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# Multi-author Review

# Biology of halophilic bacteria, Part II

## Membrane lipids of extreme halophiles: biosynthesis, function and evolutionary significance

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*Abstract.* Archaebacteria (archaea) are comprised of three groups of prokaryotes: extreme halophiles, methanogens and thermoacidophiles (extreme thermophiles). Their membrane phospholipids and glycolipids are derived entirely from a saturated, isopranoid glycerol diether, *sn*-2,3-diphytanylglycerol ('archaeol') and/or its dimer, dibiphytanyldiglyceroltetraether ('caldarchaeol'). In extreme halophiles, the major phospholipid is the archaeol analogue of phosphatidylglycerolmethylphosphate (PGP-Me); the glycolipids are sulfated and/or unsulfated glycosyl archaeols with diverse carbohydrate structure characteristic of taxons on the generic level. Biosynthesis of these archaeol-derived polar lipids occurs in a multienzyme, membrane-bound system that is absolutely dependent on high salt concentration (4 M). The highly complex biosynthetic pathways involve intermediates containing glycerol ether-linked $C_{20}$-isoprenyl groups which are reduced to phytanyl groups to give the final saturated polar lipids. In methanogens, polar lipids are derived both from archaeol and caldarchaeol, and thermoacidophiles contain essentially only caldarchaeol-derived polar lipids. The function of these membrane polar lipids in maintaining the stability, fluidity and ionic properties of the cell membrane of extreme halophiles, as well as the evolutionary implications of the archaeol and caldarchaeol-derived structures will be discussed.
*Key words.* Archaea (archaebacteria); extreme halophiles; archaeol phospholipids; archaeol glycolipids; membrane function; evolution.

### *Introduction*

It has long been postulated that the structure and composition of membrane lipids might be characteristic of related organisms under their particular growth conditions[16]. This generalization has been found to apply well to bacteria, on the level of the family and even the genus, so that lipid structure and composition, or the lipid 'profile', can serve as a bacterial taxonomic marker[2,19,20]. For the archaebacteria (archaea)[45,46], membrane lipid patterns[7,17,20,25,37], along with their 16S ribosomal RNA sequences[45,46] and their cell wall structures[14], are particularly useful taxonomically to distinguish between the three groups of archaebacteria (extreme halophiles, methanogens and thermoacidophiles) and to clearly delineate the archaea from all other organisms. Archaeal membrane lipids are unique in consisting of derivatives of a $C_{20}$-$C_{20}$-isopranyl glycerol diether, *sn*-2,3-diphytanylglycerol[17] (termed 'archaeol'[33]) and its dimer, dibiphytanyldiglycerol tetraether[7,25] (termed 'caldarchaeol'[33]) (see fig. 1). These 'core' ether lipids are completely absent in eubacteria and eukarya, which contain predominantly diacylglycerol-derived lipids. The presence of plasmalogen (monoacyl-monoalk-1-enyl-glycerol) forms of glycolipids in some obligate anaerobic eubacteria[19], and dialkylether glycerolipids in a eubacterial, anaerobic thermophile[25], as well as the presence of a geranylgeranyl glycerol monoether in a brown algae[1],

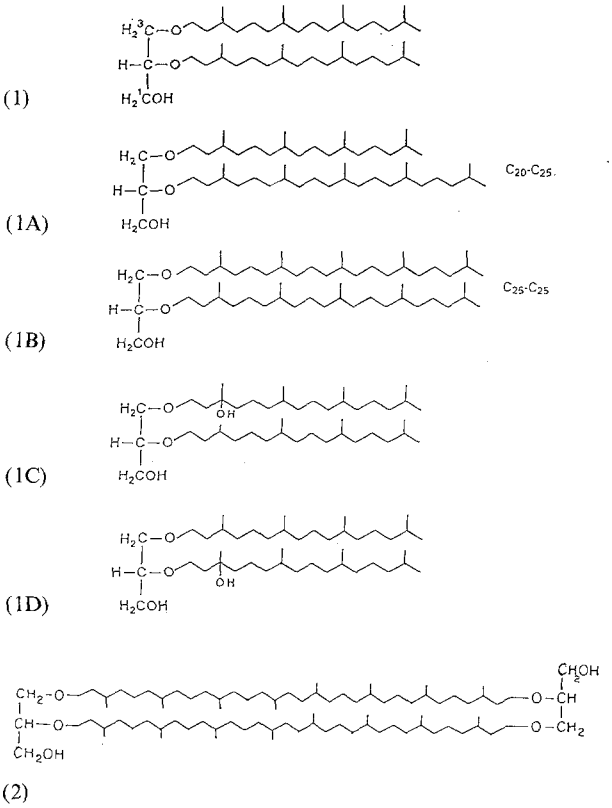



Figure 1. Structures of core lipids: diphytanylglycerol diether (**1**, archaeol) and variants (**1A–1D**); and dibiphytanyldiglycerol tetraether (**2**, caldarchaeol).

should not detract from the reliability of the above-mentioned criteria for recognition of archaebacterial lipids, so long as lipids derived from archaeol and caldarchaeol or their variants have *not* been detected in eubacteria or eukarya.

Within the archaea, the extreme halophiles are distinguished by their containing only archeol-derived lipids, while the methanogens contain both archaeol- and caldarchaeol-derived lipids and the thermoacidophiles contain predominantly caldarchaeol-derived lipids[7,19,20,25,37].

The existence of unusual ether lipid structures in archaea raises questions concerning the biosynthetic pathways for these lipids and their function in archaeal membranes, and also the evolutionary relationships within the archaea and between archaea, eubacteria and eukarya. This review will deal primarily with the membrane lipids of extreme halophiles, and will cover their chemical structures and biosynthetic pathways, their role in membrane structure and function, and the taxonomic and evolutionary implications of their unique structures.

### *Polar lipid structure and composition of extreme halophiles*

The structure and configuration of the core lipid, diphytanylglycerol (archaeol) was established as *sn*-2,3-di-*O*-3R,7R,11R,15-tetramethylhexadecylglycerol ($C_{20}$-$C_{20}$, structure **1**, fig. 1) and confirmed by chemical synthesis[17]. Variants of the diphytanylglyceroldiether (see fig. 1) found in some extreme halophiles are as follows:

1) both the $C_{20}$-$C_{25}$ diether (**1A**) and $C_{25}$-$C_{25}$ diether (**1B**) core lipids occur in alkaliphilic (*Natronobacterium* and *Natronococcus*) species of extreme halophiles[4,5];

2) the $C_{20}$-$C_{25}$ diether (**1A**) core lipid also occurs in lipids of *Halococci* from salt flats in Spain[32], in a non-alkaliphilic halobacterium (strain 172) from Japan[15,29] and in *Halobacterium*, *Haloferax* and *Natronobacterium* strains from hypersaline environments in India[43];

3) novel 3-hydroxydiether core lipids (**1C** and **1D**), which have been identified in some methanogens[37], have now been found in haloalkaliphiles (*Natronobacterium*) from hypersaline environments in India[43].

### *Phospholipids*

The phospholipids of extreme halophiles (see fig. 2) consist of one major and two or more minor acidic phospholipids. The major phospholipid was reported to be an archaeol analogue of phosphatidylglycerophosphate[15,17,32] (PGP, structure **4**, fig. 2). However, recent studies[11,21,42] have now identified the structure of the major phospholipid as the monomethylated derivative of PGP (PGP-Me, **4A**). PGP-Me and PGP can be distinguished by mass spectrometry (FAB-MS), nuclear magnetic resonance (NMR), spectroscopy and thin layer chromatography (TLC); TLC in an alkaline solvent system greatly retards the mobility of PGP but not that of PGP-Me[21]. PGP-Me (**4A**) is most likely the major phospholipid in all known extreme halophiles, including several species of *Halobacteria*, *Haloarcula*, *Haloferax*, *Halococci*, *Natronobacteria* and *Natronococci*[21,43].

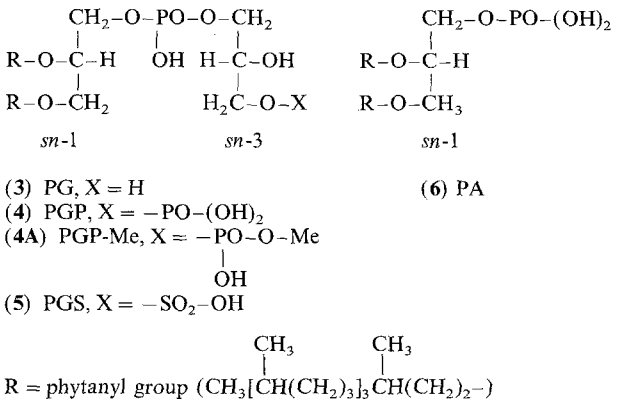



Figure 2. Structures of archaeol phospholipids in extreme halophiles.

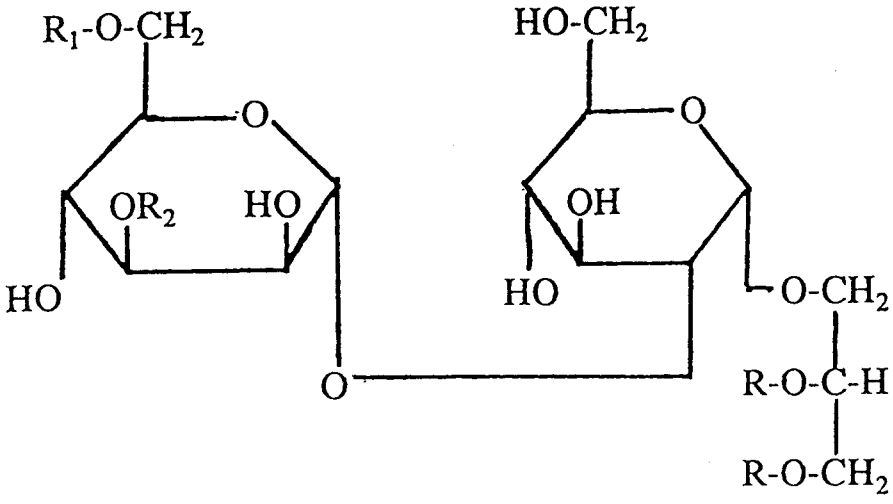
The minor phospholipids[7,15,17,32] have been identified as the archaeol analogues of phosphatidylglycerol (PG, structure **3**, fig. 2), phosphatidylglycerosulfate (PGS, **5**) and phosphatidic acid (PA, **6**).

PGP, as well as PA, are probably present as biosynthetic intermediates[20,30,31] (see fig. 5). Another minor phospholipid, that has been identified in *Natronococcus ocultis*, is the cyclic form of PGP[27]. It is possible that this component may also be a biosynthetic intermediate in the formation of PGP-Me.

Stereochemically, the structures of the archaeol analogues of PG, PGP, PGP-Me and PGS are unusual in that both glycerol moieties have the opposite configuration to those in the correponding diacylglycerol forms of PG and PGP found in eubacteria and eukaryotes[17] (see fig. 2).

### *Glycolipids*

The glycolipids of *Halobacteria* (see fig. 7) include a major sulfated triglycosyl archaeol, galactosyl-3-sulfate-mannosyl-glucosyl-archaeol[17] (S-TGA-1, structure **7**), and several minor glycolipids[36], such as: the desulfated TGA-1 (**7A**); a sulfated tetraglycosyl archaeol (S-TeGA, structure **8**) and its desulfated product TeGA (**8A**). *Haloarcula* species contain a major triglycosyl archaeol, glucosyl-mannosyl-glucosylarchaeol (TGA-2, structure **9**), and a minor diglycosyl archaeol (mannosyl-glucosyl archaeol, DGA-2) of unidentified structure[9]. *Haloferax* species contain a sulfate diglycosylarchaeol, mannosyl-6-sulfate-glucosyl-archaeol[24] (S-DGA-1, structure **10**) and its desulfated product, DGA-1 (**10A**). It is of interest that *Hb. saccharovorum*[26,39] and a *Halococcus* strain, *Hc. saccharolyticus*[32], both contain the *Haloferax* marker glycolipid, S-DGA-1, but not DGA-1.




| Genus: | | | | |
|---|---|---|---|---|
| *Halobacterium* | (7) | S-TGA-1, | $R_1 = 3\text{-}SO_3\text{-}\beta\text{-Gal}p$ | $R_2 = H$ |
| *Halobacterium* | (7A) | TGA-1, | $R_1 = \beta\text{-Gal}p$ | $R_2 = H$ |
| *Halobacterium* | (8) | S-TeGA, | $R_1 = 3\text{-}SO_3\text{-}\beta\text{-Gal}p$ | $R_2 = \alpha\text{-Gal}f$ |
| *Halobacterium* | (8A) | TeGA, | $R_1 = \beta\text{-Gal}p$ | $R_2 = \alpha\text{-Gal}f$ |
| *Haloarcula* | (9) | TGA-2, | $R_1 = \beta\text{-Glc}p$ | $R_2 = H$ |
| *Haloferax* | (10) | S-DGA-1, | $R_1 = -SO_2OH$ | $R_2 = H$ |
| *Haloferax* | (10A) | DGA-1, | $R_1 = H$ | $R_2 = H$ |

R = phytanyl group $(CH_3[\overset{CH_3}{\overset{|}{C}}H(CH_2)_3]_3\overset{CH_3}{\overset{|}{C}}H(CH_2)_2\text{-})$

Figure 3. Structures of archaeol glycolipids in extreme halophiles.

The structures of the glycolipids (**7**–**10A**) appear to be derived from a basic diglycosyl archaeol, mannosyl-glucosyl-archaeol (DGA-1, **10A**) by substitution of sugar or sulfate groups at the 3 or 6 positions of the mannose residue[23,39] (see fig. 3). However, such a structural relationship between the known halophilic glycolipids does not necessarily hold for some newly discovered glycolipids (see fig. 4), for example:

1) the 'S-DGA-2' of the extreme halophile from Japan[15], which appears to be a mannosyl-2,6-disulfate-(1-2)-glucosyl archaeol[29] (structure **11**);

2) the mannosyl-2-sulfate-(1-2)-glucosyl-archaeol (S-DGA-5, structure **14**) from *Hb. trapanicum*[41];

3) the mannosyl-2-sulfate-(1-4)-glucosyl-archaeol (S-DGA-3, structure **12**) from *Hb. sodomense*[40]; and

4) an unsulfated glucopyranosyl-1,6-glucopyranosyl-archaeol (DGA-4, structure **13**) from a *Natronobacterium* strain SSL1 in salt locales in India[43]. This latter glycolipid appears to be present in traces also in other *Natronobacterium* strains (SSL2-SSL6) from India, and in *Nb. gregoryi*[43].

*Taxonomic relations*

The polar lipid composition of extreme halophiles, particularly the glycolipid composition, appears to be correlated with the taxonomic classification of these archaea on the level of the genera so far delineated[7,20,23,39]: *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Natronobacterium* and *Natronococcus*. Species of all of these genera were found to contain the $C_{20}$-$C_{20}$-archaeol analogue of PGP-Me (**4A**) as the major phospholipid, $C_{20}$-$C_{20}$-archaeol PG (**3**) and PGS (**5**) as minor phospholipids, and small to trace amounts of $C_{20}$-$C_{20}$-archaeol PA (**6**)[7,17,20,32], with the following diagnostic exceptions:

1) in addition to the $C_{20}$-$C_{20}$ species of phospholipids, $C_{20}$-$C_{25}$ and $C_{25}$-$C_{25}$ species of PGP-Me and PG occur in haloalkaliphiles *Natronobacterium* and *Natronococcus*[4,5] including the *Natronobacterium* strains from salt locales in India[43];

2) the $C_{20}$-$C_{25}$ species of phospholipids also occurs in *Halococcus saccharolyticus* and other *Halococci* from Spain[32];

3) PGS is characteristically absent in *Haloferax*, *Halococci*, *Natronobacterium* and *Natronococcus* species[15,20] and it is unexpectedly absent in a *Haloarcula* strain (A4-1) from Granada, Spain (Monteoliva-Sanchez, M., Ruiz Rodriguez, C., and Kates, M., unpublished data); and

4) a cyclic form of PGP was found in *Natronococcus occultis* but not in any *Natronobacterium* species[27].

The glycolipid composition is more discriminating (see fig. 3), and the following generalizations may be made:

1) *Halobacteria* contain S-TGA-1 (**7**) as the major glycolipid, and TGA-1 (**7A**), S-TeGA (**8**), and TeGA (**8A**) as minor glycolipids;

2) *Haloarcula* species contain major amounts of TGA-2 (**9**) and minor amounts of an unidentified diglycosylarchaeol, DGA-2 (containing glucose and mannose);

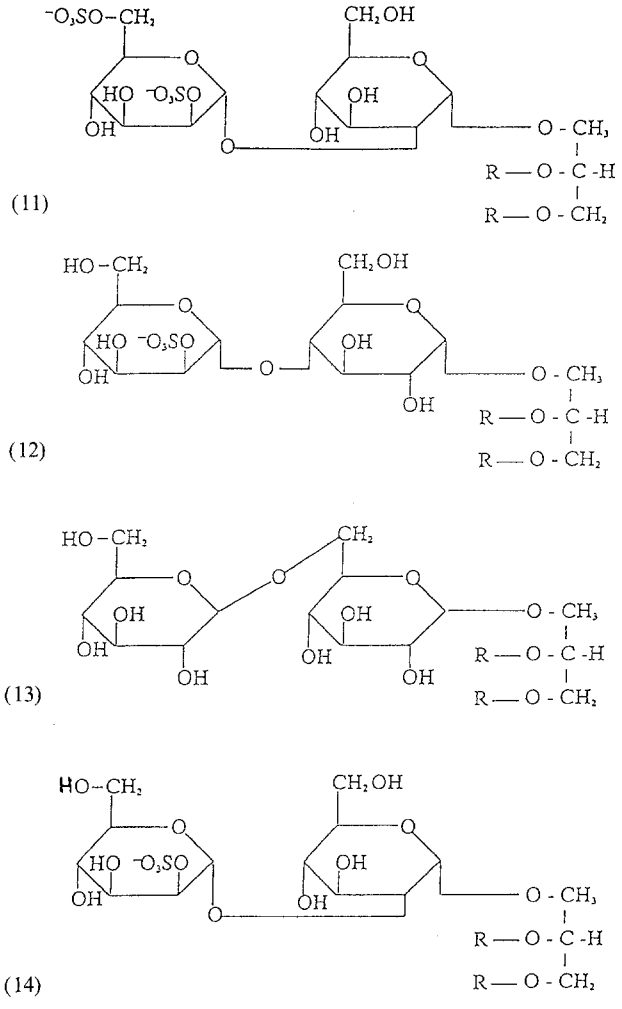


Figure 4. Structures of novel glycolipids in extreme halophiles: **11**, Manp-2,6-disulfate-α1-2-Glcpα1-1-archaeol (S-DGA-2); **12**, Manp-2-sulfate-α1-4-Glcpα1-1-archaeol (S-DGA-3); **13**, Glcp-1-6-Glcp-archaeol (DGA-4). R = phytanyl group; and **14**, Man-2-sulfate-α1-2-Glcpα1-1-archaeol (S-DGA-5).

3) *Haloferax* species contain S-DGA-1 (**10**) as major glycolipid and DGA-1 (**10A**) as minor glycolipid;
4) some *Halococci* (*Hc. morhuae*, *Hc. litoralis*, etc.)[19] are reported to contain S-TGA-1 (**7**), TGA-1 (**7A**), S-TeGA (**8**), but *Hc. saccharolyticus*[32] contains only S-DGA-1 (**10**), and trace amounts of an unidentified glycolipid and an unidentified phosphoglycolipid; and
5) neither the *Natronobacteria* nor the *Natronococci* examined[6,7,27] were reported to contain any glycolipids, but a new unsulfated glycolipid, DGA-4 (**13**) has been detected in *Natronobacteria* from India[43], and a glycolipid migrating with DGA-4 (**13**) can be detected on TLC plates of *Nb. gregoryi* lipids, when overloaded[43].
The glycolipid and phospholipid profiles described above appear to be sufficient to delineate each of the known genera of extremely halophilic bacteria. However, the lipid profiles of uncertain taxa and of some new species do not necessarily fit the pattern shown in figure 3, for example: *Halobacterium sodomense*[40] with its new glycolipid, S-DGA-3 (**12**), which is probably also present in some unidentified 'Halobacterial' strains (e.g., strains 3.5 rp 4 and 3.1 palp 4)[19,23,39], and *Hb. trapanicum*[41] with another novel glycolipid, S-DGA-5 (**14**). These strains do not contain any glycolipids characteristic of *Halobacteria*, nor are the novel glycolipids (**12, 14**) found in any known *Halobacteria*[19,40,41]; they should be further studied by 16S rRNA sequencing for possible reassignment to another or new genus[20,41]. *Halobacterium saccharovorum*[26] and *Halococcus saccharolyticus*[32], both of which contain the *Haloferax* glycolipid marker S-DGA-1 (**10**), may also require reclassification into new genera or subspecies[19,26,39,40,41].

### *Biosynthetic pathways for archaeol phospho- and glycolipids*

#### *Core lipids*

Biosynthesis of the archaeol analogues of phospholipids and glycolipids in extreme halophiles occurs in a multienzyme, membrane-bound system that is absolutely dependent on 4 M salt concentration[15,19,20]. Synthesis of the isoprenoid/isopranoid chains in the archaeol lipid core takes place via the mevalonate pathway for isoprenoids (somewhat modified)[8], which is also absolutely dependent on 4 M salt[15]. Only small amounts of normal fatty acids are synthesized by a fatty acid synthetase (FAS) which is largely (ca 80%) inhibited by 4 M NaCl or KCl[35]. However, the remaining FAS activity is sufficient for the formation of saturated fatty acids (14:0, 16:0, 18:0) which are not polar lipid-bound but are esterified to proteins of the red membrane and not to bacteriorhodopsin in the purple membrane of extreme halophiles (Pugh and Kates, unpublished data).
The synthesis of the $C_{20}$-isopranoid chains is achieved by a modified mevalonate pathway which starts from acetate and involves lysine to provide the branch-methyl and methine carbons by an unknown mechanism[8], and proceeds to the formation of geranylgeranyl-PP or a partially reduced $C_{20}$-isoprenoid[15]. The latter are then used for alkylation of a suitable glycerol derivative to form a digeranylgeranyl or di-$C_{20}$-isoprenyl glycerol ether derivative ('pre-diether'), followed by incorporation of the polar head groups to form the 'pre-phospholipids' or 'pre-glycolipids' and reduction of the geranylgeranyl or isoprenyl chains to phytanyl chains as the final step in the synthesis of the individual phospholipids and glycolipids[30,31] (see figs 5 and 6). Evidence supporting the involvement of an isoprenyl-PP as donor of the $C_{20}$-isoprenyl group is provided by the demonstration that bacitracin, which complexes with polyprenylpyrophosphates, is a powerful inhibitor of the biosynthesis of the 'pre-diether' and of phospholipids and glycolipids in whole cells of *H. cutirubrum*[31].
A pertinent question is how do the halophiles form lipids having the *sn*-1'-glycerol configuration rather than

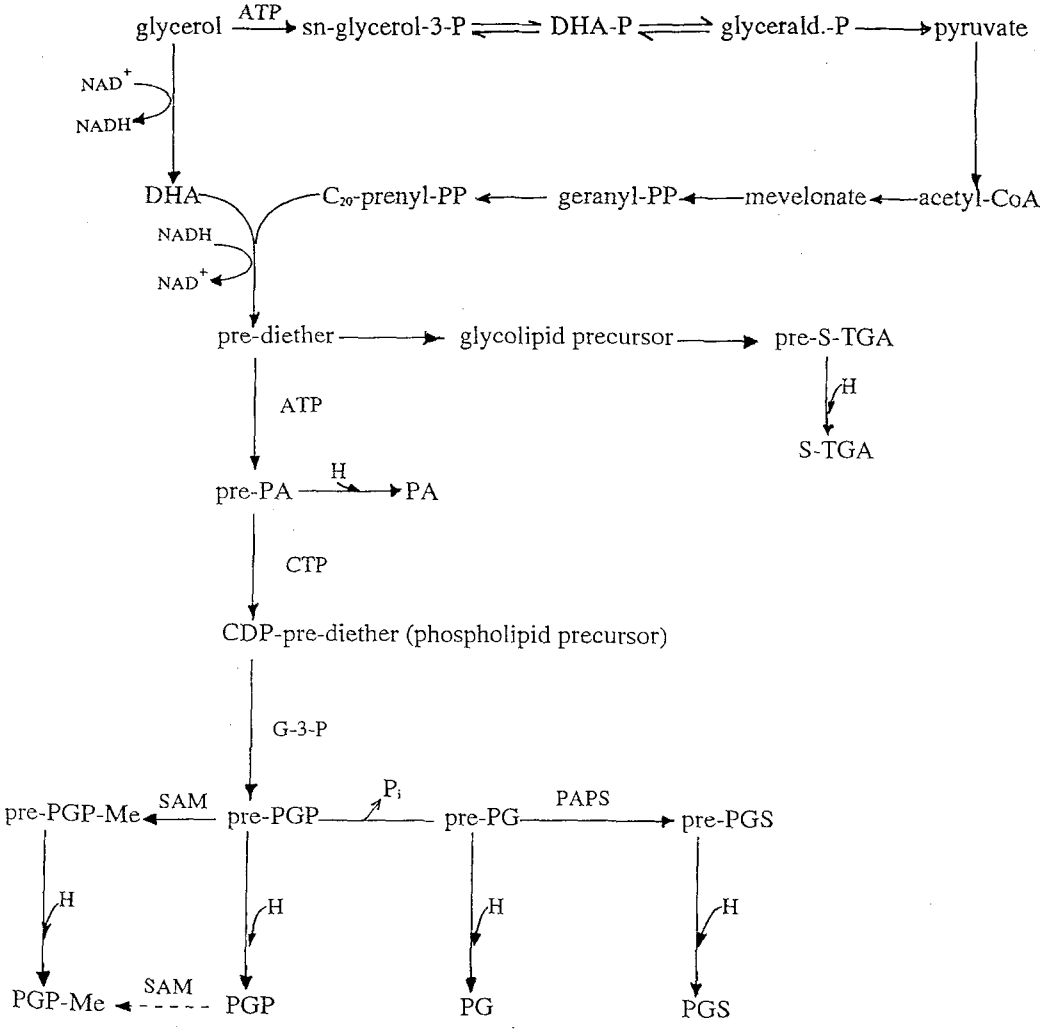
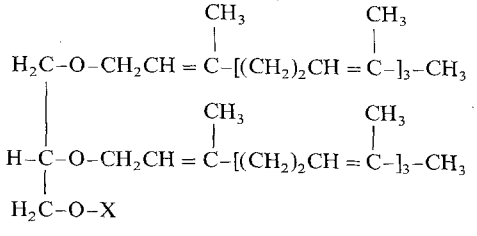
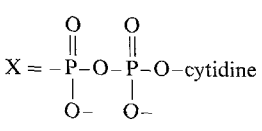



Figure 5. Proposed pathways for biosynthesis of archaeol phospholipids and glycolipids in extreme halophiles.



pre-diether X = unidentified
pre-PA X = $-PO-(OH)_2$
pre-PG X = $-PO(OH)-O-CH_2CH(OH)CH_2-OH$
pre-PGP X = $-PO(OH)-O-CH_2CH(OH)CH_2-O-PO(OH)_2$
pre-PGP-ME X = $-PO(OH)-O-CH_2CH(OH)CH_2-O-PO(OH)-OMe$
pre-PGS X = $-PO(OH)-O-CH_2CH(OH)CH_2-O-SO_3-OH$




Glycolipid precursor X = unidentified
pre-S-TGA X = –Glc-Man-Gal-3-$SO_3$–

Figure 6. Structures of isoprenyl precursors of archaeol phospholipids and glycolipids of extreme halophiles.

the ubiquitous *sn*-3-configuration? Both glycerokinase and glycerophosphate dehydrogenase activities, in the cell envelope and cytoplasm of *H. cutirubrum* form only the *sn*-3-glycerophosphate (GP) stereoisomer[44]. *sn*-3-GP could act as a direct acceptor for alkylation with geranylgeranyl-PP if it went through an oxidation (dehydrogenation) step followed by a stereospecific reduction step to form the *sn*-1-glycerol derivative[44]. Consistent with this mechanism, it was found in whole cell labelling studies that the glycerol moiety of diphytanylglycerol undergoes dehydrogenation at C-2 but not at C-1 (or C-3)[15,22]. These restraints appeared to eliminate the participation of triose phosphates (DHAP and glyceraldehyde-P) and glycerol-P as possible acceptors in the synthesis of the 'pre-diether', since they would exchange hydrogen at C-1 by aldo-keto or keto-enol isomerizations (see fig. 5). However, it would be possible for triose phosphates to act as precursors of the glycerol moiety in the archaeol lipids if they were associated with the cell membrane and kept physically separate from the cytoplasm[20,22]. With these considerations in mind, it was hypothesized[15,20,30] that the 'pre-diether' may be formed by alkylation of dihydroxyacetone (DHA) or dihydroxyacetone phosphate (DHAP) with geranylgeranyl-PP and concomitant stereoselective reduction of the ketogroup to form the *sn*-2,3-geranylgeranylglycerol derivative (figs 5 and 6); *sn*-3-GP or glycerol itself could also serve as an acceptor provided they underwent dehydrogenation at C-2 and then stereospecific reduction as described for DHA as acceptor.

Using intact cells of *H. Halobium*, Kakinuma et al.[13] have confirmed the loss/retrieval of hydrogen from C-2 of the archaeol glycerol by $NAD^+$/NADH oxidation/reduction. They have suggested that synthesis of archaeol occurs by stepwise alkylation of *sn*-3-GP with geranylgeranyl-PP to form first *sn*-3-geranylgeranyl-1-GP and then *sn*-2,3-di-geranylgeranyl-1-GP, the change of configuration being achieved by stereo-specific oxidation/reduction with $NAD^+$/NADH. Archaeol variants, such as $C_{20}$-$C_{25}$- or $C_{25}$-$C_{25}$-archaeol[4,5] could presumably be formed by substituting the $C_{25}$-isoprenyl-PP for geranylgeranyl-PP as prenyl donor at the appropriate steps in the alkylation process, or by chain elongation of the $C_{20}$-isoprenyl intermediates.

*Phospholipids*

Pulse-labelling studies with whole cells of *H. cutirubrum* showed the following product-precursor relationship of the phospholipids[30]: pre-diether → pre-PA → phospholipid precursor → pre-PGP → pre-PG → pre-PGS. The following pathway for biosynthesis of phospholipids in extreme halophiles was proposed[20,30] (figs 5 and 6). The pre-diether is phosphorylated with ATP, and the pre-PA formed is converted to the 'phospholipid precursor', most likely cytidine diphosphate archaeol[31], which is coupled with glycerophosphate to form pre-PGP. Dephosphorylation of pre-PGP by a specific phosphatase would form pre-PG, which would then be converted to pre-PGS by sulfation with phosphoadenosine phosphosulfate (PAPS). Each of the 'pre-' phospholipids is subsequently hydrogenated to give the final, saturated archaeol analogues of PGP, PG and PGS. PGP-Me may be formed by methylation of pre-PGP-Me with S-adenosylmethionine (SAM) and hydrogenation, or by methylation of PGP (fig. 5).

*Glycolipids*

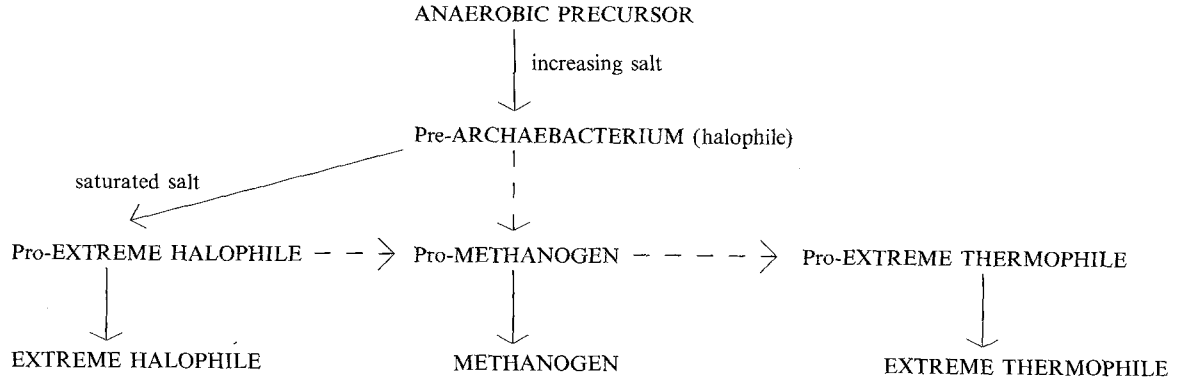
The pre-diether also serves as the precursor of the glycolipids[15,19] (figs 5 and 6), being glycosylated stepwise with glucose, mannose and galactose followed by sulfation with PAPS to give the pre-S-TGA which is finally reduced to the saturated S-TGA (**7**). Evidence in support of a stepwise glycosylation and sulfation has been provided by pulse-labelling studies with whole cells of *H. cutirubrum*, which established the following product-precursor relationship between the glycolipids of this bacterium[15,19,30] (see fig. 3): pre-diether → glycolipid precursor → Glc-archaeol (MGA-1) → DGA-1 → TGA-1 → S-TGA-1. The minor glycolipid S-TeGA (**8**) might be biosynthesized by galactofuranosylation of S-TGA-1 (**7**) or by galactofuranosylation of TGA-1 (**7A**) followed by sulfation with PAPS. The minor non-sulfated glycolipids TGA-1 (**7A**) and TeGA (**8A**) might be formed by deletion of the appropriate sulfation steps or by the action of sulfatases on S-TGA-1- and S-TeGA. Such pathways would account for the presence of each of the glycolipids found in the *Halobacteria* (fig. 3). In *Haloferax* species, the major glycolipid S-DGA-1 (**10**) might be formed by deletion of the galactosylation step of DGA-1 and insertion of a specific DGA-1 sulfation step. In *Haloarcula*, the major glycolipid TGA-2 (**9**) (fig. 3) might be formed by replacement of the galactosylation step with a glucosylation reaction using UDP-Glc, and deletion of any glycolipid sulfating enzyme system. Thus, the characteristic glycolipid composition of the three genera of *halobacteria* (fig. 3) could be achieved by deletion and/or insertion of the genes for the appropriate glycosylation enzymes and sulfating enzymes mentioned above.

*Function of archaeol lipids*

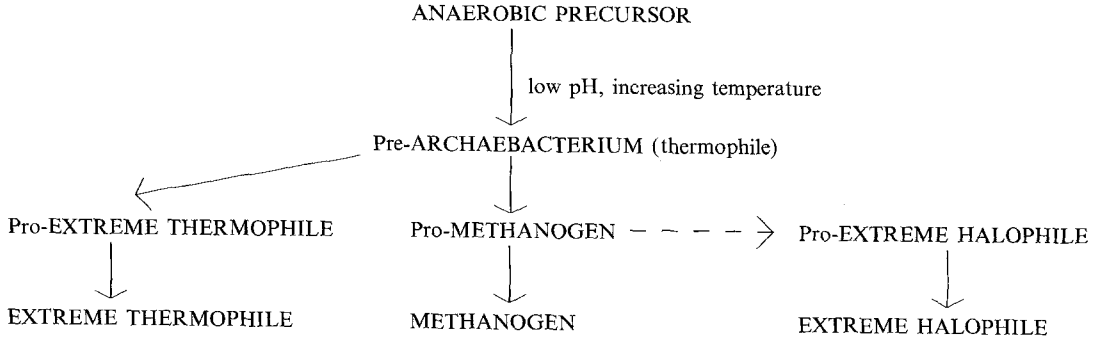
The peculiar ether lipids of extreme halophile membranes, and indeed of archaebacterial membranes in general, appear to have properties that are well suited to the rather harsh environments in which the archaebacteria live. Thus, the alkyl ether structure, in contrast to the acyl ester structure found in eukarya and eubacteria, would impart stability to the lipids over a wide range of pH and temperatures; and the saturated alkyl chains would impart stability towards oxidative degradation,

particularly for the extreme halophiles that are exposed to air and sunlight[15,19]. Also, the branched isopranyl structure of archaeol chains would ensure that the halophile membrane lipids are in the liquid crystalline state at all ambient temperatures that might be encountered (−40 to +40°C). Furthermore, the unnatural *sn*-1-configuration of the core glycerol diether would impart resistance to attack by phospholipases released by other organisms and would thus have a survival value for the extreme halophiles[19,20]. A more specific function is associated with the sulfated triglycosylarchaeol (S-TGA-1, 7), which is found entirely in the exterior surface layer of the purple membrane (PM) of *Halobacteria*[12,19]. S-TGA-1, together with the major phospholipid PGP-Me[19,20] can participate in proton conductance pathways[38] through their polar headgroup sulfate and phosphate groups, respectively. This would aid in transporting the protons transduced by light-activated bacteriorhodopsin across the outer surface of the PM to the red membrane (RM), where the PGP-Me

**Scenario 1.**




**Scenario 2.**




**Scenario 3.**

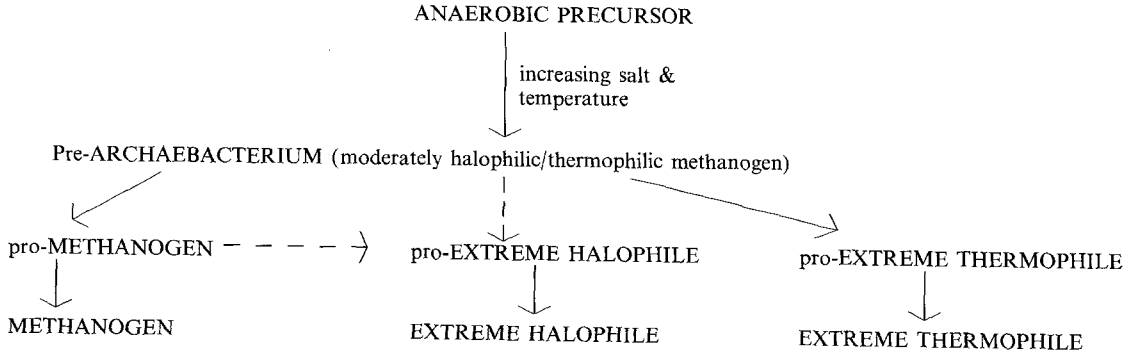



Figure 7. Proposed evolutionary relationships between halophiles, methanogens and extreme thermophiles based on polar lipid composition[18,20].

headgroup phosphates would conduct the protons to the sites of the $H^+$-ATPases in the red membrane, to drive ATP synthesis[10].

Another point that should be noted is the maintenance of a high negative charge surface density by the high concentration of acidic lipids in the membranes of *Halobacteria*, *Haloarcula* and *Haloferax*[15]. Such a high negative charge surface density would be shielded by the high $Na^+$ ion concentration (4 M), thus preventing disruption of the lipid bilayers due to negative charge-charge repulsion. A highly negatively charged membrane surface would thus appear to be required for the survival of extreme halophiles in media of high salt concentration.

The sulfated triglycosylarchaeol may have another function in the PM, related to proton pumping action of bacteriorhodopsin, since it has been demonstrated that reconstitution of bacteriorhodopsin (BR) in PGP-Me vesicles containing S-TGA-1 results in increased rates of proton pumping[28]. Similar functions might be envisaged for the sulfated diglycosylarchaeol (S-DGA, **10**) in species of *Haloferax*, which also contain the PM, but no experimental evidence is available concerning the function of this glycolipid.

*Evolutionary considerations and conclusions*

The observation that the fatty acid synthetase (FAS) in *H. cutirubrum* is strongly inhibited by high salt concentration, while the mevalonate enzyme system for isoprenoid biosynthesis has an absolute requirement for high salt concentration, may offer a clue to the mechanism of evolution of extreme halophiles from non-halophilic, or moderately halophilic precursors, and possibly also for the evolution of the methanogens and extreme thermophiles. The following hypothetical scenarios[18,20] are offered for discussion (see figs 5–7):

1) In the first scenario (fig. 7), an anaerobic precursor synthesizing acyl ester phospholipids (and perhaps also monoacylmonoalkyl(alk-1-enyl) ether phospholipids), as well as isoprenoid compounds, was exposed to gradually increasing salt concentrations such as in evaporating salt lakes or ponds. As the intracellular salt concentration increased, progressive inhibition of the FAS, the complex acyl lipid-synthesizing enzymes (particularly the acyl-CoA: sn-3-GP acyl transferase), and the mevalonate enzyme system occurred, resulting eventually in a near-lethal deficiency of membrane lipids. However, before this state was reached, a mutant arose, designated 'pre-archaebacterium' or 'pre-extreme halophile', in which the mevalonate enzyme system and the alkyl ether lipid synthetases, but not the FAS to the same extent, were modified for effective functioning in increasing salty environments. The driving force here was the increased survival value afforded by the more stable isopranyl ether phospholipids and glycolipids.

As the salt concentration of the environment approached saturation, the mevalonate enzyme system and the alkyl ether lipid synthetases of the 'pre-archaebacterium' were further modified for optimal synthesis of isoprenyl/isopranyl chains and diphytanyl glycerol diether (archaeol) lipids, respectively, in near saturated (4 M) NaCl, to form the 'pro-extreme halophile' (fig. 7). The FAS, which was only slightly modified, was now largely inhibited, but still able to produce sufficient fatty acids for membrane protein acylation. Replacement of the membrane acyl lipids by the phytanyl glycerol diether lipids rectified the deficiency in acyl polar lipids and at the same time provided a membrane lipid bilayer that was more suited to a high salt environment. Replacement of the typical eubacterial cell wall by the archaebacterial glycosylated protein cell wall may also have happened at this stage ('pro-extreme halophile'). Further mutation of the 'pro-extreme halophile' occurred to produce bacteriorhodopsin and bacterioruberin, enabling the mutant to function anaerobically in the light. The aerobic system of fermentation evolved later to form the extreme halophiles known today.

The methanogens and perhaps the extreme thermophiles may have evolved from either the pre-archaebacterium or the pro-extreme halophile to form a 'pro-methanogen' or 'pro-thermoacidophile' (fig. 7), a process that would have involved re-adaptation of the mevalonate enzyme system and the alkyl ether synthetases to function at lower salt concentration. In this connection it is of interest that a halophilic methanogen growing in 2 M salt has recently been described[34]; a halophilic thermophile has not yet been isolated[45]. At this stage, development of the novel enzyme system for head-to-head coupling of the ends of the isoprenyl chains to form tetraether lipids (see fig. 1) may have occurred in the 'pro-methanogen' and 'pro-thermoacidophile', as an adaptation to high temperatures. Finally, the cyclopentane ring-forming enzyme system developed in the 'pro-thermoacidophile', thus leading to the extreme thermophiles being able to grow at temperatures near 100 °C[7,25].

2) In scenario 2 (fig. 7), it is argued that the thermophiles could have developed independently of the halophiles as a result of inhibition of the FAS in a 'pre-archaebacterium' by low pH and/or high temperature. This possibility implies that the mevalonate enzyme system and ether lipid synthetases, but not the FAS, would have been modified appropriately to function optimally at low pH and high temperature. Further, the enzyme systems for synthesis of archaeol and caldarchaeol derived lipids would have developed first in a pro-thermophile and have then been passed on to a pro-methanogen, and then to a pro-extreme halophile, with deletion of the caldarchaeol synthetase.

3) It is also possible (scenario 3, fig. 7) that the 'pre-archaebacterium' was a moderately halophilic and thermophilic methanogen, in which the FAS was inhib-

ited by both high salt and high temperature, while the mevalonate and ether lipid enzyme systems were modified to function optimally under these conditions, and the caldarchaeol synthetase could also have evolved at high temperatures. These enzyme systems would then have been passed on to a pro-extreme thermopile, and then to a pro-extreme halophile with deletion of the caldarchaeol synthetase.

On the basis of parsimony, it would appear feasible that extreme halophiles, which only have the enzyme system for diether synthesis, were the precursors of archaeal methanogens and extreme thermopiles, which have both diether and tetraether synthesizing enzyme systems. The evolutionary relationships between these archaea would then be as shown in scenario 1 (fig. 7). Alternatively, but less feasible, the pre-archaebacterium could have been a thermophilic anaerobe with the resulting evolutionary relationships shown in scenario 2 (fig. 7), or a moderately halophilic/thermophilic methanogen with the evolutionary relationships shown in scenario 3 (fig. 7).

It would be of interest to test these hypotheses by examining the methanogens and thermophiles for the presence of FAS and acyl transferases and studying the effect of salt concentration, low pH and high temperature on their activities and on those of the mevalonate enzyme system. It may be noted that preliminary studies with *Mb. thermoautotrophicum* have revealed the presence of a functional FAS producing fatty acids for acylation only of membrane proteins (Pugh and Kates, unpublished data). Studies of halophilic methanogens and halophilic extreme thermophiles would also be of great interest.

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