatty acid composition (Adler and Liljenberg, 1981).

Mechanisms of Salt-Dependent Lipid Changes

The *mechanisms* of the changes in lipid composition that are triggered by a rise in NaCl concentration have been studied in only two bacterial species, both Gram-negative moderate halophiles, viz. *Pseudomonas halosaccharolytica* and *Vibrio costicola*.

In *P. halosaccharolytica*, the phospholipids comprise phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and a glucosylphosphatidylglycerol, and growth in high salinity results in an increase in the proportion of phosphatidylglycerol and a decrease in phosphatidylethanolamine (Hiramatsu *et al.*, 1980). In subsequent studies of the mechanism, growing cultures were harvested by centrifugation and the cells resuspended in buffer containing 2 M NaCl; after starvation for 90 min, the suspension was centrifuged and aliquots of cells resuspended in buffers

containing a range of NaCl concentrations. Pulse-chase labeling of lipids with various radioactive precursors showed that the rate of synthesis of phosphatidylethanolamine was inhibited by an increase in salt concentration, but the rate of phosphatidylglycerol synthesis was unaffected (Hara and Masui, 1985). The lack of stimulation of phosphatidylglycerol synthesis by salt does not accord with the compositonal data. This may well reflect the fact that radiolabeling experiments were performed with nongrowing, starved cells, whereas lipid compositions were determined directly on cells harvested from culture media. The authors argue that the inhibition of phosphatidylethanolamine synthesis leads to an increase in phosphatidylglycerol content because of the branched phospholipid biosynthetic pathway. While this is a logical deduction, it is not supported by the radiolabeling data. Similarly, although NaCl concentration has little or no effect on the amount of glucosylphosphatidylglycerol in growing bacteria (Ohno *et al.*, 1979), there is a very marked stimulation of glucosylphosphatidylglycerol synthesis in nongrowing, starved bacteria (Hara and Masui, 1985). These discrepancies make it difficult to interpret the experiments in terms of a mechanism for haloadaptive changes in membrane lipid composition.

In the second mechanistic study, by Russell and coworkers, phospholipid synthesis has been measured in *V. costicola* at different salinities, and following a sudden increase (shift-up) or decrease (shift-down) in salinity. In the shift experiments, cultures are centrifuged carefully, washed, and the bacteria resuspended with minimal force at the new salt concentration in order to avoid perturbing the cells; prewarmed growth media or buffers are used, and the cells are not cooled during centrifugation. Control experiments, in which cultures are centrifuged, washed, and resuspended at the same salinity, resume growth immediately at the expected rate, showing that bacterial metabolism has not been perturbed. Therefore, it is possible to study phenotypic adaptation to altered salt concentration.

In *V. costicola* the rate of phosphatidylethanolamine synthesis is faster than that of phosphatidylglycerol in cultures growing in 1 M NaCl media, whereas in 3 M NaCl media the synthesis of phosphatidylglycerol is faster than that of phosphatidylethanolamine (Russell *et al.*, 1985). These biosynthetic data correspond with compositional data showing that bacteria grown in 1 M NaCl contain nearly twice as much phosphatidylethanolamine than phosphatidylglycerol, in contrast with those grown in 3 M NaCl which contain less phosphatidylethanolamine than phosphatidylglycerol (Hanna *et al.*, 1984; Kogut and Russell, 1984). In addition, the rates of synthesis of both phospholipids are up to fivefold faster in 1 M compared with 3 M NaCl, which is in line with growth rates in the respective media (cf. Russell *et al.*, 1985; Adams *et al.*, 1987).

The phospholipid metabolic events in *V. costicola* following a shift-up in salinity can be divided into three temporal phases, which correspond with those described by Brown (1978) in discussing haloadaptation by *Dunaliella*. In the first place, during the minutes immediately after the shift-up, there is a drastic inhibition of all phospholipid synthesis; this inhibition is reversible in that, if the culture is "shifted back" (i.e., restored to the starting NaCl concentration), the original phospholipid synthesis rates are resumed (Russell *et al.*, 1985). It is not known what causes this inhibition, but one possibility that we are testing is that fatty acid synthesis is inhibited—thus leading to secondary inhibition of phospholipid synthesis by depletion of the pool of CDP-diacylglycerol common to phosphatidylethanolamine and phosphatidylglycerol synthetic routes (Harwood and Russell, 1984). Alternatively, the inhibition could result from the plasmolysis that occurs when cultures are shifted up; this could inhibit phospholipid synthesis through leakage of metabolites (e.g., ATP) from cells, or by altering the physical environment of the synthetic enzymes in the membrane, possibly via lipid conformational changes.

In addition to the overall inhibition of phospholipid synthesis during the first phase of haloadaptation, there is a "switch" in the rates of phosphatidylethanolamine and phosphatidylglycerol synthesis—i.e., before the shift-up, phosphatidylethanolamine is greater than phosphatidylglycerol synthesis, but, after the shift-up, phosphatidylglycerol is greater than phosphatidylethanolamine synthesis (Russell *et al.*, 1985). Therefore, differential effects of the shift-up on the synthesis of the two major phospholipids must occur, perhaps via membrane effects, as well as the overall inhibition. During the second phase of haloadaptation, which lasts a few hours, this differential effect on phosphatidylglycerol and phosphatidylethanolamine synthesis is maintained. There is a large and rapid rise in the rate of phosphatidylglycerol synthesis, accompanied by a smaller and more gradual rise in the rate of phosphatidylethanolamine synthesis. In the third phase of haloadaptation, the rates settle to those characteristic of isotonically grown cultures in the appropriate salt concentration (e.g., 3 M), with the rate of phosphatidylglycerol synthesis being greater than that of phosphatidylethanolamine synthesis (Russell *et al.*, 1985).

The major haloadaptive changes that occur in the second phase do so largely during the lag in growth that lasts for 1–2 hr after a shift-up from 1 M to 3 M NaCl (Kogut and Russell, 1984; Russell *et al.*, 1986). Whether there is a lag in growth following shift-up, and the extent of the lag, depends on the magnitude of the shift (Russell *et al.*, 1986), but the phospholipid compositional changes and the changes in phosphatidylglycerol and phosphatidyltehanolamine synthesis always occur within the first few hours after the shift-up (Adams *et al.*, 1987). Although it has not been proved

that the phospholipid compositional changes are a necessary adaptation for growth at higher salinity, the observed changes in phosphatidylglycerol and phosphatidylethanolamine synthesis rates during many different experiments that we have performed in which the magnitude of the shift-up (or shift-down) is varied with either a constant starting or final NaCl concentration are consistent with such an interpretation.

The phospholipid changes do not require protein synthesis and are mediated by changes in biosynthetic rate, not, for example, by selective degradation of phosphatidylethanolamine (Russell *et al.*, 1985). We have reasoned that the cell membrane is a likely site for the sensor and trigger mechanism of the haloadaptive response, since this membrane is in contact with the external ionic environment (Russell and Kogut, 1985). This is also indicated by our recent demonstration using NMR that the intracellular levels of *free* Na^+ in *V. costicola* are much lower than the extracellular concentration, i.e., 200–300 mM even when bacteria are grown in up to 2 M NaCl (H. Gilboa, Y. Avi-Dor, M. Kogut, and N. J. Russell, unpublished results). The enzymes of phospholipid synthesis respond to salt concentration when assayed in isolated membranes, in that phosphatidylglycerol synthesis is enhanced over that of phosphatidylethanolamine synthesis at higher salinities (R. L. Adams and N. J. Russell, unpublished results). Moreover, the extent of the differential effect depends on the NaCl concentration of the medium in which the bacteria were grown before membrane isolation. Since this would affect the phospholipid composition of the membrane, it supports the hypothesis that both the lipid environment and the effects of external salinity on membrane properties are involved in regulating the relative rates of phosphatidylglycerol and phosphatidylethanolamine synthesis. The effects of salt on phospholipid conformation are discussed below.

Osmotic Effects on Membrane Phospholipid Composition

A rise in external salinity will inevitably stress cells osmotically, because the plasma membrane is not freely permeable to Na^+, and, following a sudden shift-up in salt concentration, temporary plasmolysis occurs. Since the phospholipid synthesis enzymes are located in the bacterial plasma membrane (Raetz, 1978), they could respond to changes in lateral pressure within the membrane caused by the plasmolysis.

In order to explore the role of osmotic effects in the haloadaptive changes in phospholipid composition in *V. costicola*, we repeated the salt shift-up experiments using sucrose instead as a nonpenetrating, but non-ionic, solute; as before, the magnitude of the shift-up was varied, keeping the starting

or final solute concentration constant. We found that the responses of phospholipid composition and synthetic rates of phosphatidylglycerol and phosphatidylethanolamine to a shift-up in sucrose concentration are very similar to those observed using NaCl (Adams *et al.*, 1987). Although there are differences of detail, sucrose shift-up causes a drastic initial inhibition of overall phospholipid synthesis, a "switch-over" in rates of phosphatidylethanolamine and phosphatidylglycerol synthesis, and a rapid rise in phosphatidylglycerol synthesis relative to that of phosphatidylethanolamine synthesis during the second phase of "haloadaptation." These synthetic changes are mirrored by phospholipid compositional changes that are very similar to those observed using NaCl (Russell *et al.*, 1986; Adams *et al.*, 1987). It appears, therefore, that osmotic pressure rather than alterations in ion concentration are responsible for triggering the haloadaptive phospholipid changes. In support of this are the results of similar experiments using glycerol, a penetrating and therefore non-osmotically-stressful non-ionic solute, which does not elicit the same response of phospholipid composition or synthesis (Adams *et al.*, 1987).

More recently, we have investigated the effects of non-ionic solutes on phospholipid synthesis *in vitro* using cell lysates and washed membrane preparations. These membrane systems respond to sucrose concentration, giving the same "switch-over" in rates of phosphatidylethanolamine and phosphatidylglycerol synthesis as found with NaCl (R. L. Adams and N. J. Russell, unpublished results). This observation raises the possibility that the effects of NaCl and sucrose are not osmotic, but instead are mediated through effects of the solutes on the membrane—perhaps directly on the phospholipid-synthesizing enzymes (e.g., by removal of water) or indirectly via alterations in lipid phase state/conformation (e.g., localized formation of nonbilayer-phase lipids).

Effect of High Salinity on Membrane Lipid Phase Properties

Bilayer and Nonbilayer Lipid Phases

The most common microbial phospholipids bear a negative charge from the phosphate residue, and are either anionic (e.g., phosphatidylglycerol and diphosphatidylglycerol) or zwitterionic (e.g., phosphatidylethanolamine and phosphatidylcholine), depending on the head-group constituent (Harwood and Russell, 1984). Some glycolipids (e.g., diacylgalactosylglycerol and diacyldigalactosylglycerol) are uncharged; others are anionic due to sulfate (in sulpholipids) or phosphate (in phosphoglycolipids) residues. Another feature of such lipid molecules, which is particularly relevant, is the relative

sizes of the polar head group and the nonpolar acyl chains. This gives the molecule an overall shape that can be regarded as a cylinder (equivalent sizes of polar and nonpolar moieties) or a cone (head group smaller or larger than the acyl chains). A consideration of the effects of molecular charge and size, and the thermodynamic and steric constraints that they impose, has formed the basis of an explanation of the various types of aggregates that lipids form when they are dispersed in an excess of water (Luzzati and Tardieu, 1974; Israelachvili *et al.*, 1980). The most familiar of these phases is the lamellar, in which the molecules are arranged in a bilayer that forms the ground structure of membranes. Lipids such as phosphatidylglycerol, phosphatidylcholine, and diacyldigalactosylglycerol adopt a lamellar phase. In contrast, other common microbial lipids such as unsaturated phosphatidylethanolamine, diphosphatidylglycerol, and diacylgalactosylglycerol adopt a hexagonal-II phase; in this conformation, the lipid molecules are arranged as cylinders with their head groups facing the water-filled interior (Cullis and de Kruijff, 1979). In addition, other phases are possible, including micellar, cubic, and hexagonal-I (in which the head groups face the outside of cylindrical structures). Cubic phases are formed by dioleoylphosphatidylethanolamine when the lipid is cycled between -5 and 15°C many times, and once formed is stable at 4°C for several weeks (Shyamsunder *et al.*, 1988). Such temperature cycling occurs in many natural environments, so this observation could be relevant to biomembranes.

It should be emphasized that such nonbilayer phases have not been observed directly in natural membranes, but only with pure lipid preparations or with simple mixtures. When nonbilayer-phase-forming lipids are mixed with a large enough proportion of bilayer-phase-forming lipids, the mixture adopts the lamellar phase. This is the situation in microbial membranes, particularly those of bacteria that may contain large proportions (i.e., $> 50\%$ of the total) of nonbilayer-phase-forming lipids, often unsaturated phosphatidylethanolamine (Cullis and de Kruijff, 1978; Harwood and Russell, 1984). The presence of proteins in natural membranes can also destabilize the bilayer phase, and this destabilization in model systems can be prevented by the addition of some nonbilayer-forming lipids (van Hoogevest *et al.*, 1984). It has been suggested that one of the functions of nonbilayer-forming lipids is to provide a choice of molecular geometries so as to minimize packing defects around the irregular surfaces of proteins, thereby preventing deleterious permeability "leaks" (Quinn and Williams, 1983).

It has also been proposed that nonbilayer-forming lipids in natural membranes may have functions in membrane fusion, and the transbilayer transport of proteins and lipids, all processes that require disruption of the bilayer structure (de Kruijff *et al.*, 1984; Rietveld and de Kruijff, 1986). In

addition, different lipid "shapes" are required on the two sides of a bilayer in regions where the membrane is highly curved, and this requirement can contribute to lipid asymmetry (Rothman and Lenard, 1977). In this respect, lipids with a propensity for forming hexagonal phases may be particularly relevant, because their head groups are smaller (hexagonal-II-forming) or larger (hexagonal-I-forming) relative to the size of the nonpolar acyl chains.

Many lipids, when dispersed alone or in simple mixtures, display a transition between lamellar and hexagonal-II phases under conditions that can be regarded as physiological, and therefore of biological, interest. The transition to a hexagonal-II phase is generally favored by an increase in temperature, a decrease in pH, dehydration, and the addition of cations. Since halotolerant and halophilic microorganisms live in high concentrations of NaCl, it is specially relevant to consider the effects of monovalent cations on lipid phase behavior in relation to the phenotypic and genotypic adaptations of membrane lipid composition discussed in previous sections of this review.

Effects of Cations on Lipid Phase Properties in vitro

The packing of lipids in the lamellar phase is altered by relatively low concentrations of cations, and studies of the effects of NaCl have been restricted largely to concentrations in the range 10–100 mM. Although such studies provide interesting information about lipid conformation, they are of little use in relation to halotolerant and halophilic microorganisms that grow in molar concentrations of salt. However, two investigations of the effects of molar NaCl concentrations up to saturation on the thermal phase behavior of purified saturated phosphatidylethanolamines have been reported by Harlos and Eibl (1981) and Seddon *et al.*, (1983). An increase in NaCl concentration from a few millimolar up to saturation has a relatively small effect on the gel to liquid-crystalline transition temperature, increasing it by $\sim 10\%$; there is a larger effect in the opposite direction on the lamellar to hexagonal transition temperature, which is decreased by nearly 20%. Both of these effects are due to increased stabilization of interactions between the phosphatidylethanolamine head groups, which is probably not due to simple electrostatic screening by the salt. It is more likely to result from the displacement of water of hydration from the head group by cations, which in turn would reduce head-group surface area and therefore favor hexagonal-II phase formation. This interpretation is consistent with the observation that the head group of phosphatidylcholine, which forms a lamellar phase, binds more water than does the head group of phosphatidylethanolamine (Yeagle and Sen, 1986). Changes in head-group hydration and hydrogen-bonding capacity are believed to be responsible for the reduced fluidity of phosphatidylglycerol

and phosphatidylcholine vesicles in the presence of molar concentrations of sucrose (Uso and Rossignol, 1984). However, it should be said that such hydration effects and the function of the hydrogen-bonding potential of water molecules bound to phospholipid head groups are poorly understood (Gruner *et al.*, 1988).

Unsaturation of the acyl chains produces a dramatic reduction in the lamellar to hexagonal-II phase transition temperature of phosphatidylethanolamine (Tilcock and Cullis, 1982), effectively lowering it from nonphysiological (except for a few extreme thermophiles) to physiological temperatures for the majority of microorganisms. For example, the value for dioleoylphosphatidylethanolamine is 5–10°C (Tilcock and Cullis, 1982; Gruner *et al.*, 1988). Therefore, the effect of high salinity on phosphatidylethanolamines having unsaturated acyl chain lengths of 14–18 carbons is to cause them to form the hexagonal-II phase when dispersed alone in an excess of aqueous solvent. Using freeze-fracture electron microscopy and X-ray diffraction, we have shown this to be so for the phosphatidylethanolamine extracted from the moderately halophilic bacterium *V. costicola*, which contains $>60\%$ of unsaturated fatty acids, mainly C16 and C18, when grown in 1 M or 3 M NaCl (G. C. Sutton, P. J. Quinn, and N. J. Russell, unpublished results). On the basis of experiments with simple mixtures of purified unsaturated phosphatidylethanolamines (Tilcock and Cullis, 1982), one can predict that, for a natural membrane, there would be a tendency for the unsaturated phosphatidylethanolamine in microbial membranes to phase separate as a hexagonal-II domain when the salinity of the culture medium was increased. While small microdomains of hexagonal-II phase lipids may well have specific functions in natural membranes (see above), the separation of a bulk domain would have disastrous consequences on the permeability properties of the membrane. Even if phase separation does not occur, the interactions responsible for the transition from lamellar to hexagonal-II phase are still present in the membrane, and will affect the properties of the bilayer and may have physiological consequences (Shyamsunder *et al.*, 1988).

Another phospholipid found abundantly in microbial membranes is phosphatidylglycerol, which forms a lamellar phase under a variety of conditions of temperature, pH, and cation concentration (Blaurock and McIntosh, 1986; Wilkinson and McIntosh, 1986; Pascher *et al.*, 1987). It appears that phosphatidylglycerol can maintain a lamellar phase under these different conditions by altering the conformation of its head group and/or acyl chains, and by varying the bilayer thickness (Watts *et al.*, 1981). Moreover, phosphatidylglycerol is able to suppress the transition of phosphatidylethanolamine to the hexagonal-II phase (Farren and Cullis, 1980). This property is probably related to the requirement for an anionic lipid, which has been known for a long time to be indispensable for the preparation of

osmotically active liposomes using egg-yolk phospholipids (Bangham *et al.*, 1967); the same is also true of phospholipids extracted from *Escherichia coli* (Yoshikawa *et al.*, 1985). In relation to natural membranes, it may well be significant that it has been demonstrated using mutants that phosphatidylglycerol is necessary for the viability of *E. coli* (Nishijima and Raetz, 1979). It has been suggested that phosphatidylglycerol plays a vital role in stabilizing the plasma membrane of *E. coli* against osmotic pressure (Yoshikawa *et al.*, 1985). In this context, it is interesting that it is often phosphatidylglycerol that increases in halotolerant and halophilic microorganisms subjected to osmotic stress by increased salinity (Table I).

Despite the interest in membranes of extremely halophilic archaebacteria, surprisingly little is known about the conformation of their characteristic diphytanylglycerol diether lipids. In a recent freeze-fracture electron-microscopic study of the major lipids purified from *H. cutirubrum*, Quinn *et al.*, (1986) showed that, in the presence of 5 M NaCl, the glycolipid sulfate formed a lamellar phase whereas phosphatidylglycerol phosphate adopted a nonbilayer phase. Mixtures of the lipids tended to phase separate in salt, and it was inferred that *in vivo* it was interaction with proteins that imposed a bilayer structure on the ether lipids. Compared with membranes in eubacteria, those in halobacteria have an unusual structure, in which there is a crystalline packing of the lipid and protein (Unwin and Henderson, 1975). However, it would seem that, in halobacterial membranes, just as in eubacteria, there are lipid components that prefer to adopt a nonbilayer phase in high-salinity media and that these predisposing forces are present in the membrane *in vivo*.

Effects of Cations on Lipid Phase Properties in vivo

The first hypothesis put forward to explain why halotolerant and halophilic microorganisms increase their proportion of anionic lipids in response to raised salinity was that it was a mechanism to achieve charge balance at the membrane surface (Hiramatsu *et al.*, 1980). However, Russell and Kogut (1985) pointed out that, on theoretical grounds, the actual increase in *amount* of anionic lipids could only account for negative-charge shielding by cations of the order of millimolar, not molar salt concentrations. This was supported experimentally by the observation in *V. costicola* (Adams *et al.*, 1987) and a range of halotolerant food-spoilage bacteria (Bygraves and Russell, 1988) that the same changes in anionic lipid composition occur in response to non-ionic solutes. Therefore, the lipid changes are not related specifically to NaCl or ionic effects.

An alternative hypothesis considered that a rise in external solute concentration enhances the predisposition for membrane lipids such as

unsaturated phosphatidylethanolamine to form nonbilayer phases, which acts as a trigger for subsequent events both in the membrane and the cell interior. Initially the membrane may increase its passive permeability, or the activity of ion pumps could be affected by the lipid conformational changes, which would alter the internal ionic environment. Some suggestions for how the effector systems could be triggered have been described by Russell and Kogut (1985). Within the membrane, the tendency to form nonbilayer phases could activate specific phospholipid-synthesizing enzymes, which are located in the membrane, resulting in a rise in the proportion of lipids, such as phosphatidylglycerol, that prefer the lamellar phase. This would counteract the disruptive forces of the nonbilayer-phase-forming lipids, thereby maintaining the bilayer integrity and restoring normal passive permeability properties. The system, therefore, would be a self-balancing one because the stimulus for activation of, for example, phosphatidylethanolamine synthesis would be counteracted. In *V. costicola*, the pattern of phosphatidylglycerol synthesis after a sudden shift-up in solute concentration is consistent with this hypothesis in that, following the initial inhibition, there is a sharp rise in synthetic rate, which then slows to a new steady-state level (Russell *et al.*, 1985; Adams *et al.*, 1987).

The hypothesis also explains why ionic and non-ionic solutes have similar effects, as long as they stimulate the formation of nonbilayer phases —possibly through a common mechanism involving dehydration of the head group. Currently we are testing this hypothesis by determining the phase behavior of purified phospholipids from *V. costicola* in different solute concentrations, and relating this to their effects on membranes *in vivo*. This should show whether the common changes in membrane lipids that are observed when microorganisms adapt to high salinity represent a mechanism for preventing deleterious structural reorganization of membrane constituents.

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