Structural features of ether lipids in the archaeobacterial thermophiles *Pyrococcus furiosus*, *Methanopyrus kandleri*, *Methanothermus fervidus*, and *Sulfolobus acidocaldarius*[1]

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Abstract: The ether lipids of several thermophilic archaea (archaeobacteria) were compared by negative-ion fast atom bombardment mass spectrometry. The major polar lipids in extracts of *Pyrococcus furiosus* were assigned as archaeol lipids (phosphatidylglycerol diether, *m/z* 805; phosphatidylinositol diether, *m/z* 893; and diglycosyl diether, *m/z* 975) and caldarchaeol lipids (diglycosyl phosphatidylglycerol tetraether, *m/z* 1778; and diglycosyl phosphatidylinositol tetraether, *m/z* 1866). The polar lipids of *Methanopyrus kandleri* were primarily glycolipids consisting of a series of archaeol lipids with one to six hexose units, composed primarily of mannose (mannose:glucose 9:1); phospholipids consisting of archaeol lipids (phosphatidylinositol diether; and a novel phosphatidylcholine diether, *m/z* 802.7), and phosphoglycolipids as minor caldarchaeol lipids (primarily diglycosyl phosphatidylglycerol tetraether). *Methanothermus fervidus* extracts contained archaeol lipids (phosphatidylinositol diether; diglycosyl diether; and acetyldiglycosyl diether, *m/z* 1016), and caldarchaeol lipids (glycosyl phosphatidylinositol tetraether, *m/z* 1704; diglycosyl phosphatidylinositol tetraether; and acetyldiglycosyl phosphatidylinositol tetraether, *m/z* 1907). Acetylation of a sugar residue occurred commonly in this thermophile and increased as cells entered the stationary growth phase. Lipid extracts of *Sulfolobus acidocaldarius* contained detectable amounts of archaeol and hydroxyarchaeol analogs of phosphatidylinositol, phosphatidylglycerol, and phosphatidylethanolamine lipids, in addition to the dominant caldarchaeol lipids already reported. All four thermophiles contained both archaeol and caldarchaeol lipids and phosphoinositol head groups, but no single structural entity uniquely separated their lipids from those found previously in mesophilic archaea. By contrast, extremely halophilic archaea appear to be distinguished from the thermophilic archaea by the presence of a major phosphatidylglyceromethylphosphate lipid.

Key words: ether lipids, mass spectrometry, hyperthermophiles, extreme halophiles, Archaea.

Résumé : Les éthers lipidiques de quelques archae (archéobactéries) thermophiles ont été comparés par spectrométrie de masse par bombardement atomique rapide d'ions négatifs. Les principaux lipides polaires retrouvés dans des extraits de *Pyrococcus furiosus* ont été identifiés comme des lipides d'archéols (phosphatidylglycérol diéther, *m/z* 805; phosphatidylinositol diéther, *m/z* 893; et diglycosyl diéther, *m/z* 975) ou comme des lipides de caldarchéols (diglycosyl phosphatidylglycérol tétraéther, *m/z* 1778; et diglycosyl phosphatidylinositol tétraéther, *m/z* 1866). Les lipides polaires de *Methanopyrus kandleri* étaient surtout des glycolipides composés d'une série des lipides d'archéols possédant un à six unités d'héxoses comprenant surtout du mannose (mannose:glucose 9:1); des phospholipides composés des lipides d'archéols (phosphatidylinositol diéther; et une nouvelle phosphatidylcholine diéther, *m/z* 802,7), et des phosphoglycolipides comme les lipides mineurs de caldarchéols (surtout le diglycosyl phosphatidylglycérol tétraéther). Des extraits de *Methanothermus fervidus* contenaient des lipides d'archéols (phosphatidylinositol diéther; diglycosyl diéther; et acétyldiglycosyl diéther, *m/z* 1016) ainsi que des lipides de caldarchéols (glycosyl phosphatidylinositol tétraéther, *m/z* 1704; diglycosyl phosphatidylinositol tétraéther; et acétyldiglycosyl phosphatidylinositol tétraéther, *m/z* 1907). L'acétylation d'un résidu sucré se produisait régulièrement chez ce thermophile et elle augmentait lorsque les cellules entraient en phase de croissance stationnaire. Des extraits lipidiques du *Sulfolobus acidocaldorius* contenaient des quantités détectables des analogues archéols et hydroxyarchéols des lipides phosphatidylinositol, phosphatidylglycérol et phosphatidyléthanolamine en plus des lipides dominants de caldarchéols déjà rapportés. Ces quatre bactéries thermophiles contenaient toutes des lipides de type archéols et caldarchéols et des groupements phosphoinositol, mais aucune entité structurale particulière n'a permis de distinguer ces lipides de ceux déjà connus chez les archéobactéries mésophiles. Par contre, les archéobactéries halophiles extrêmes ont pu être différencier des archéobactéries thermophiles par la présence d'un lipide principal, le phosphatidylglycérométhylphosphate.

Mots clés : éthers lipidiques, spectrométrie de masse, hyperthermophiles, halophiles extrêmes, Archaea.
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Introduction

One of the features linked to life in the extreme habitats associated with the Archaea is the structural properties of their membrane lipids. Archaeal lipids are characterized by saturated phytanyl chains in ether linkage to glycerol carbons with *sn*-2,3 configuration, forming diether lipids (Kates 1978), tetraether lipids (Langworthy 1985), or their variations (Fig. 1).

Although the lipid head groups have been determined for several archaea (see Kates 1993), little is known about the structures of the polar lipids in the extreme thermophiles. Most of what is known is from studies of moderate thermophiles including *Sulfolobus* species (Gulik et al. 1988), *Thermoplasma* species (Langworthy et al. 1982; Swain et al. 1997), *Methanobacterium thermoautotrophicum* (Nishihara et al. 1989), and *Methanococcus jannaschii* (Ferrante et al. 1990).

A major deterrent in these studies with hyperthermophiles is the difficulty in obtaining sufficient biomass to purify each lipid species for the usual nuclear magnetic resonance and chemical analyses (Sprott et al. 1994*b*). However, fast atom bombardment mass spectrometry (FAB MS) has been applied successfully to the analysis of total polar lipid extracts from *Halobacterium cutirubrum* (Fredrickson et al. 1989) and *Methanosarcina* species (Sprott et al. 1994*a*). The method has the potential advantages of obtaining the molecular ions for each lipid species without prior purification and with very minimal material being required (less than 40 μg of total polar lipids from *Methanosarcina mazei*). Here, we apply this technique to provide an initial structural assessment of the lipids synthesized by several archaeal thermophiles.

Materials and methods

Growth of archaea

Methanothermus fervidus V24S (DSM 2088) isolated from a hot solfataric spring was cultivated in 50 L of DSM 203 medium (DSM catalogue of strains) in a 75-L Chemap AG fermenter at pH 6.5 and 78°C under an 80% H_2 – 20% CO_2 gas phase. Late logarithmic phase cultures were harvested with a Pellicon cell harvester and the cell paste was stored at −20°C until needed. To study the effect of growth phase on the membrane lipid composition of *Methanothermus fervidus*, cells were cultivated (78°C, pH 6.5, 80% H_2 – 20% CO_2 gas phase) in 100 mL medium contained in 1-L Pyrex bottles. Twice a day, the bottles were repressurized to 34.5 kPa (5 psi) at room temperature, and the incubation continued at 100 rpm. At 24-h intervals, the entire head space in the bottle was replaced with fresh H_2–CO_2 and the medium was supplemented with 0.5 mL cysteine–Na_2S (1.25% w/v each, adjusted to pH 10 and autoclaved under N_2 gas phase) reducing solution. The bottles were incubated in steel boxes described below, and cells were harvested by centrifugation.

Sulfolobus acidocaldarius 98-3 (ATCC 33909) originating from an acidic hot spring was cultivated in 50 L of ATCC medium 1723 (ATCC catalogue of strains) in a 75-L Chemap AG fermenter at pH 3.0 and 70°C. The culture was sparged with air at 26 L/min. Late logarithmic growth phase cultures were harvested and stored at −20°C.

Methanopyrus kandleri AV19 (DSM 6324) from a hydrothermal vent was cultivated in DSM medium 511 (10 mL medium/160 mL serum vial) at pH 6.5 and 98°C. Extreme caution was taken because the glass growth vessels were pressurized to 207 kPa (30 psi) with 80% H_2 – 20% CO_2 and because the pressure increased further upon incubation at 98°C. The vessels were pressurized at room temperature while in a stainless steel box, where each vessel was partitioned from the adjacent one by stainless steel plates. Holes at the bottom of the steel container facilitated heat transfer during static incubation in an oven. The vessels were cooled to room temperature and repressurized twice daily during the 2-day incubation period. Cells in late logarithmic growth phase were harvested by centrifugation and frozen at −20°C.

Pyrococcus furiosus (DSM 3638, obtained from Dr. K. Jarrell, Queen's University, Kingston, Ont.) isolated from hot marine sediment was cultivated in a medium based on stock solutions prepared according to Balch et al. (1979); namely, 980 mL mineral solution 3, 10 mL trace minerals containing Na_2SeO_3 and $NiCl_2$, 10 mL trace vitamins, 20 g NaCl, 1 g NH_4Cl, 1 g yeast extract, 5 g peptone, and 5 g maltose. The medium (pH 7.0) was reduced under a 20% CO_2 in N_2 gas phase with cysteine sulfide solution and dispensed in aliquots of 100 mL/1-L Pyrex bottle. Elemental sulfur was added (0.5 g/bottle), the bottles were sealed with butyl rubber stoppers, and following inoculation the culture was incubated statically for 24 h at 96°C.

Halobacterium cutirubrum (ATCC 33170) was grown aerobically in a synthetic medium (Grey and Fitt 1976). Complex medium 1590 (ATCC catalog of bacteria and bacteriophage, 1989, p. 362) was used to grow *Natronobacterium magadii* (ATCC 43099).

Lipid extraction and MS analysis

Samples of total lipids, extracted from frozen–thawed cell pastes by the method of Bligh and Dyer, were dissolved in $CHCl_3$–CH_3OH (2:1, v/v), and when required the total polar lipids were obtained by acetone precipitation (Sprott et al. 1995). Positive- and negative-ion FAB MS were performed with a JEOLJMS-AX 505H instrument using triethanolamine plus kryptofix® (Sigma-Aldrich Canada, Mississauga, Ont.) matrix. Most probable structures were assigned based on data from archaeal lipids where structures are known.

Fractionation of the lipids of *Methanopyrus kandleri*

Total lipid extracts from *Methanopyrus kandleri* were fractionated by thin-layer chromatography (TLC) using silica gel G-25 (0.25 mm) and developed with $CHCl_3$ – CH_3OH – 7 M NH_4OH (180:105:24, by volume). Bands were visualized by iodine vapour and the lipids recovered from the adsorbent were subjected to analysis by negative-ion FAB MS. Dragendorff staining was used to detect lipids with a quaternary ammonium ion (Kates 1986).

Carbohydrate and core lipid identification

Total lipids extracted from *Methanopyrus kandleri* were hydrolyzed with 2.5% methanolic HCl at 70°C over 2 h, and head groups were separated from the core lipids by solvent partitioning as previously described (Ferrante et al. 1989). Core lipids in the petroleum ether fraction were separated by TLC (Sprott et al. 1990) and identified using as standards the archaeol and caldarchaeol core lipids prepared from *Methanospirillum hungatei* (Kushwaha et al. 1981). Carbohydrates in the aqueous phase were demethylated, acetylated, and analyzed by gas chromatography – mass spectrometry (GC–MS), as described before (Ferrante et al. 1989).

Results

Pyrococcus furiosus

Both archaeol and caldarchaeol lipids were found by negative-ion FAB MS analysis of lipid extracts from *Pyrococcus furiosus* (Fig. 2), as reported for *Pyrococcus woesei* (Lanzotti et al. 1989) and *Pyrococcus abyssi* (Erauso et al. 1993). The fragment of *m*/*z* 731.6 is typical of phosphoarchaeol lipids (Fig. 1).

Lipid structures corresponding to the *m*/*z* of the molecular anion $[M-H]^-$ for the various lipids of *Pyrococcus furiosus*

Fig. 1. Structures of archaeol (standard diether), hydroxyarchaeol (hydroxydiether), and caldarchaeol (standard tetraether) lipids, where X, X_1, and X_2 correspond to protons. In the case of polar lipids the head groups would be at positions X, X_1, and X_2. Diversity in head group structure occurs among archaeal genera, to include sugars, phosphopolyols, phosphoamino groups, or phosphoserine groups. When subjected to negative-ion FAB MS, diether and hydroxydiether phospholipids form the characteristic fragment ions shown as m/z 731.6 and 747.6. Not shown are the 3-hydroxyarchaeol core lipid (Sprott et al. 1990), the nonitolcaldarchaeol lipids first found in *Sulfolobus* species, nor the presence of cyclopentane rings sometimes formed in the chains of certain archaea (Gulik et al. 1988; Trincone et al. 1992).

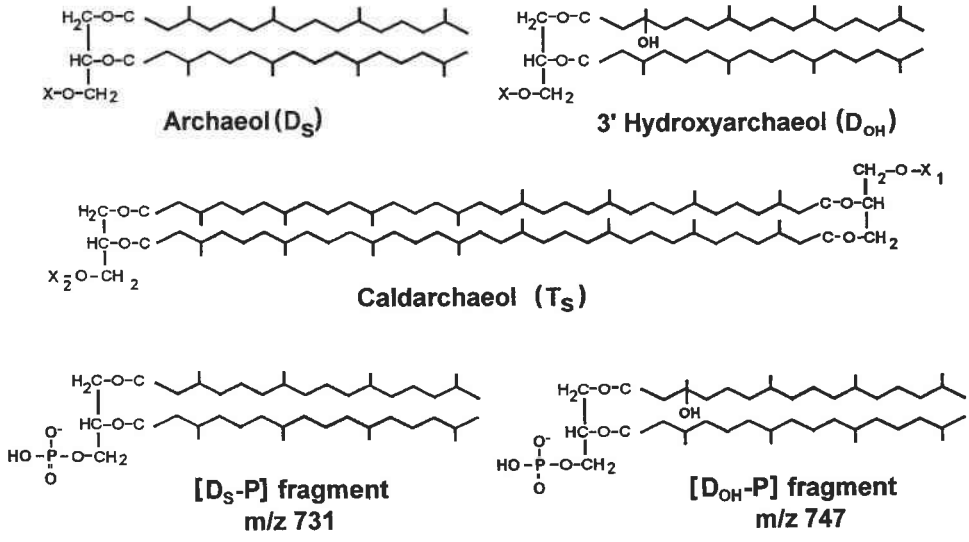
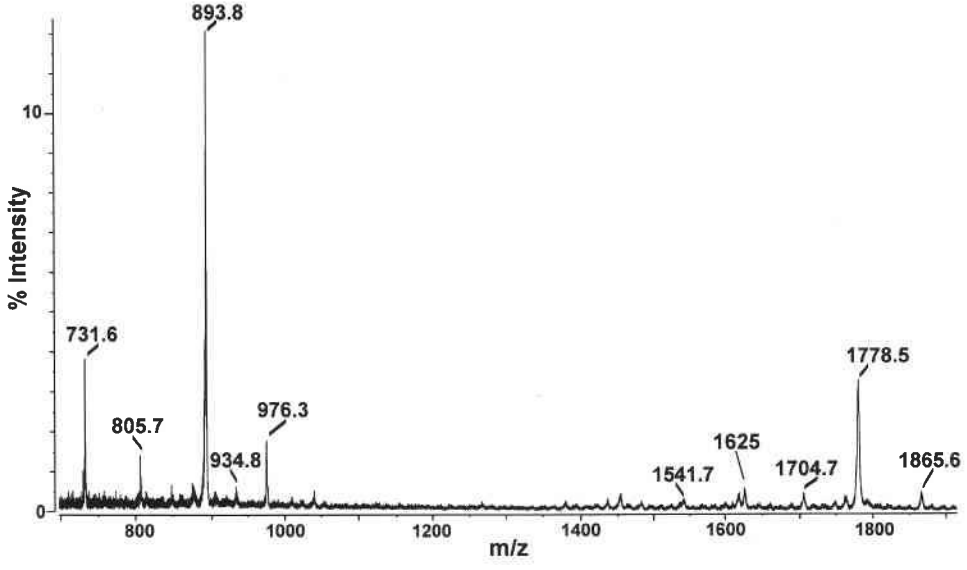


Fig. 2. Negative-ion FAB MS spectrum of the total polar lipids extracted from *Pyrococcus furiosus*.



are shown in Table 1. An ether lipid of m/z 1778 is typically a diglycosyl phosphatidylglycerol tetraether, as found in *Methanospirillum hungatei* (Kushwaha et al. 1981; Sprott et al. 1994*b*). In certain other cases, more than one structure could account for the observed negative-ion FAB MS m/z signals, and (or) it was not possible to distinguish whether the signal represented a true metabolite or a fragmentation product of a lipid of higher mass. For example, an inositol containing tetraether lipid is the most likely possibility to explain the signal of m/z 1865, as in *Sulfolobus* (Trincone et al. 1992), *Desulfurococcus mobilis* (Lanzotti et al. 1987), and *Methanobacterium thermoautotrophicum* (Nishihara et al. 1989); however, an exception was found in the single case of *Methanospirillum hungatei*. In this case, negative-ion FAB MS resulted in a signal of m/z 1865, through generation of the negative ion by loss of a CH_3 from a unique diglycosyl tetraether lipid with a *N*,*N*,*N*-trimethylpentanetetrol head group (Ferrante et al. 1987). Other signals of m/z 1541.7 and 1704.7 could not be discounted as fragmentation products of this lipid of theoretical molecular weight 1866.4 (Table 1). First, the lipid corresponding to a m/z of 1541.7 could be assigned several structures, but in *Sulfolobus* (Trincone et al. 1992) and *Methanobacterium thermoautotrophicum* (Nishihara et al. 1989) the correct assignment is

Fig. 3. Negative-ion FAB MS spectrum of the total lipid extract from *Methanopyrus kandleri*.

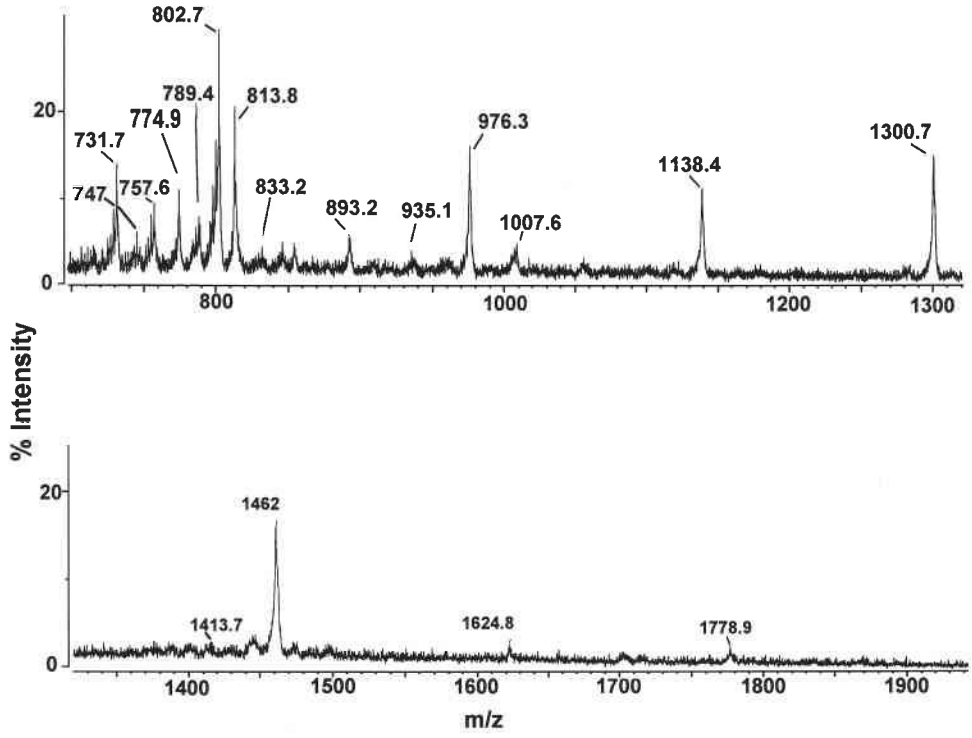


Table 1. Most probable structures for the polar lipids of *Pyrococcus furiosus* based on negative-ion FAB MS analysis.

Observed $[M-H]^-$ signals (m/z)	Structure	Calculated $[M-H]^-$ (m/z)
1865.6	$(Hexose)_2$-T_S-PI	1866.4
1778.5	$(Hexose)_2$-T_S-PG	1778.4
1704.7[a]	Hexose-T_S-PI	1704.4
1541.7[a]	T_S-PI	1542.3
976.3	$(Hexose)_2$-D_S	975.8
934.8	Acetyl-hexose-P-D_S[b]	934.7
893.8	D_S-PI or P-hexose-D_S	893.7
805.7	D_S-PG	805.7

Note: T_S, caldarchaeol; D_S, archaeol; PI, phosphoinositol; PG, phosphoglycerol; P, phosphate.

[a]Could originate as fragment ions during negative-ion FAB MS analysis. Hexose-T_S-P or P-hexose-T_S are also consistent with the observed m/z.

[b]The position of the phosphate is unknown. To calculate $[M-H]^-$ *N*-acetylation was assumed.

a phosphatidylinositol tetraether of theoretical molecular weight 1542.3 for $[M-H]^-$. Second, the signal of m/z 1704.7 corresponded in *Desulfurococcus mobilis* (Lanzotti et al. 1987) and *Thermoproteus tenax* (Thurl and Schäfer 1988) to a tetraether with head groups of phosphoinositol and a hexose sugar (theoretical $[M-H]^-$ of 1704.4). Finally, a small signal (m/z 1625; Fig. 2) may correspond to a diglycosyl tetraether of theoretical m/z of 1624 for $[M-H]^-$, as was found in *Methanobacterium thermoautotrophicum* (Nishihara et al. 1989).

Table 2. Structures for the polar lipids of *Methanopyrus kandleri*, based on negative-ion FAB MS analysis.

Observed $[M-H]^-$ signals (m/z)	Structure	Calculated $[M-H]^-$ (m/z)
Fragment ions		
747	$[D_{OH}$-P-H]	747.6
731.7	$[D_S$-P-H]	731.6
Glycolipids		
1624.8	$(Hexose)_6$-D_S	1624.0
1462.9	$(Hexose)_5$-D_S	1462.0
1300.7	$(Hexose)_4$-D_S	1299.9
1138.4	$(Hexose)_3$-D_S	1137.9
976.3	$(Hexose)_2$-D_S	975.8
813.8	Hexose-D_S	813.8
Phosphoglycolipids		
1778.9	$(Hexose)_2$-T_S-PG	1778.4
935.1	NAcGlc-P-D_S[a]	934.7
Phospholipids		
893.2	D_S-PI	893.7
833.2	D_{OH}-PS	834.7
802.7	D_S-PC	802.7[b]
789.4	D_{OH}-PE or D_{gg}-PG[c]	789.7
757.6	D_{gg}-PE[c]	757.7
774.9	D_S-PE	773.7

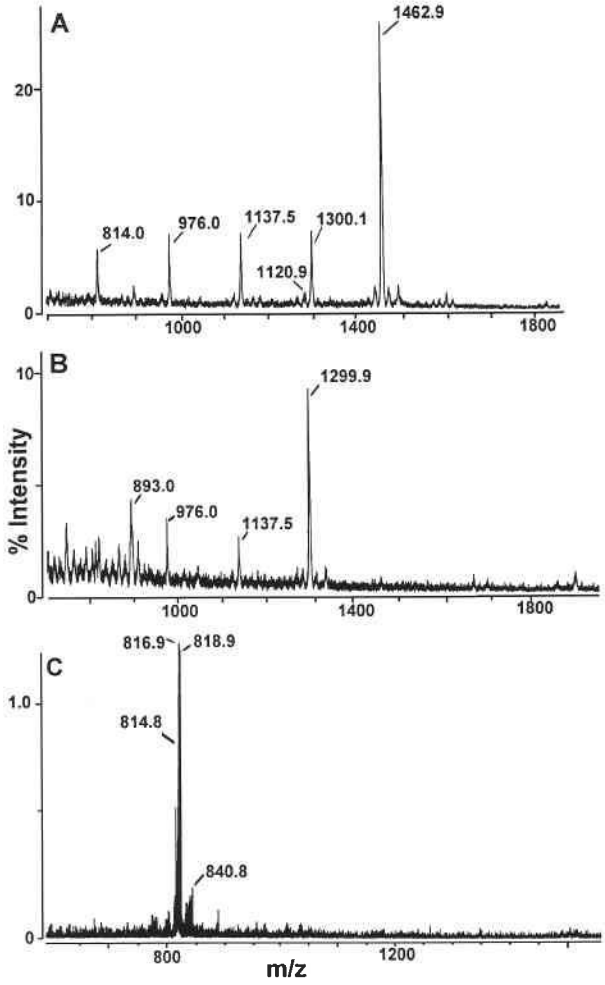
Note: D_{OH}, hydroxyarchaeol; D_S, archaeol; D_{gg}, geranylgeranyl archaeol; T_S, caldarchaeol; NAcGlc, *N*-acetylglucosamine; PI, phosphoinositol; PS, phosphoserine; PC, phosphocholine; PE, phosphoethanolamine; PG, phosphoglycerol.

[a]Position of phosphate not determined.

[b]Loss of CH_3 from m/z 817.7.

[c]Proposed to be the D_{gg} form of D_S-PG or D_S-PE, respectively.

Fig. 4. FAB MS spectra of subfractions of *Methanopyrus kandleri* lipids. (A) Negative-ion FAB MS of a glycolipid fraction of R_f 0.11 enriched for *m/z* 1462 lipid. (B) Negative-ion FAB MS of a glycolipid fraction of R_f 0.17 enriched for *m/z* 1300 lipid. (C) Positive-ion FAB MS of the total lipid extract.



A *m/z* of 893 is likely to represent the phosphatidylinositol diether found in several archaea and observed in *Pyrococcus woesei*. Yet in *Pyrococcus* strain AN1, this lipid is found along with another of identical *m/z*, corresponding to glucosyl-3-phosphate diether (Lanzotti et al. 1989).

Methanococcus voltae synthesizes an *N*-acetylglucosamine-1-phosphate diether (Ferrante et al. 1986) of calculated *m/z* 934.7 for $[M-H]^-$. In *Pyrococcus furiosus* a similar lipid anion occurs, although the position of the phosphate could not be established by negative-ion FAB MS analysis of unpurified mixtures. Hydroxydiether lipids were not observed, as seen by the absence of the fragment signal of *m/z* 747 (Fig. 1).

Methanopyrus kandleri

The polar lipids of *Methanopyrus kandleri* have been reported to consist exclusively of diethers (Kurr et al. 1991). However, the negative-ion FAB MS spectrum revealed a minor lipid of *m/z* 1778.9 (Fig. 3), which corresponds in mass to the diglycosyl phosphatidylglycerol tetraether lipids found in *Methanospirillum hungatei* (Kushwaha et al. 1981). The relative intensity of the 1778 signal varied in different extracts, suggesting either a difficulty in reproducibly solvating or a variation in amounts synthesized dependent on conditions of cell culture. A core lipid analysis by TLC confirmed the presence of a major component corresponding to authentic 2,3-di-*O*-phytanyl-*sn*-glycerol (archaeol) and minor amounts of caldarchaeol. A third, minor core lipid was identified as hydroxydiether by negative-ion FAB MS analysis, which detected a *m/z* 747 fragmentation product characteristic of phosphohydroxydiether lipids (Fig. 3).

Table 3. Most probable structures of the polar lipids of *Methanothermus fervidus*, based on negative-ion FAB MS analysis.

Observed $[M-H]^-$ signals (*m/z*)	Structure	Calculated $[M-H]^-$ (*m/z*)
1906.5	Acetyl-(hexose)$_2$-T_S-PI	1907.4
1865.7	(Hexose)$_2$-T_S-PI	1866.4
1745.7[a]	Acetyl-hexose-T_S-PI	1745.4
1704.3[a]	Hexose-T_S-PI	1704.4
1542.0[a]	T_S-PI	1542.3
1380.8[a]	T_S-P	1380.2
1016.9	Acetyl-(hexose)$_2$-D_S	1016.8
975.6	(Hexose)$_2$-D_S	975.8
934.4	*N*-Acetyl-hexose-P-D_S[b]	934.7
893.5	D_S-PI	893.7
813[a]	Hexose-D_S	813.8
774.7	D_S-PE	773.7
805	D_S-PG	805.7

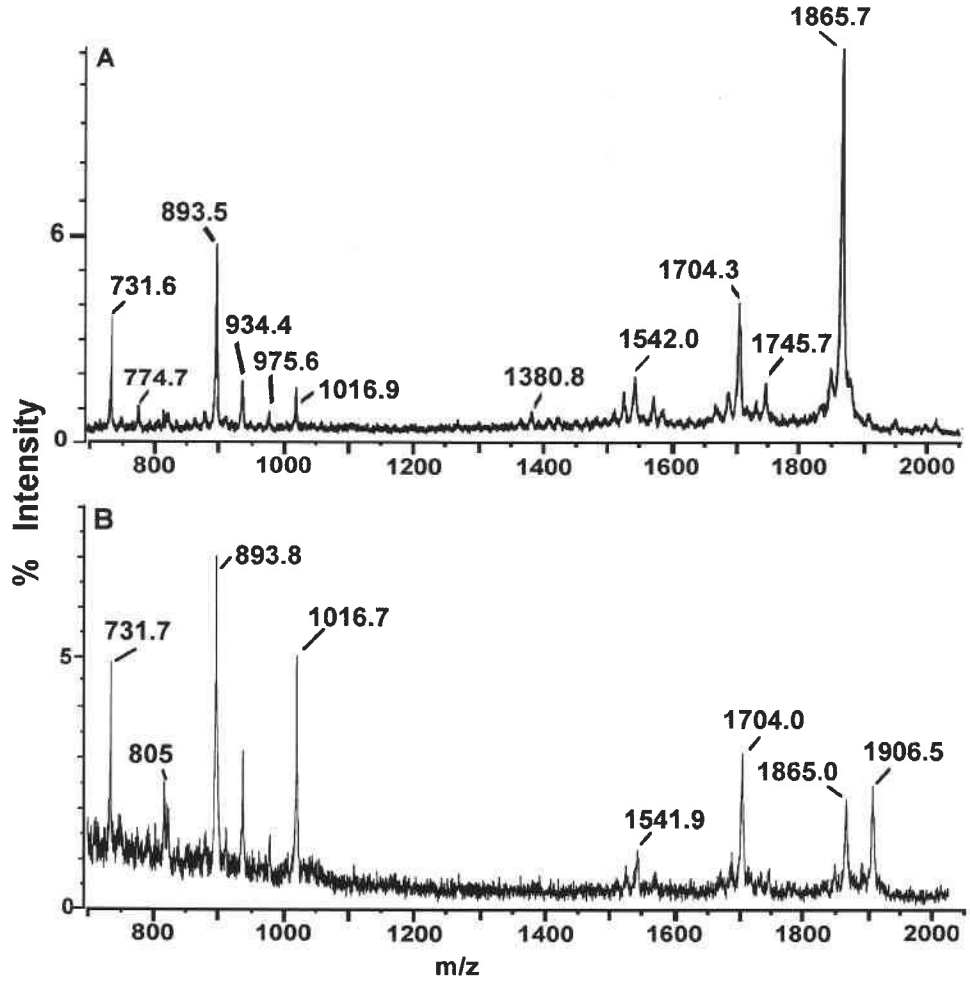
Note: The only carbohydrate head groups found were glucose and *N*-acetylglucosamine (Koga et al. 1993). T_S, caldarchaeol; D_S, archaeol; PI, phosphoinositol; PE, phosphoethanolamine; PG, phosphoglycerol.

[a]These signals could arise, at least in part, as fragment ions rather than representing true metabolites.

[b]Position of phosphate not determined.

In *Methanopyrus kandleri* the polar lipid fraction represents about 50% w/w of the total lipids, and of the polar lipids, more than 92% are glycolipids (Hafenbradl et al. 1996). This is seen in the negative-ion FAB MS spectrum of the polar lipid region (Fig. 3), considering that glycolipids in general are detected with less sensitivity than are phospholipids. The glycolipids were a series of diethers containing from one to six hexose residues (Table 2), confirming and extending the diglycosyl, triglycosyl, and pentaglycosyl diethers observed by Hafenbradl et al. (1996). The pentaglycosyl archaeol (*m/z* 1462) and the tetraglycosyl archaeol (*m/z* 1299) were not fragmentation products of higher mass lipids, as determined from partially purified lipid fractions (Fig. 4). Also, the similar peak heights of the remaining members in the series indicates that these lipids are metabolites rather than fragmentation products. The glycolipid head groups were identified following hydrolysis of the total lipid extract and preparation of alditol acetate derivatives. GC–MS analysis of these derivatives established that the sugars consisted of mannose, glucose, galactose, and *N*-acetylglucosamine, with mannose dominant and corresponding to a relative abundance of 86%, on the basis of peak areas. Glucose, galactose, and mannose have been reported earlier to be components of the lipids from *Methanopyrus kandleri* (Hafenbradl et al. 1996). The lipid of

Fig. 5. Negative-ion FAB MS spectra of the total polar lipids of *Methanothermus fervidus*. (A) Mid-logarithmic growth phase. (B) Late logarithmic growth phase.



theoretical *m/z* 934.7 was classified as an *N*-acetylglucosamine archaeol with a phosphate moiety, based on the identification of *N*-acetylglucosamine in lipid hydrolysates and the observed signal of *m/z* 935.1 (Table 2). A lipid structure was not assigned to the *m/z* 1007.6 signal.

The nonpolar lipid fraction of *Methanopyrus kandleri* is unusual in having a large amount of 2,3-di-*O*-geranylgeranyl-*sn*-glycerol (Hafenbradl et al. 1993). Interpreting the negative-ion FAB MS spectrum of the phospholipids was complicated by the possible presence of geranylgeranyl archaeol polar lipids and by the presence of hydroxyarchaeols (Table 2). Notably, the *m/z* 789.4 signal could be interpreted either as hydroxyarchaetidylethanolamine or as the geranylgeranyl archaeol analog of phosphatidylglycerol. The signal of *m/z* 757.6 appeared to be a geranylgeranyl archaeol variation of the phosphatidylethanolamine archaeol also present in these extracts.

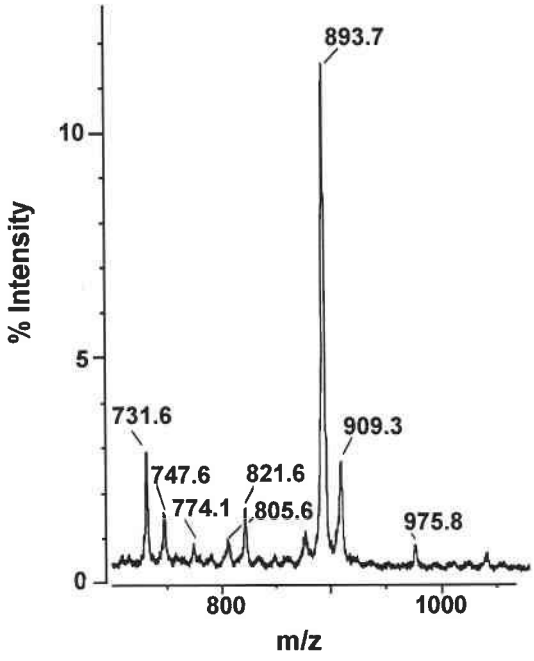
A signal of *m/z* 802.7 in the negative-ion FAB MS spectrum suggested the presence of a novel phosphatidylcholine archaeol as the most abundant phospholipid (Fig. 3, Table 2). During negative-ion FAB MS of a phosphatidylcholine lipid the negative-ion is expected to occur through the loss of a methyl group (Ferrante et al. 1987; Sprott et al. 1994*b*), in this case to yield a *m/z* of 817.7 − 15 = 802.7. A positive-ion FAB MS analysis of total lipid extracts detected signals primarily of *m/z* 818.9 (theoretical 817.7 + 1 H) and 840.8 (theoretical 817.7 + Na). Evidence for unsaturated forms of this lipid were seen in both negative- and positive-ion FAB MS (Figs. 3 and 4). A lipid positive for choline by Dragendorff staining migrated on TLC plates with an R_f of 0.53, compared with 0.52 for dimyristoyl phosphatidylcholine as a positive control, thus confirming the identification of archaetidylcholine. This is the first instance, to our knowledge, of a phosphatidylcholine lipid in an archaeobacterium, although *Methanospirillum hungatei* forms archaeol and caldarchaeol lipids with an *N*,*N*,*N*-trimethylpentanetetrol analog of phosphatidylcholine (Ferrante et al. 1987; Sprott et al. 1994*b*).

These results are in keeping with staining reactions which revealed the presence of glycolipids, aminophospholipids, phosphoglycolipids, and phospholipids (Hafenbradl et al. 1996).

Methanothermus fervidus

The negative-ion FAB MS spectrum of the polar lipids of *Methanothermus fervidus* reveals both archaeol and caldarchaeol lipids (Table 3). These results are consistent with the core lipid analysis of Lauerer et al. (1986), except no unassigned signals were found to support the presence of an unknown core lipid. A key feature of the lipids of this thermophile was the extent of acetylation of the glycolipids,

Fig. 6. Negative-ion FAB MS of the total polar lipids of *Sulfolobus acidocaldarius* showing the archaeol lipids region of the spectrum. Structural data for the tetraethers, which are the bulk of the lipid, were reported by Gulik et al. (1988).



which became more pronounced as the cells entered the late logarithmic growth phase (Fig. 5). Several lipids appeared to be *N*-acetylated during growth gaining a *m/z* of 41; most notable was the decline in the *m/z* 1865.0 signal and formation of 1906.5. Removal and analysis of the lipid head groups of this archaeobacterium have yielded glucose and *N*-acetylglucosamine as the only sugars (galactose and mannose negative) and inositol (Koga et al. 1993), supporting the assignments given. Small amounts of phosphatidylethanolamine and phosphatidylglycerol archaeols were detected by the negative-ion FAB MS analysis.

Sulfolobus acidocaldarius

The caldarchaeol and nonitolcaldarchaeol polar lipid structures for a number of strains of *Sulfolobus* are similar (Gulik et al. 1988; Trincone et al. 1992). The minor, diether lipids were readily detected by negative-ion FAB MS (Fig. 6), corresponding in *m/z* to archaeols and hydroxyarchaeols (Table 4) typical of the phospholipids found in *Methanosarcina* species (Sprott et al. 1993).

Extreme halophiles

Most of the polar lipid structures present in lipid extracts prepared from *Halobacterium cutirubrum* and *Natronobacterium magadii* have been determined (Kates 1993). Furthermore, assignments have been made for the *m/z* signals obtained in negative-ion FAB MS spectra of the glycolipid and phospholipid fractions from *Halobacterium salinarium* (Klöppel and Fredrickson 1991). Based on the above information, assignments of lipid structures were made for the negative-ion FAB MS spectrum of the total polar lipids of *Halobacterium cutirubrum* (Fig. 7). As expected, phospholipids were archaeol lipids of phosphatidylglycerol, sulfated phosphatidylglycerol,

Table 4. Structures of the archaeol polar lipids of *Sulfolobus acidocaldarius*, based on negative-ion FAB MS analysis.

Observed $[M-H]^-$ signals (*m/z*)	Structure	Calculated $[M-H]^-$ (*m/z*)
975.8	$(Hexose)_2$-D_S	975.8
909.3	D_{OH}-PI	909.7
893.7	D_S-PI	893.7
821.6	D_{OH}-PG	821.7
805.6	D_S-PG	805.7
774.1	D_S-PE	773.7

Note: D_S, archaeol; D_{OH}, hydroxyarchaeol; PI, phosphoinositol; PG, phosphoglycerol; PE, phosphoethanolamine.

phosphatidylglycerophosphate, and phosphatidylglycerolmethylphosphate, and glycolipids were sulfated triglycosyl and sulfated tetraglycosyl archaeol lipids. Glycolipids lacking sulfate were not detected in this analysis, probably because of lower detection limits. The presence of a *m/z* 773.8 peak typical of phosphatidylethanolamine diether indicates that small amounts of this amino lipid may be present, in contrast to results with less-sensitive methods (Kates 1993). An unidentified new peak of *m/z* 787.8 could correspond to an increase in the phosphoethanolamine head group by addition of a methylene moiety to form either a phosphatidylpropanolamine or a monomethylphosphatidylethanolamine diether. The spectrum for *Natronobacterium magadii* lipids confirms the structural differences reported to exist between *Halobacterium* species and *Natronobacterium* species (Kates 1993); glycolipids and sulfated lipids are absent, and the lipid core is composed of the standard $C_{20,20}$-diether plus the $C_{20,25}$-diether. (Fig. 8).

Discussion

FAB MS is a useful tool to rapidly characterize the polar lipids present in the lipid mixtures extracted from archaeobacterial thermophiles. This method requires very little material and is very powerful in detecting such modifications as acetylation (*m/z* increase of 41 or 42, for *N*- or *O*-acetylation) and hydroxylation (*m/z* increase of 16). However, supporting data is always needed to unequivocally assign a structure, i.e., more than one structure may fit the *m/z* of the molecular ion. For example, phosphatidylinositol diether (D_S-PI) and glucose-3-phosphate diether (D_S-Glc-3-P) are both *m/z* 893, and both are reported in *Pyrococcus* strain AN1 (Lanzotti et al. 1989). Other methods, such as permethylation analysis and NMR spectral analysis, are required for glycolipids and phospholipids to determine specific structural details, such as anomeric configuration, linkage positions, and substitution positions of the glycosyl groups. Another consideration is that the response factor varies with the lipid species and may be affected by the presence of certain other lipids (Fenselau et al. 1989). Therefore, each ether lipid in a mixture will have its own detection limit, which is not always easily defined. To detect all of the main phospholipid species present in a lipid extract from *Methanosarcina mazei* (Sprott et al. 1994*a*) by our negative-ion FAB MS analysis, less than 40 μg of the total polar lipid extract was required. Phospholipids are generally detected better than glycolipids, and some lipids

Fig. 7. Negative-ion FAB MS of the total polar lipids of *Halobacterium cutirubrum*. D_S, archaeol; PG, phosphoglycerol; PGS, phosphoglycerosulfate; PGP, phosphoglycerophosphate; Hex, hexose.

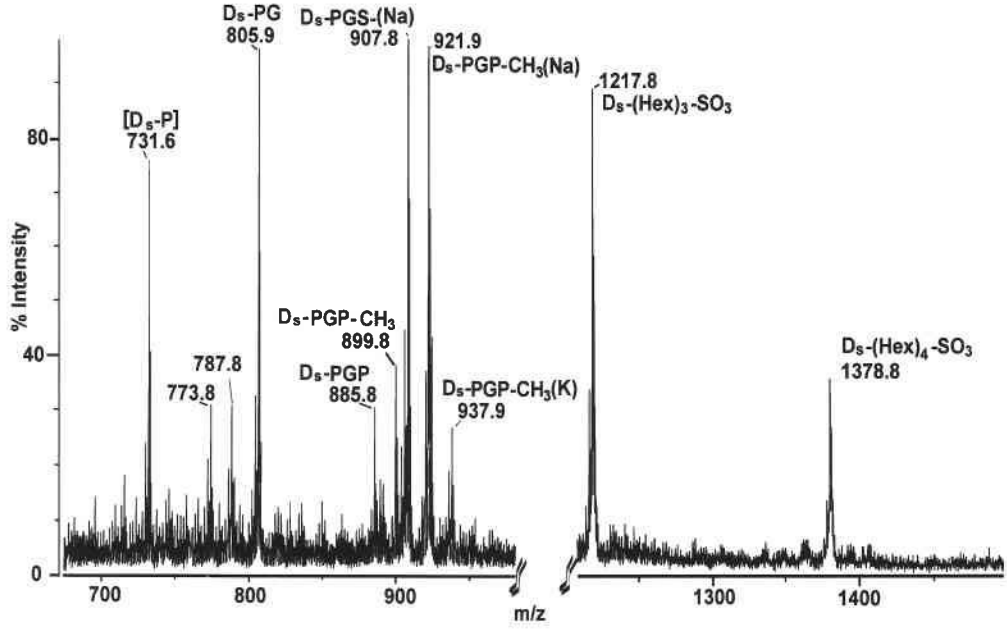
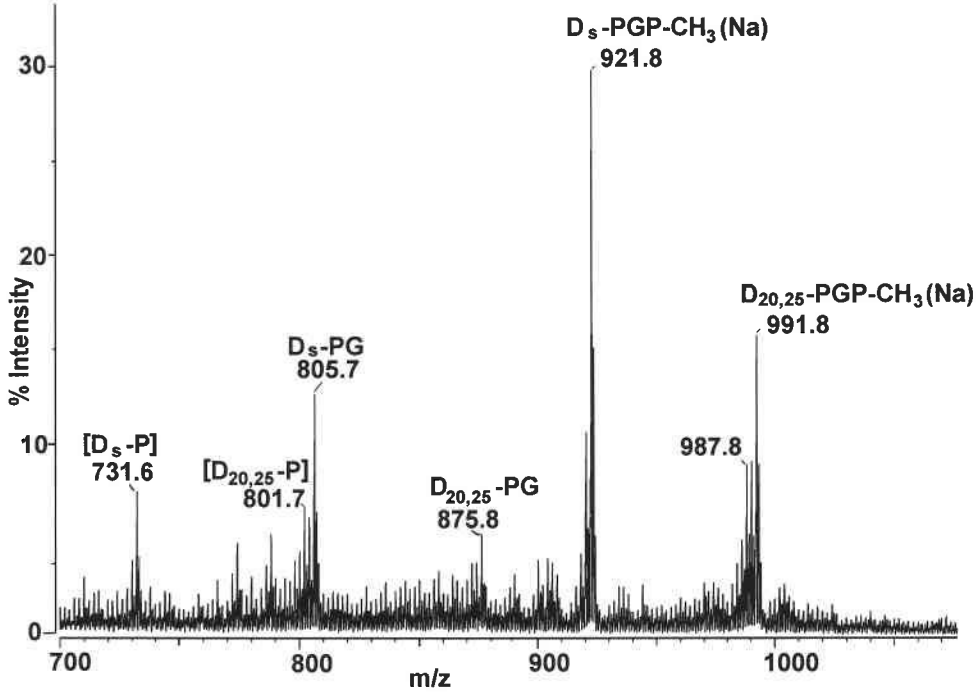


Fig. 8. Negative-ion FAB MS of the total polar lipids of *Natronobacterium magadii*. D_S, archaeol; $D_{20,25}$, archaeol with C_{20} and C_{25} alkyl chain lengths; PG, phosphoglycerol; PGP, phosphoglycerophosphate.



with very low response in negative-ion FAB MS may be missed altogether. Also, in a mixture of lipids, some *m/z* signals may arise by fragmentation of larger molecules, making it difficult to distinguish these signals from molecular ions of true metabolites. However, the method does allow a rapid assignment of structure for some archaeal ether lipids and at least allows postulation of the set of possible structures. By combining both negative- and positive-ion FAB MS analysis it was possible to determine that the main phospholipid signal in lipid extracts of *Methanopyrus kandleri* was an archaetidylcholine, novel in representing the first archaeal ether lipid found with this head group.

From the summary of properties found for the ether lipids in the archaeal thermophiles explored in this study (Table 5), no single structure clearly distinguishes these lipids from those found in archaeal mesophiles. Inositol lipids and glycolipids are common, unsaturation is very rare, and acetylation of carbohydrate moieties sometimes occurs. A membrane

Table 5. General characteristics for the polar ether lipids synthesized by the thermophilic archaea used in this study.

Archaeon	Archaeols	Hydroxyarchaeols	Caldarchaeols	Unsaturation	Acetylated sugars	Inositol	Phospho- or glyco-lipid
Pyrococcus furiosus	+	–	+	–	+	+	Both
Methanopyrus kandleri	+	+(minor)	+(minor)	+	–	+(minor)	Mainly glycolipid
Methanothermus fervidus	+	–	+	–	+	+	Both
Sulfolobus acidocaldarius	+	+(minor)	+(major)	–	–	+	Both

functional at high temperatures apparently does not require high tetraether contents, as seen for *Methanopyrus kandleri* and *Thermococcus celer* (De Rosa et al. 1987). In the latter case, glycolipids were minor and phosphatidylinositol diether was the dominant lipid species, whereas in *Methanopyrus kandleri*, glycolipid archaeols were the dominant feature. It would appear that, in Archaea within which ether lipids are a common distinguishing feature, the heat stability of other essential nonlipid molecules must determine the upper temperatures for growth.

FAB MS analysis of lipid extracts can clearly distinguish genera within the extreme halophilic archaea. The method also appears to readily detect the major lipid found in extreme halophiles, namely phosphatidylglyceromethylphosphate (Fredrickson et al. 1989), and thus distinguish extreme halophiles from other members of the archaea where it is absent.

The importance of the Archaea to global ecology is now thought to have been greatly underestimated, with recent discoveries of wide distributions of these bacteria in both extreme and nonextreme habitats. For example, Archaea appear to be widely distributed in oceans, making up to 30% of the submicrometre picoplankton in cold surface waters of Antarctica and Alaska (Olsen 1994). Detection of archaeal ether lipids using sensitive, diagnostic methods such as described here may supplement molecular biology methodologies to answer questions on the global importance of the Archaea.

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