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Unsaturated diether lipids in the psychrotrophic archaeon *Halorubrum lacusprofundi*

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Abstract

The major phospholipids of *Halorubrum lacusprofundi* grown at 25 °C were archaeol phosphatidylglycerol, archaeol phosphatidylglycerylsulphate and archaeol phosphatidylglycerylphosphate methyl ester. Glycolipids included a monoglycosyl archaeol and the sulphate ester of a diglycosyl archaeol. Cultures grown at 12 °C contained the same suite of phospho- and glycolipids, with the addition of a series of unsaturated analogues with up to six double bonds. The patterns of unsaturation were similar for all the phospholipid series, but a different pattern occurred in the glycolipids. The analytical techniques used in this study allow facile detection of unsaturated archaeal cell membrane lipids that are degraded by commonly used chemical derivatization procedures.

Keywords: Archaea; Unsaturated ether lipids; Halophile; Temperature-dependence; Antarctica; Psychrotroph; APCI; Electrospray

Introduction

The cell membranes of all archaea are characterized by the presence of phospho- and glycolipids containing archaeol, a compound containing two saturated C_{20} polyisoprenoid chains ether linked to the *sn*-2 and -3 positions of a glycerol molecule (Fig. 1). There have been scattered reports of the presence in the lipids of certain archaea of compounds containing unsaturated analogues of archaeol [7,9,11,16,17,19–22,25]. The existence and role of these compounds have been largely ignored in discussions of archaeal ether lipids. Most reports of unsaturated diether lipids nominally derived from archaeol have been limited to the phylum Euryarchaeota, with the great majority of records amongst the halophilic archaea [7,11,16,17,20–22,25]. The confirmed presence of unsaturated diether lipids in a methanogen, *Methanopyrus kandleri* [19] and a member of the Thermococcales, *Thermococcus* sp. S557 [9] suggests that the ability to synthesise phospho- and glycolipids based on unsaturated diether lipids may be a feature of the phylum.

Abbreviations: ArPG, Archaeol phosphatidylglycerol; ArPGS, Archaeol phosphatidylglycerylsulphate; ArPGPMe, Archaeol phosphatidylglycerylphosphate methyl ester; ArMG, Monoglycosyl archaeol; ArDGS, Diglycosyl archaeol sulphate

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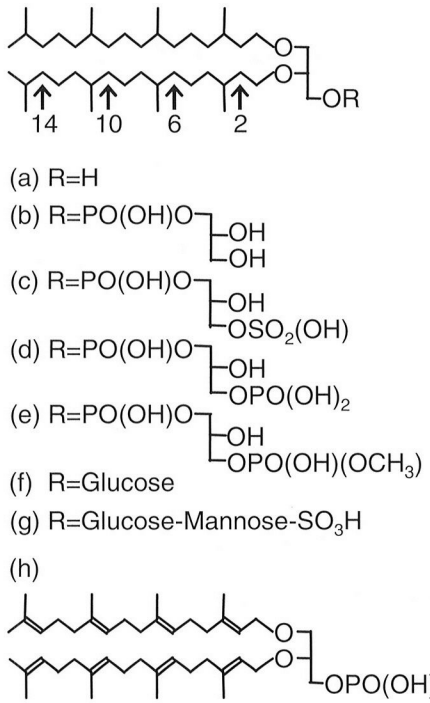

Fig. 1. Structures of: (a) archaeol, (b) ArPG; (c) ArPGS; (d) ArPGP; (e) ArPGPMe; (f) ArMG; (g) ArDGS and (h) phosphorylated digeranylgeranyl glycerol, an intermediate phospholipid in the biosynthesis of saturated diether lipids. The precise structures of ArMG and ArDGS were not determined in the current study, but are assumed to have the same constituents found in previous studies of *H. lacusprofundi* (see text). The carbon numbering system used in this paper is indicated.

Confidence in reports of unsaturated diether lipids has been clouded by suggestions that these compounds occur in other methanogens [18]; these unsaturated diether lipids are now known to be artefacts that resulted from the dehydration of hydroxy diether lipids under acidic conditions used to release the core diether compound from the complex lipid [5]. *M. kandleri* remains the only methanogen in which unsaturated diether lipids have been unambiguously identified [19]. A further confounding problem has been the possible presence of macrocyclic diether lipid, resulting from the formation of a carbon–carbon bond between the terminal carbons of the two hydrocarbon chains in archaeol, which is found in some thermophilic methanogens [4,14]. This ether lipid has the same molecular weight as a monounsaturated diether lipid and could be confused for it, though details of the mass spectrum are quite different. Further macrocyclic ether lipids containing one or two intrachain cyclopentyl rings have been identified in environmental samples [23]. Again, the molecular weight of these species is the same as di- and tri-unsaturated unsaturated diether lipids, but the mass spectra will be significantly different.

In this study we describe the use of LC–MS to identify unsaturated diether lipids in both the phospho- and glycolipid fractions of lipid extracted from *Halorubrum lacusprofundi*, and confirm the presence of unsaturation. We further discuss the role of temperature in determining the distribution of unsaturated diether lipids in the phospho- and glycolipid series.

Materials and methods

Cultures were grown in modified Halobacteriaceae Medium 2 [1], which contained 200.0 g NaCl, 20.0 g $MgSO_4 \cdot 7H_2O$, 10.0 g yeast extract, 7.5 g casamino acids, 3.0 g trisodium citrate, 2.0 g KCl and 2.3 mg $FeCl_2 \cdot 6H_2O$ dissolved in 1000 ml distilled water. Cultures were incubated for 30 days at 12 °C or for 6 days at 25 °C, the biomass was harvested into a sterile polypropylene tube and pelleted by centrifugation at 10,000*g* for 15 min.

Lipid extraction and isolation

Lipid in freeze-dried biomass was extracted by a modified Bligh–Dyer method [2,26]. Samples were placed into a separatory funnel and chloroform, methanol and water added in the ratio 5:10:4 by volume. The mixture was shaken, and allowed to stand with occasional further shaking overnight. The following morning further chloroform and water were added in a ratio of 5:5 by volume, giving a final ratio of chloroform:methanol:water of 10:10:9. Two phases were formed; the lower, organic phase was drained into a round bottom flask, and the solvent removed under reduced pressure. The total lipid extract was stored at −20 °C.

Separation of the lipid into classes based on polarity was accomplished by column chromatography on 1 g silica gel (Sigma: mesh size 100–200, dried for 1 h at 100 °C and cooled prior to use). The total lipid extract was fractionated by stepwise elution with chloroform (hydrocarbons—not studied further), acetone (termed hereafter the 'glycolipid fraction') and methanol ('polar lipid fraction').

Liquid chromatography–mass spectrometry

Analysis of phospholipids, sulfatophospholipids and sulfatoglycolipids was undertaken by LC–MS using negative ion electrospray ionization. A Waters Alliance 2690 HPLC fitted with a Waters Nova-Pak C_{18} column (150 mm × 3.9 mm) was coupled to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source. An isocratic mobile phase containing 95:5 methanol:0.1 M ammonium acetate was used at a flow

rate of 0.8 ml min^{-1}. The mass range from m/z 700 to 1200 was monitored, with data-dependent MS/MS product ion scans alternating with normal scans. The MS/MS scans isolated the strongest ion observed in the m/z 700–1200 range with an isolation window of 5 m/z units, applied a collision energy of 40% and monitored all product ions down to the ion trap cutoff point of 25% of the m/z value of the isolated precursor ion. The capillary temperature was 275 °C, sheath gas pressure was 585 kPA, auxiliary gas pressure was 275 kPa and needle voltage was 4.8 kV.

Glycolipids were analysed by negative ion atmospheric pressure chemical ionization–mass spectrometry (APCI-MS) using the same chromatographic system. Peaks corresponding to the acetate adducts of the neutral lipids were observed. The capillary temperature was 180 °C, the vaporizer was set at 460 °C, the sheath gas pressure was 380 kPa, and the auxiliary gas pressure was 70 kPa. The range from m/z 300 to 2000 was monitored. MS/MS was performed using the same conditions as given above.

Isolation and characterisation of core ether lipids

Core ether lipids (i.e. with the phospho- or glycohead groups removed) were obtained from the total lipid extract or the polar lipid fraction by base hydrolysis. The sample was dissolved in 3 ml methanol and placed in a round bottom flask. 1 ml 10% KOH (w/v) was added, and the mixture heated at reflux for 3 h. After cooling to room temperature the reaction mixture was extracted with three 1 ml portions of 4:1 hexane:chloroform to isolate the core ether lipids. The solvent was removed under reduced pressure, and the core ether lipids stored at −20 °C.

The core ether lipids were converted to their acetate derivatives as follows: the crude core ether lipid fraction was transferred to a test tube and the solvent removed. Three millilitres of 5:1 acetic anyhydride: pyridine was added and the reaction mixture left to stand for 24 h at room temperature. Water (2 ml) was added to stop the reaction, and, after cooling, the acetate derivatives isolated by extraction with three 1 ml portions of 4:1 hexane:chloroform. The extracts were pooled and the solvent removed under a stream of dry dinitrogen.

The saturated and unsaturated core ether lipids were separated by argentation thin layer chromatography (Ag^+-TLC). The Ag^+-TLC plates were prepared by pre-cleaning silica gel plates (Merck, silica gel 60, 5 × 20 cm, 0.25 mm layer thickness) by development with chloroform:methanol, and then steeping in 5% $AgNO_3$ in methanol:water (20:80) for 1 h. The plates were dried at 90 °C and then allowed to equilibrate at room temperature for 24 h. The acetate derivatives of the core ether lipids were transferred to a band at the bottom of the plate and the plates developed with hexane:diethyl ether (80:20) for 90 min. Ether lipids were detected as yellow bands under UV light after spraying with 2′,7′-dichlorofluoroscein (0.2% in methanol) and drying.

Individual unsaturated core ether lipids were isolated by scraping bands from the plates into a round bottom flask containing 3 ml of 15% NaCl in water to precipitate the silver ions. The mixture was allowed to stand overnight, and the unsaturated core ether lipids isolated by extraction with three 1 ml portions of hexane:chloroform (4:1 v:v). The three extracts were pooled and filtered through a glass wool plug.

The isolated unsaturated core ether lipids were analysed by electron ionization mass spectrometry using a Vacuum General Micromass 7070F magnetic sector mass spectrometer operating at a source temperature of 250 °C and an electron energy of 70 eV. NMR spectra were recorded on thoroughly dried samples dissolved in $CDCl_3$ using a Bruker AM-300 MHz spectrometer.

Results

The LC chromatograms of the phospholipid fraction of the total lipid extract obtained from *H. lacusprofundi* grown at 25 °C contained three major peaks at retention times 11.5, 14.0 and 27.2 min attributable to archaeol-based phospholipids. Negative ion electrospray mass spectrometry indicated that the $[M–H]^-$ of these peaks were at m/z 885.8, 899.8 and 805.8, respectively, consistent with the presence of archaeol phosphatidylglycerylsulphate (ArPGS); archaeol phosphatidylglycerylphosphate methyl ester (ArPGPMe); and archaeol phosphatidylglycerol (ArPG). Structures of these compounds are shown in Fig. 1. The peak at m/z 885.8 could also be assigned to ArPGP (Fig. 1), as this molecule and ArPGS would not be separated with the mass spectrometer used due to their identical nominal molecular weights. The peak was assigned to ArPGS on the basis that there was a reproducible 4% increase in the relative abundance for the +2 isotope ion compared to that in the sulfur-free ArPGPMe, as predicted for the presence of the ^{34}S isotope. Furthermore, previous reports on the lipids of *H. lacusprofundi* indicated the presence of ArPGS and the near absence of ArPGP [13,24].

The MS/MS data supported these assignments, with characteristic intense product ions 74 mass units lower (loss of $C_3H_6O_2$) for ArPG, 80 mass units (loss of SO_3) and 154 mass units lower (loss of SO_3 and $C_3H_6O_2$) for the ArPGS, and 112 mass units (loss of $H_2PO_4CH_3$) and 186 mass units lower (loss of $H_2PO_4CH_3$ and $C_3H_6O_2$) for ArPGPMe.

The chromatogram contained a further peak, the mass spectrum of which had $[M–H]^-$ at m/z 1056, that

was attributable to the sulphate ester of a diglycosyl archaeol (ArDGS) (Fig. 1). The identities of the hexoses in ArDGS and the precise nature of the linkages in this molecule were not determined.

No identifiable peaks were observed in the LC–ES–MS spectrum of the glycolipid fraction. This was not unexpected, as in the absence of a deprotonatable phosphate or sulphate group no $[M-H]^-$ ions would be produced from any glycolipids present. By analogy with the relatively easy detection of carbohydrates by negative ion APCI through the formation of anionic adducts with formate or acetate ions (N.W. Davies, unpublished data), negative ion APCI using the acetate ions already present in the mobile phase was expected to allow the detection of glycolipids, and proved successful. LC-APCI-MS of the glycolipid fraction revealed a single prominent peak with retention time 20.2 min that had m/z 873.8 $[M+CH_3COO^-]$ that was attributable to a monoglycosyl archaeol (ArMG) (Fig. 1). The dominant MS/MS product of this ion was at m/z 813.8, which was attributable to ArMG $[M-H]^-$ ion. The identity of the hexose residue in the lipid was not determined. No diglycosyl or triglycosyl archaeols were observed.

The total ion currents of the LC–ES–MS runs of the phospholipid fraction of the total lipid extract of biomass grown at 12 °C were more complex (Fig. 2), but by plotting specific mass chromatograms it was possible to clearly identify and separate the members of three series of compounds related to the saturated phospholipids (Fig. 3). In each case a sequence of peaks was identified that had m/z 2, 4, 6, 8, 10 and 12 mass

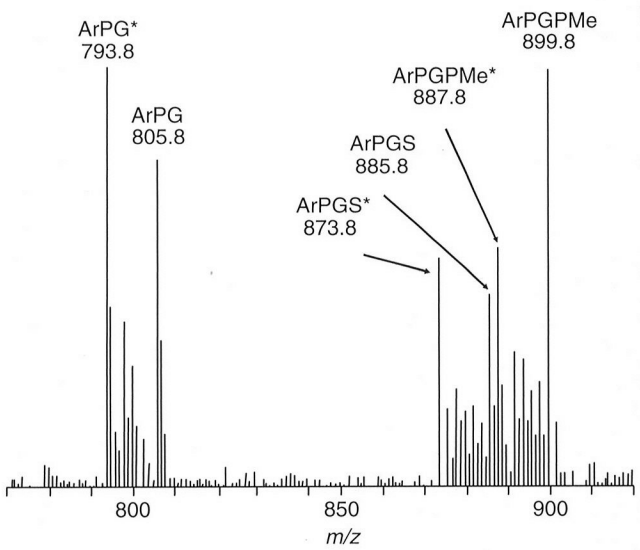

Fig. 2. Negative-ion electrospray-mass spectrum of the polar lipid fraction of *H. lacusprofundi* grown at 12 °C summed over an entire chromatographic run. The identities of $[M-H]^-$ peaks for the saturated and hexaunsaturated (asterisked) ether lipids are indicated.

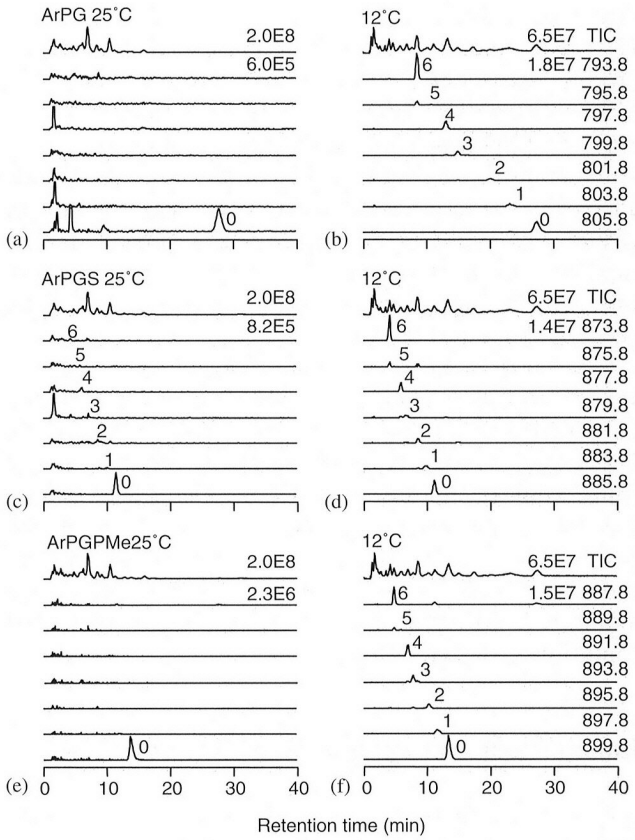

Fig. 3. Total ion current (TIC) and selected single ion channels for phospholipids isolated from *H. lacusprofundi*: (a) ArPG (grown at 25 °C); (b) ArPG (12 °C); (c) ArPGS (25 °C), (d) ArPGS (12 °C), (e) ArPGPMe (25 °C) and (f) ArPGPMe (12 °C). The m/z value used to generate each chromatogram is indicated, along with the relative scale for each plot. All the single ion channels are plotted at the same vertical scale. The number of double bonds in each unsaturated phospholipid is also indicated.

units lower than the ArPG, ArPGS and ArPGPMe. The retention times of these compounds decreased regularly with decreasing mass, suggesting that a coherent series of compounds was present. The ArPGS series was also present in trace amounts in samples grown at 25 °C (Fig. 3). As discussed further below, we assign these peaks to analogues of the saturated phospholipids with 1–6 double bonds in the isoprenoid chains of the ether lipid. The MS/MS data for these were homologous to their saturated equivalents, with the same neutral losses observed in each case. The +2 isotope data for the stronger members of the ArPGS series again supported the presence of one sulfur atom.

Similar series of peaks at lower m/z and shorter retention times related to ArMG and ArDGS were also observed in the LC–ES–MS and LC–APCI–MS chromatograms, respectively, although in these cases only the compounds with molecular weights lower by 2 and 6

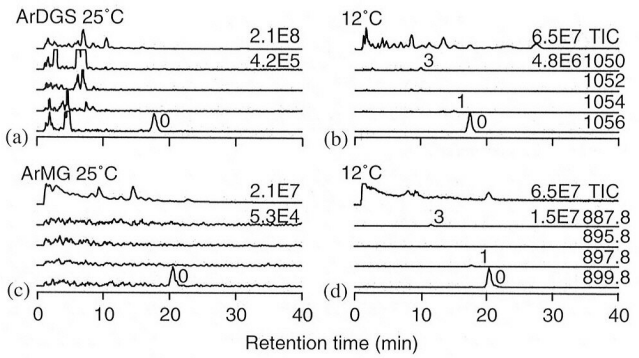

Fig. 4. Total ion current (TIC) and selected single ion channels for individual glycolipids isolated from *H. lacusprofundi*: (a) ArDGS (LC–ES–MS) (grown at 25 °C); (b) ArDGS (12 °C); (c) ArMG (LC–APC–MS) (25 °C); and ArMG (12 °C). The *m/z* value used to generate each chromatogram is indicated, along with the relative scale for each plot. All the single ion channels are plotted at the same vertical scale. The number of double bonds in each unsaturated glycolipid is also indicated. The large peaks at shorter retention time in some plots were not related to phospho- or glycolipids.

mass units occurred, and then only at low concentration (Fig. 4).

The patterns of unsaturation of the three main phospholipid series were similar (Table 1), although the relative concentration of saturated ArPGPMe appeared to be higher, and its hexaunsaturated congener lower, than for the ArPG and ArPGS series. The most abundant compounds were those with 0, 4 and 6 double bonds. The corresponding compounds with 1, 2 and 3 double bonds were less abundant, and the compound with 5 double bonds was present at ca. 1% of the total in all cases. The unsaturation patterns in the two glycolipids were quite similar (Table 1), but were markedly different to those in the phospholipids in that only lipids having one or three double bonds were present.

Confirmation of unsaturation in the ether lipids came after removal of phospho- and glyco- head groups by base hydrolysis to release the core ether lipid. Ag^+-TLC separated the saturated and unsaturated archaeols into a series of components. Seven distinct bands were observed; the relative mobilities (with respect to saturated archaeol) of the bands and the compounds detected by mass spectrometry in each band are given in Table 2. Ag^+-TLC separates *cis* and *trans* olefinic compounds, with compounds with a single trans double bond eluting with saturated species [3]. Archaeol analogues containing inter- or intrachain cyclisation would elute with archaeol, as they lack unsaturation. The co-chromatography of archaeol and monounsaturated archaeol indicates that the stereochemistry around the double bond in the monunsaturated archaeol(s) was *trans*. The separation of the triunsaturated and tetraunsaturated archaeols into three distinct, but multi-

Table 1. Estimated relative proportions of each unsaturated phospho- and glycolipid in *H. lacusprofundi* grown at 12 °C

No. of double bonds	ArPG	ArPGS	ArPGPMe	ArMG	ArDGS
0	30	28	40	88	91
1	8	8	9	4	4
2	4	8	7	0	0
3	9	7	11	8	4
4	14	14	14	0	0
5	1	1	1	0	0
6	34	35	18	0	0

The proportions were estimated from the peak areas of the single ion current for each lipid shown in Figs. 3 and 4.

Table 2. Mobilities (relative to that of archaeol) of bands isolated by Ag^+-TLC from the core ether lipids of *H. lacusprofundi*, along with the number of double bonds in the ether lipids in these bands as determined by mass spectrometry

Band no	Relative mobility	No. of double bonds.
1	0.29	6
2	0.49	5
3	0.59	3, 4
4	0.74	3, 4
5	0.80	3
6	0.89	2
7	1.00	0, 1

component, bands indicated that a mixture of compounds containing multiple double bonds with both *cis* and *trans* stereochemistry was present. Alternatively, positional isomers may have been present. Separation of these compounds by GC was not achieved; the triunsaturated archaeol from each of the three bands in which it was present eluted as a single sharp peak. Similar observations were made for the tetraunsaturated archaeols.

Further confirmation of unsaturation in the archaeols came from ^{1}H NMR spectroscopy. All the putative unsaturated archaeols isolated through Ag^+-TLC had spectra in which distinct multiplets attributable to olefinic protons were observed, one at ca. 5.1 ppm attributable to olefinic protons attached to carbons 6, 10 and/or 14 on either chain, and a second centred at ca 5.35 ppm attributable to olefinic protons attached to carbon 2 (Fig. 1) [9]. Relative peak areas of the multiplets were 0:1 for the monounsaturated archaeol, 1:1 for the tetraunsaturated archaeol and 2:1 for the hexaunsaturated archaeol.

Discussion

The phospholipids found in *H. lacusprofundi* at 25 °C in this study were consistent with those reported earlier

for this species [15,24]; previous studies had indicated the presence of both ArPGP and ArPGS, but it has since been shown that the phospholipid originally identified as ArPGP was actually ArPGPMe [13], and that ArPGP is present in halophilic archaea as a minor component only [13]. The glycolipids were also similar. The precise identity of ArDGS was not determined in the current study, but was most likely the mannosyl-2-sulphate-(1-4)-glycosyl archaeol reported previously in the species [15,24]. The presence of an unidentified glycolipid in *H. lacusprofundi* has also been noted [24]; it is probable that this lipid was ArMG. The structure of ArMG most likely involves a glucose residue bound to the *sn*-1 of carbon the glycerol backbone, as all other glycolipids presently reported for the genus *Halorubrum* have this basic unit [15]. Monoglycosyl archaeols have not been recorded before in halophilic archaea, but may have been overlooked. Previous records of monoglycosyl archaeols appear to be limited to methanogens [8,12]. The absence of diglycosyl archaeol, which is widespread in other halophilic archaea and which might be expected to occur in *H. lacusprofundi* considering the presence of ArDGS, was again consistent with earlier studies [13,15].

This study confirmed the ability of *H. lacusprofundi* to biosynthesise and utilise unsaturated ether lipids observed in the original description of this species [7]. When grown at 12 °C this species contained phospholipids with up to six double bonds in the polyisoprenoid chains. These double bonds are likely to occur at carbons 2, 6, 10 and/or 14 [9,19], although in most cases no firm conclusions can be drawn. In theory there are numerous possible isomers for each of the unsaturated archaeols [19]. The NMR studies on isolated unsaturated arcaheols indicated that double bonds occurred at carbon 2 in each case, and that in the tetra- and hexaunsaturated archaeols both chains of the ether lipid contained this feature. The double bond positions in a hexaunsaturated archaeol have been shown previously [9] to be at carbons 2, 6 and 10 on each chain, and we consider it likely that the structure of the hexaunsaturated core ether lipid in this study was the same.

The biosynthesis of diether lipids occurs via phosphorylated digeranylgeranyl glycerol [13,16], an unsaturated archaeol derivative with eight double bonds (Fig. 1). We suggest that the unsaturated diether lipids observed in *H. lacusprofundi* are the result of incomplete saturation of unsaturated precursors, although it is also possible that they are formed through secondary desaturation after the formation of fully saturated diether lipids. Formation of the phosphate-based portion of the phospholipid occurs prior to the saturation of the hydrocarbon chains. If the unsaturated phospholipids in *H. lacusprofundi* are the result of only partial saturation of the phosphorylated digeranylgeranyl glycerol, the implication of the similar patterns of unsaturation amongst the phospholipids is that the saturation process is not affected by the nature of the phosphate-based portion of the molecule. It also suggests that there is a high biosynthetic control on the saturation process.

A similar argument applies to the glycolipids. Mechanistic studies indicate that the pathways that lead to the formation of phospholipids and glycolipids separate relatively early in the biosynthetic process [13,16]. The polar head group is again bound to the diether lipid core prior to saturation, with the implication that the saturation process has similar specificity for all the glycolipids. It is clear, however, that the specificity is very different for glycolipids than for the phospholipids. This difference may be the result of the involvement of different enzymes or reflect the function of these molecules in the cell membrane.

Unsaturated diether lipids were detected in trace amounts in biomass grown at 25 °C, but at 12 °C accounted for approximately 65% of the total. These data support the earlier contention that the presence of unsaturated diether lipids in the cell membrane of *H. lacusprofundi* is an adaptation to growth at low temperature [7]. This conclusion has been questioned recently by Nishihara et al. [19], who suggested (from the presence of unsaturated diether lipids in the thermophilic methanogen *M. kandleri*) that unsaturated diether lipids could not be an adaptation to cold conditions. However, this evidence does not preclude the original conclusion [7], but rather indicates that unsaturated diether lipids can be utilised by Archaea for different physiological functions within the cell membrane under different circumstances. The occurrence of unsaturated diether lipids in thermophilic archaeal species *per se* does not negate their potential role in adaptation to growth at low temperature. The distribution of phospho- and glycolipids in *M. kandleri* is very different to that in *H. lacusprofundi*, including the presence of phosphoamino lipids absent from halophilic archaea [10,19]. It is probable that significant differences in the physical properties of the particular complex lipids from each species result in membranes of different characteristics that will explain in part the apparent paradox of the presence of unsaturated diether lipids in both thermophilic and psychrophilic archaea. Furthermore, the dominant lipids in *M. kandleri* are glycolipids (>90% [10]), and, as for *H. lacusprofundi* (in which glycolipids made up only a small percentage of the total), the pattern of unsaturation in this fraction may be significantly different from that in the phospholipid.

As mentioned in the introduction, unsaturated diether lipids have been reported sporadically and generally at low concentration in a range of halophilic archaea. This suggests that the presence of unsaturated diether lipids in archaeal cell membranes is widespread, but has not been recognised due to the slow growth rate of many of

these species at low temperatures. Furthermore, use of acidic conditions to isolate the core diether lipid from the phospho- and glycolipids results in the loss of the unsaturated chain(s) from the molecule, forming monophytanyl glycerols if a combination of unsaturated and saturated chains was originally present [17]. Therefore, the presence of unsaturated diether lipids could be missed if acidic conditions are used to isolate the core ether [9,10]. *Halorubrum. lacusprofundi* was originally isolated from hypersaline Deep Lake in Antarctica, in which the temperature ranges on an annual cycle from about −15 °C to +10 °C [6]. This species is adapted to growth at low temperature (as low as 4°C [7]), and therefore is a good model for the investigation of the effect of temperature on the make up of the cell membrane lipids in halophilic archaea. If other species of halophilic archaea were grown at lower temperatures and the lipids analysed by the methods described here, it may well be that greater amounts of unsaturated diether lipids would be observed.

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