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Review

Recent Advances in Structural Research on Ether Lipids from Archaea Including Comparative and Physiological Aspects

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A great number of novel and unique chemical structures of archaeal polar lipids have been reported. Since 1993, when those lipids were reviewed in several review articles, a variety of core lipids and lipids with unique polar groups have been reported successively. We summarize new lipid structures from archaea elucidated after 1993. In addition to lipids from intact archaeal cells, more diverse structures of archaea-related lipids found in environmental samples are also reviewed. These lipids are assumed to be lipids from unidentified or ancient archaea or related organisms. In the second part of this paper, taxonomic and ecological aspects are discussed. Another aspect of archaeal lipid study has to do with its physiological significance, particularly the phase behavior and permeability of archaeal lipid membranes in relation to the thermophily of many archaea. In the last part of this review we discuss this problem.

Key words: archaea; ether polar lipid; structure determination; chemotaxonomic marker; heat-tolerant membrane

Since 1962, when Kates *et al.* found a diether-type phospholipid in an extremely halophilic microorganism, *Halobacterium cutrirubrum*,[1] over 100 ether-type polar lipids (phospholipids, glycolipids, and phosphoglycolipids) have been identified in various archaea. The time when the first diether-type phospholipid was found in *Halobacterium* was far before the concept of archaea or archaebacteria was proposed. Soon after Woese *et al.*[2] proposed that methanogens should be classified in a completely separate group as archaebacteria (later renamed archaea[3]) in 1977, ether-type lipids were recognized as one of the most characteristic markers of archaeal cells. Since lipids of thermoacidophiles (*Sulfolobus* and *Thermoplasma*) and methanogens as well as extreme halophiles were identified as isoprenoid-glycerol ethers, these microorganisms were also classified in archaea. Throughout the 1980s and the 1990s, a great number of novel and unique chemical structures of archaeal polar lipids were reported and reviewed in several review articles.[4–7] After the reviews were published, a variety of core lipids and lipids with unique polar groups were reported successively. The lipid structures which have been identified in archaea up to now are summarized in Table 1, which shows the distribution of lipid component parts but not exact structures in all the known families of archaea. Archaeal lipids are generally composed of a core lipid (archaeol or caldarchaeol) and phosphodiester bonded polar head groups or glycosides that are linked to one of the core lipids. Therefore, if one knows the lipid component parts in a given species of archaea, one can assume the lipid structures present in that species. Because the composition of lipid component parts is widely shared by archaea species belonging to the same family, Table 1 shows the family-level distribution of lipid component parts, even though the phylogenetic relationship of the lipid composition of species of crenarchaeota is not unambiguously established. Up to now, minimal information on lipid components has been reported for at least one organism of all but one archaeal family (*Thermofilaceae*). Recent studies on methanogens lipids revealed that they reflect the phylogenetic relationship of archaeal organisms and that they can be a tool for taxonomic and ecological studies of Archaea.[8]

In this article, first we summarize recently elucidated structures of new core lipids and polar lipids from archaea since publication of the reviews in 1993.[6,7] In addition to lipids from intact archaeal cells, more diverse structures of archaea-related lipids were found from environmental samples, which are also reviewed, because they are assumed to be lipids from unidentified or ancient archaea or related organisms. In the next part of this review, taxonomic and ecological aspects are discussed. The physiological significance of archaeal lipids is discussed in relation to the heat-tolerance of archaeal lipids in the last part of this review.

I. New Core Lipids

Isocaldarchaeol

The structure of caldarchaeol was first proposed to be an antiparallel arrangement of the two glycerol units for *Thermoplasma acidophilum* tetraether lipid shown in

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Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography

Table 1. Distribution of Lipid Component Parts in Archaea

A line in which only a family name is shown represents common distribution of lipid componet parts in a number of species of the family.

Archaeal family	Core lipid									Phospholipid Head Group						Glycolipid Sugar						Reference
	Archaeol					Caldarchaeol																
	Ar	HO	C25	Cyc	Usat	CA	Ring	H	Ctol	Ino	Et	Ser	Gro	APT	Cho	Glc	Gal	Man	GN	Gul	Sul	
Halobacteriaceae	+	−	(−)	−	−	−	−	−	−	−	−	−	+	−	−	+	+	+	−	−	+	6
Methanobacteriaceae	+	−	−	−	−	+	−	−	−	+	+	+	−	−	−	+	−	−	−	−	−	8
Methanothermaceae	+	−	−	−	−	+	−	+	−	+	+	−	−	−	−	+	−	−	+	−	−	8, 33
Methanococcaceae	+	+	−	−	−	(−)	−	−	−	−	(+)	+	−	−	−	+	−	−	+	−	−	8
Methanocaldococcaceae	+	−	−	+	−	(+)	−	−	−	−	+	+	−	−	−	+	−	−	+	−	−	8
Methanomicrobiales	+	−	−	−	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	8
Methanopalnaceae	+	−	−	−	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	8
Methanocorpusculaceae	+	−	−	−	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	8
Methanospirillaceae	+	−	−	−	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	8
Methanosarcinaceae	+	(+)	−	−	(−)	−	−	−	−	+	+	(+)	+	−	−	+	−	−	+	−	−	8
Methanosaetaceae	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+	+	−	−	−	8
Methanopyraceae	+	+	−	−	+	+	−	−	−	+	+	+	?	−	+	+	+	+	+	−	−	32, 33, 34
Thermoplasmataceae	+	+	−	−	−	+	+	−	−	+	−	−	+	−	−	+	+	+		+	−	45, 46, 47, 48
Thermococcaceae	+	−	−	−	−	+	(−)	(+)	−	+	−	−	+	−	−	+					−	15, 16, 33, 82
Archaeoglobaceae	+					+	−	−		+	+	−	−	−	−	+	+	+	−			Tarui, unpublished
Sulfolobaceae	+	+	−	−	−	+	+	−	+	+	+	−	+	−	−	+	+	−	−	−	+	5, 6, 21, 22, 33
Thermoproteaceae	+					+	+			+						+						83
Thermofilaceae																						
Desulfurococcaceae									−													
Aeropyrum pernix	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−		−	−	39
Desulfurococcus mobilis	+					+	−		−	+						+	+					84
Ignicoccus islandicum	+					+																85
Syaphylothermus marinus	+					+																86
Sulfophobococcus zilligii	−					+	−															87
Pyrodictiaceae	+					+																88

(+), present in the type species of the type genus but absent in some other species
(−), absent in the type species of the type genus but present in some other species
Ar, archaeol; HO, hydroxyarchaeol; C25, C25-chain containing archaeol; Cyc, macrocyclic archaeol, Usat, unsaturated archaeol
CA, caldarchaeol; Ring, cycropentane ring-containing caldarchaeol; H, H-shaped caldarchaeol; Ctol, calditol
Ino, inositol; Et, ethanolamine; Ser, serine, Gro, glycerol; APT, aminopentanetetrol; Cho, choline
Glc, glucose; Gal, galactose; Man, mannose; GN, (*N*-acetyl)glucosamine; Gul, gulose; Sul, sulfate or sulfono group

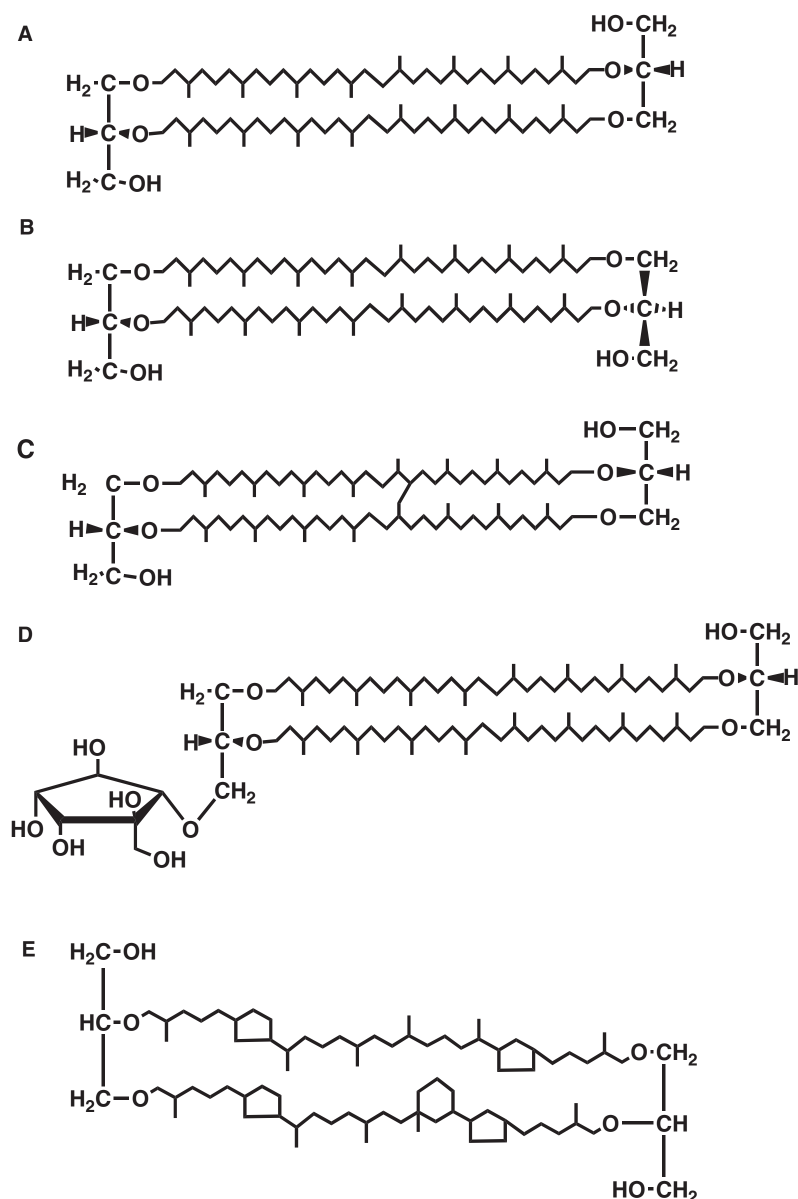

Fig. 1. New Tetraether-Type Core Lipids from Archaea.

A, Caldarchaeol; B, isocaldarchaeol;[12)] and C, H-shaped caldarchaeol from *Methanothermus fervidus*;[14)] D, calditoglycerocaldarchaeol;[22)] E, crenarchaeol.[26)]

Fig. 1A by Langworthy,[9)] and later NMR investigation by De Rosa *et al.* on the tetraether core lipid from *Caldariella acidophila* (later reclassified as *Sulfolobus solfataricus*[10)]) supported such an arrangement.[11)] This proposal has been tacitly extrapolated to all other tetraether cores from other archaea. Gräther *et al.*[12)] found that caldarchaeol isolated from *Methanobacterium thermoautotrophicum* strain Marburg (recently renamed *Methanothermobacter marburgensis*), apparently homogeneous by thin-layer chromatography (TLC), was a mixture of regioisomers with antiparallel (Fig. 1A) and parallel (Fig. 1B) arrangements of two glycerol moieties based on two independent elaborate degradation procedures. The principle of one of the reactions is shown in Fig. 2. The ratio of the two isomers was 45:55, estimated by respective methods. The parallel isomer was named isocaldarchaeol. “Caldarchaeol”, isolated from *Thermoplasma acidophilum* and *Sulfolobus solfataricus*, is also a mixture of the two isomers. The two isomers might be formed by chance during head-to-head condensation of C20 precursor isoprenoid chains (possibly geranylgeranyl chains) of a diether-type polar lipid precursor of tetraether polar lipid. If this is the case, it is possible that caldarchaeol isolated from other archaea is a mixture of these isomers.

H-shaped caldarchaeol from Methanothermus fervidus and related hyperthermophilic archaea

Methanothermus fervidus is a hyperthermophilic methane-producing archaeon isolated from a hot spring

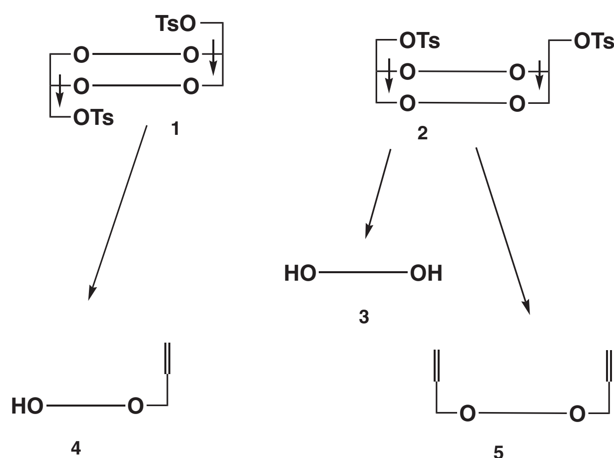

Fig. 2. Demonstration of the Presence of Regioisomers of Caldarchaeol and Isocaldarchaeol.

Tosylated caldarchaeol **1** and isocaldarchaeol **2** were submitted to the Boord haloalkoxy elimination reaction[89] (the reaction sites are shown by arrows).[12] Monoallyl ether **4** and diallyl ether **5** as well as diol **3** were formed, indicating that apparently homogeneous tetraether core lipid is a mixture of **1** and **2**.

in Iceland that grows fastest at 83 °C.[13] During analysis of core lipids of *M. fervidus* cells, Morii *et al.*[14] found a new core lipid that migrated more slowly than ordinary caldarchaeol on TLC, and hydrocarbon prepared from it was not eluted from a column of gas-liquid chromatography (GLC) at 350 °C. They assumed that it might be a compound with high molecular weight. This assumption was confirmed by MS of the new core lipid and the derived hydrocarbon. That is, although the molecular weight of the core lipid was 2 mass unit less than that of ordinary caldarchaeol, the molecular weight of the hydrocarbon derived from the core lipid coincided with the molecular weight of C80 hydrocarbon. This means that the new core lipid has a covalent C–C bridge between two C40 hydrocarbon chains. Although the exact location of the bridge has not been established by MS fragmentation analysis, a most likely structure was proposed based on data from MS and NMR (Fig. 1C). Morii *et al.* named it H-shaped caldarchaeol. Sugai *et al.* successfully detected an H-shaped hydrocarbon by GLC at a higher temperature from the lipid of *Pyrococcus horikoshii*[15] and a further 3 species out of 16 species of the family *Thermococcaceae*.[16] This result indicates that the occurrence of H-shaped caldarchaeol is not family-specific. It was confirmed that H-shaped caldarchaeol is, in fact, a core portion of polar lipids by preparing it from a polar lipid fraction, although the complete structure of a polar lipid with H-shaped caldarchaeol core is not known.

Correct structure of calditol in Sulfolobus spp.

De Rosa *et al.*[17] reported that two kinds of tetraether core lipids were found in *Sulfolobus solfataricus*, ordinary diglycerocaldarchaeol (Fig. 1A) and glyceronitocaldarchaeol. In the latter core lipid, two polyol backbones bound with C40 isoprenoid diol are glycerol and nonitol (or calditol; hereafter the structure proposed by De Rosa *et al.*[17] is called nonitol, and the structure of the natural product and its isomers are called calditol). The structure of nonitol originally proposed is a branched open chain nonahydroxynonane (2-(1′,2′,3′-trihydroxypropyl)-1,2,3,4,5,6-hexahydroxyhexane, $C_9H_{20}O_9$; 272, Fig. 3A). Nonitol or calditol has a structure in which a glycerol moiety is linked to C6 polyol. Isoprenoid hydrocarbon chains are bound at two hydroxyl groups of the glycerol moiety *via* ether bonds. Therefore, nonitol or calditol resembles a glycosylglycerol backbone of glycosyl caldarchaeol. But because the C6 polyol portion cannot easily be removed from the glycerol moiety, nonitol or calditol is assumed to be a polyol backbone of the lipid core as a whole, like glycerol.

Several questions were raised by synthetic studies and instrumental reinvestigation of the structure. Jeganathan *et al.*[18] synthesized nonitol and found that its NMR spectrum was different from that of the natural compound. They tentatively concluded that the natural and synthetic nonitols were diastereomers. Fairbanks *et al.*[19] concluded that the structure proposed by De Rosa *et al.*[17] was erroneous based on the discrepancies in NMR spectra between another chemically-synthesized isomers of nonitol and calditol isolated from *Sulfolobus* cells, and stated that reinvestigation of the structure was required. Sugai *et al.*[20] questioned the nonitol structure from the mass spectral data (m/z of the molecular ion $[M - H]^- = 253$) of calditol isolated from *S. acidocaldarius*. They proposed a new structure for calditol, shown in Fig. 3B, based on the results obtained by ^{1}H- and ^{13}C-NMR, negative and positive FAB-MS of native and acetylated calditol, and chemical degradation analysis. The new structure is an ether of glycerol and 2-hydroxymethyl-1,2,3,4,5-pentahydroxycyclopentane ($C_9H_{18}O_8$; 254). Although there should be 32 stereoisomers of calditol due to the five chiral centers on the ring other than the *sn*-2 carbon of the glyceryl moiety, they went no further than to show that a *cis*-configuration between two proton pairs (H3 and H4) on the ring from the results of NOE. Gräther *et al.*[21] independently proposed a different isomeric structure (Fig. 3D) for the calditol structure based mainly on NMR studies. Finally, Blériot *et al.*[22] synthesized four stereoisomers of calditol around the chiral centers of C-2 and C-3 on the ring, and one of them (Fig. 3D) was found to be fully identical to the natural product in ^{1}H and ^{13}C-NMR and optical rotation. All three proposals for the calditol structure are consistent in the planar structure in which 2-hydroxymethyl-1,2,3,4,5-pentahydroxycyclopentane is linked *via* an ether bond with the glycerol moiety at the *sn*-1 position. The difference among the three proposals is the configuration around the five chiral centers on the cyclopentane ring. Blériot *et al.* depicted the calditol structure proposed by Sugai in their paper as structure 5 (Fig. 3C). This is in fact an

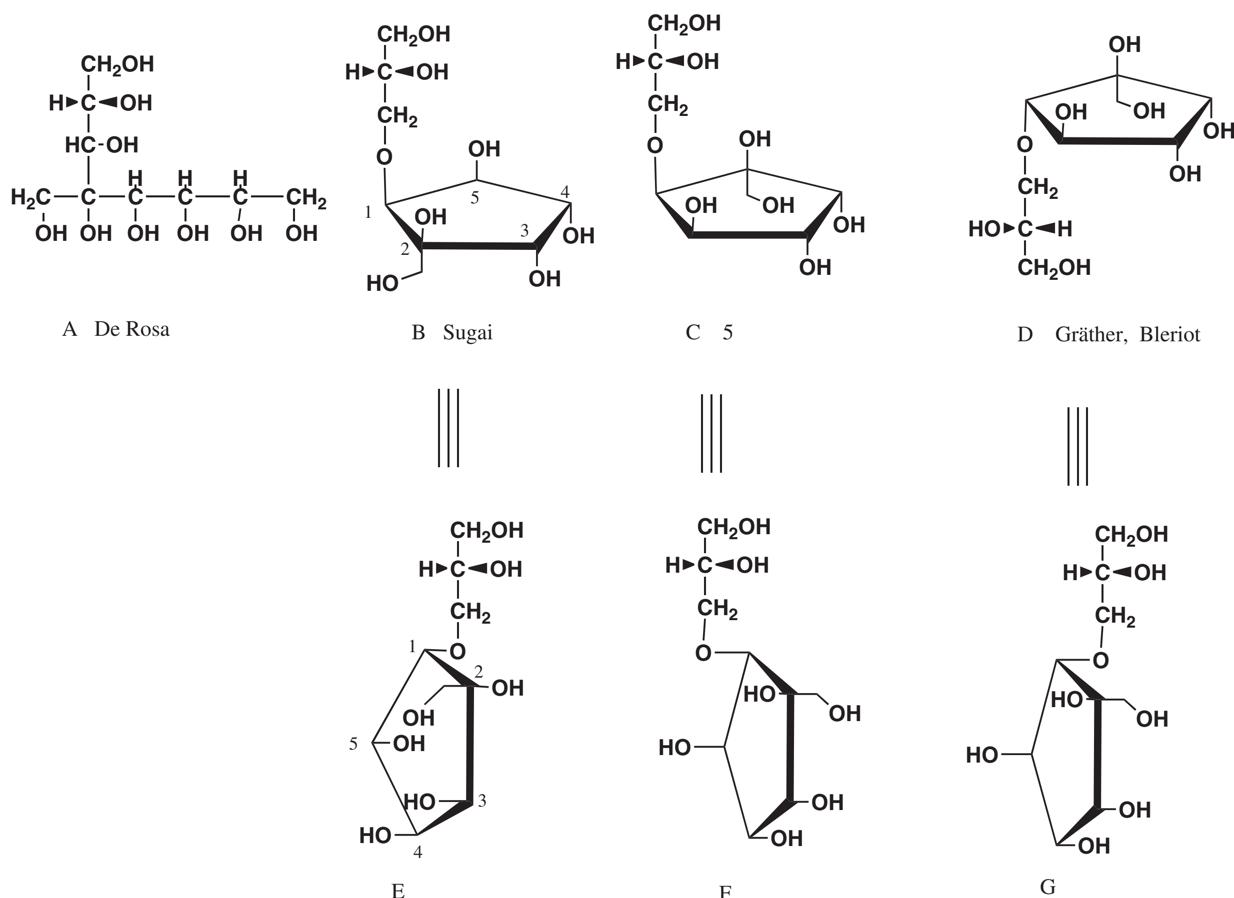

Fig. 3. Proposed Structures of Nonitol and Calditol.

A, Open-branched chain structure proposed by De Rosa.[17] B and E, Proposed by Sugai *et al.*[20] C and F, Structure 5 depicted in Blériot's paper[22] as the Sugai's proposal. D and G, Correct structure of calditol, proposed by Gräther[21] and Blériot *et al.*[22]

diastereomer of Sugai's calditol. As shown in Fig. 3E, F, the ring configurations are in mirror images, while the glycerol moieties of the two structures are absolutely the same. Blériot's caldtitol structure is different from Sugai's in the configuration at the four positions of the ring, not at a single stereocenter as they mentioned. Structure 5 (in the Blériot's paper) is, therefore, unfounded. The correct structure of calditol has been elucidated to be the structure shown in Fig. 3D (= G). The structure of calditol-containing caldarchaeol (calditoglycerocaldarchaeol) is shown in Fig. 1D. According to Sugai *et al.*, the intramolecular ether linkage is resistant to BCl_3 treatment, probably due to steric hindrance, but it can be cleaved by heating with 57% HI at 100 °C for 60 h.

Crenarchaeol and archaea-related lipids in environment samples

Analyses of rRNA and caldarchaeol or its component C40 isoprenoid are two main markers to detect the presence of archaea in environmental samples, especially marine picoplanktons and sediments. In addition to ordinary C40 biphytane (C40 H–H), various new hydrocarbons have been found. Hoefs *et al.*[23] detected, from a polar lipid fraction, C40 isoprenoid hydrocarbon with three cyclopentane rings at unusual position in particulate and sedimentary organic matter samples of water column from several marine environments (Fig. 4A). The same unusual cyclopentane ring containing biphytane C40 3Ri was also detected in picoplankton collected from sea water in Antarctica.[24] Because these hydrocarbons were prepared by HI-$LiAlH_4$ treatment of the extracted samples, they were probably present as an ether lipid form in the samples. rRNA analysis of the same sample revealed that they were nonthermophilic crenarchaeotes. Hoefs *et al.* discussed the fact that planktonic archaea have thrived in marine environments for at least the past 50 million years judging by the occurrence of the characteristic lipids in the fossil record.[23] Schouten *et al.*[25] found a variety of tetraether core lipids with isoprenoid-derived and non-isoprenoid branched hydrocarbons from various environmental samples by use of the HPLC/MS technique. Among them, novel and unique structures were found. For example, a C31 chain with three methyl branches (Fig. 4B), a C30 chain with one methyl branch and one cyclopentane ring (Fig. 4C), and C35 and C37 chains in which a C20 isoprenoid chain and an C15 and C17 iso-

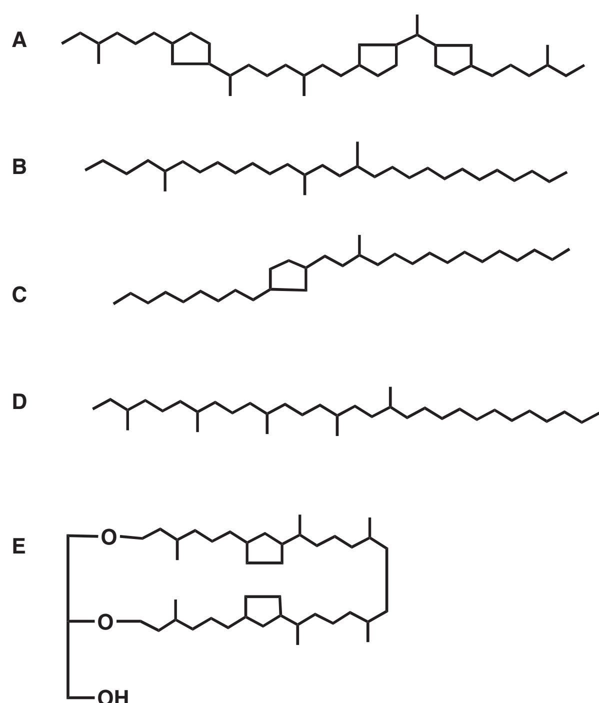

Fig. 4. Archaea-Related and Non-Archaeal Hydrocarbons from Environmental Samples.

A, C40 isoprenoid with three cyclopentane rings at an unusual position;[23] B, C31 non-isoprenoid hydrocarbon with three methyl branches;[25] C, C30 non-isoprenoid hydrocarbon with a cyclopentane ring;[25] D, C35 hybrid hydrocarbon apparently formed by combination of C20 isoprenoid and iso-branched C15 non-isoprenoid hydrocarbon;[25] E, cyclic archaeol with two cylopentane rings.[28]

branched fatty chain, respectively, are conjugated (Fig. 4D), are especially unique. A cyclohexane ring-containing lipid (Fig. 1E) added a new member to the ring-containing lipid family. The original tetraether lipid containing a cyclohexane ring was named crenarchaeol.[26] Mono- or di-methyl branched hydrocarbons might be derived not from archaea but from thermophilic bacterial members, such as *Thermotoga*, which contains 15,16-dimethyl-30-glyceryloxitriacontanoic acid.[27] Two latter conjugated hydrocarbons can be regarded as hybrid lipids between archaeal isoprenoid lipids and bacterial fatty chains. It is not known whether the hybrid lipids occurred in an assumed hybrid organism or were formed abiotically in an environment after the death of the parent organisms. It may be expected that new archaeal organisms will be isolated and proved to contain these new lipids in their cells.

Stadnitskaia *et al.*[28] identified a new macrocyclic archaeol with one or two cyclopentane rings in the biphytanediyl chain (Fig. 4E) in carbonate crust taken from the Black Sea. This new compound resembles cyclic archaeol without a cyclopentane ring of *Methanocaldococcus jannaschii* on the one hand, and also resembles cyclopentane ring-containing caldarchaeol in *Sulfolobus* spp. on the other.

Allyl ether type core lipids from Methanopyrus kandleri and Methanococcoides burtonii

Methanopyrus kandleri is another hyperthermophilic methanogenic archaeon, whose optimal growth temperature is 98 °C.[29] The organism is phylogenetically the most distantly related with other methanoarchaea.[30] Digeranylgeranylglycerol (unsaturated archaeol) was first discovered by Hafenbradl *et al.*[31,32] in the nonpolar lipid fraction of *Methanopyrus kandleri*. They reported in the same paper,[32] however, that the core lipid prepared by HCl-methanolysis of polar lipids of the organism was composed only of saturated archaeol. But Sprott *et al.*[33] have shown the presence of unsaturated archaeol-based phospholipids, the most abundant of which is unsaturated archaetidylcholine, in the same archaeon by MS of total lipids. Nishihara *et al.*[34] reexamined HCl-methanolysis for choline-containing phospholipids, and they found that most of the phospholipids were degraded during methanolysis but that a small amount of the lipid remained intact, composed of saturated archaeol as a core. They assumed that the acid-degraded portion of the phospholipids was composed of acid-labile allyl ether core lipids. To confirm this assumption, they developed a $LiAlH_4$ reductive cleavage method of allyl ether bonds.[34] Allyl ether-bonded hydrocarbon was released as intact hydrocarbon by their method. The products were identified as unsaturated isoprenoid hydrocarbons with one, two, three, or four double bonds by GLC–MS. In this way acid-labile phospholipid with allyl ether-bonded hydrocarbons was first chemically characterized (Fig. 5). $LiAlH_4$ does not attack saturated ether, alk-1′-enyl ether (plasmalogen), or reduce an ethylene double bond, but cleaves an ester bond or a phosphodiester bond.[35]

The hydrocarbon was proportionally released from the original allyl ether lipid by this method even if absolute recovery was not achieved. Quantitation of allyl ether-bonded hydrocarbon by GLC, therefore, was achieved by measuring the ratio of the sample to an internal standard (*n*-tetracosane), which gave a hydrocarbon composition of archaetidylcholine of total allylic hydrocarbon of 78%, non-allylic unsaturated hydrocarbon of 16%, and saturate hydrocarbon of 6%. Of the allylic hydrocarbon, the composition was four double bond-species, 47%; three double bond-species, 11%; two double bond-species, 14%; and one double bond-species, 7%. On the basis of the acid lability of allyl ether lipids, the molecular species of archaetidylcholine were estimated by phosphate determination of HCl-degradation products of archaetidylcholine (see Ref. 36 for the method). The results were almost consistent with the hydrocarbon composition described as above.[34] This was the first example of estimation of molecular species composition of archaeal ether phospholipid. In addition to archaetidylcholine, six phospholipids, including phosphorylated *N*-acetylglucosaminyl archaeol, archaetidylinositol, hydroxyarchaetidylserine, hydroxyarchaetidylethanolamine or unsaturated archaetidylglycerol,

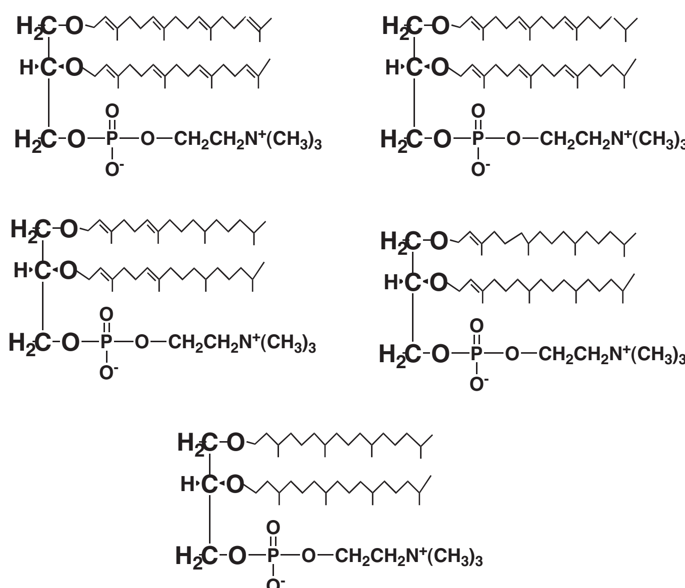

Fig. 5. Some of the Molecular Species of Archaetidylcholine from *Methanopyrus kandleri* with Different Number of Double Bonds. Top four structures shown are ally ether lipids.[34)]

unsaturated archaetidylethanolamine, and archaetidylethanolamine, six archaeol-based glycolipids with one to six hexose units, and one tetraether type phosphoglycolipid, dihexosyl caldarchaetidylglycerol, have been identified, mainly by FAB-MS.[33)] Total lipid of *M. kandleri* is rich in non-polar lipid (the polar lipid fraction represents only 50% of the total lipids), and more than 92% of the polar lipids are glycolipids.[32)] Of the four kinds of glycolipid-sugar, mannose is predominant (86%). Such membrane lipid composition appears to be unique compared with that of other microorganisms.

Nichols *et al.*[37)] have also identified unsaturated archaeol- and unsaturated hydroxyarchaeol-based phospholipids with glycerol and inositol as polar head groups by HPLC–MS in a psychrotrophic methanoarchaeon, *Methanococcoides burtonii*, isolated at Ace Lake in Antarctica. They reported that the unsaturated archaeol core contains one to five double bonds, although the location of the double bonds could not be determined. They also described that the unsaturated hydroxyarchaeol core lipid contained one to four double bonds. These unsaturated ether phospholipids are all new ones. Although they depicted the structural formula of α-hydroxyarchaetidylglycerol and α-hydroxyarchaetidylinositol in their paper, no evidence for the α-isomer of hydroxyarchaeol was presented. Koga *et al.*[8)] have reported the presence of β-hydroxyarchaeol in the same organism based on lipid component parts analysis. Hydroxyarchaetidylglycerol and hydroxyarchaetidylinositol in *M. burtonii*, therefore, are most likely β-hydroxyarchaetidylglycerol and β-hydroxyarchaetidylinositol.

Long chain archaetidyl-myo-inositol and archaetidylglucosyl-myo-inositol from Aeropyrum pernix

Aeropyrum pernix was the first absolutely aerobic, hyperthermophilic archaeon isolated at a coastal solfataric thermal vent in Kodakara-Jima island in Japan. It grows optimally at 90–95 °C.[38)] Total polar lipid is composed of two major phospholipids, archaetidyl-*myo*-inositol and archaetidyl-(glucosyl)-*myo*-inositol,[39)] in addition to one minor phosphoglycolipid and two glycolipids.[38)] Each of the major phospholipids contains only one kind of core lipid with two C25 isoprenoid chains. Although an archaeol core lipid with two C25 isoprenoid chains had been found in haloalkaliphilic Archaea,[40)] it also contains a C20 chain. It is the first example that all isoprenoid chains in polar lipids are C25 isoprenoid without a C20 chain in the cells of one species of archaeal organism. Archaetidyl-*myo*-inositol is not very novel a lipid except for the longer isoprenoid chains. Archaetidyl-(glucosyl)-*myo*-inositol, however, has a unique structure, in which an α-D-glucosyl moiety is bound at the 2 position of *myo*-inositol of archaetidylinositol (Fig. 6). Archaetidyl-(glucosyl)-inositol resembles archaetidyl-(glucosaminyl)-*myo*-inositol in *Methanosarcina barkeri*, but is different in the glycosyl moiety and in the position of inositol at which glycoside is linked.

II. New Ether Polar Lipids

β-hydroxyarchaetidylethanolamine and β-hydroxyarchaetidylglycerol from Methanosarcina barkeri

Total lipid of *Methanosarcina barkeri* is composed of nine major phospholipids with archaeol or β-hydroxyarchaeol as a core lipid. Four kinds of polar head groups,

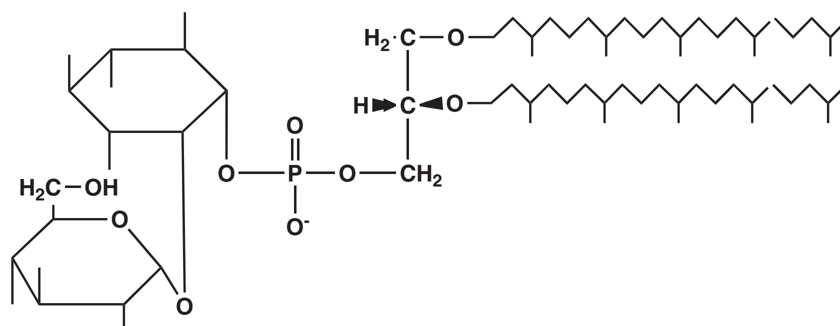

Fig. 6. Archaetidyl-(glucosyl)-*myo*-inositol from *Aeropyrum pernix*.[39]

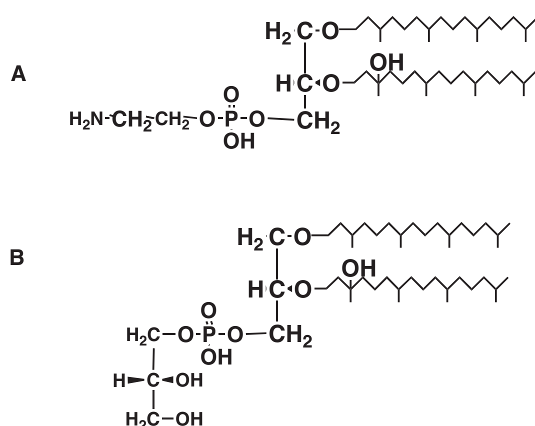

Fig. 7. New Polar Lipids from *Methanosarcina barkeri*.[39]
A, β-hydroxyarchaetidylethanolamine; B, β-hydroxyarchaetidylglycerol.

L-serine, *myo*-inositol, ethanolamine, and glycerol, are attached to archaeol and β-hydroxyarchaeol through a phosphodiester bond (eight phospholipids). Another one is archaetidyl-(glucosaminyl)-*myo*-inositol. No β-hydroxyarchaeol derivative of the last one was found. Among these nine lipids, seven phospholipids were reviewed in a previous paper.[7] β-hydroxyarchaetidylethanolamine and β-hydroxyarchaetidylglycerol were newly reported[41] (Fig. 7A, B). Cells of the genus *Methanosarcina* contain little glycolipid compared with other methanogens. Hydroxyarchaeol core and *myo*-inositol, ethanolamine, and glycerol as phosphodiester-linked polar groups are the common markers of members of the family *Methanosarcinaceae*. This conclusion was derived from analysis of the lipid component parts of 13 strains from 10 species of the family.[8]

Ether analog of cardiolipin (bisarchaetidylglycerol) and two phosphosulfoglycolipids from extreme halophiles

Although halobacterial lipids had been extensively studied, mainly by Kates group,[6] a new phospholipid and a new phosphoglycolipid have been identified from purple membrane of a genetically engineered strain of *Halobacterium salinarum*. The purple membrane of a *Halobacterium* cell contains only one protein, bacteriorhodopsin, a photo-driven energy-transducing protein, associated with special lipids. The phospholipid is an ether analog of cardiolipin (Fig. 8A), and the phosphoglycolipid is archaetidylated S-TGA-1[42] (Fig. 8B). An ether analog of cardiolipin is bisarchaetidylglycerol. By this finding, ether-type analogs of all kinds of bacterial/eukariotic ester phospholipids (ethanolamine-, serine-, glycerol-, *myo*-inositol-, choline-phospholipids, and cardiolipin) have been found in archaeal phospholipids, except for monomethyl- and dimethyl-ethanolamine-phospholipids. S-TGA-1 is a major glycolipid of halobacteria, the structure of which is sulfogalactosyl-mannosyl-glucosyl archaeol. An additional archaetidyl group is bound at position 6 of the glucosyl moiety of S-TGA-1 in the new lipid. It is interesting that these new lipids have four phytanyl chains in their molecules. Corcelli *et al.*[42] discussed the possible role of these lipids in the energy-transducing apparatus, as in eukaryotic mitochondria. The ether analog of cardiolipin and another phosphoglycolipid which has archaetidylated 6-sulfomannosyl-glucosyl archaeol (archaetidylated S-DGA-1) have also been identified from cells of *Haloferax volcanii*, which lacks purple membranes[43] (Fig. 8C).

Polar lipids from Thermoplasma acidophilum

The main polar lipid (MPL) of *Thermoplasma acidophilum* was characterized incompletely as glycosyl caldarchaetidylglycerol.[44] In 1997, Swain *et al.* identified β-L-gulose as the sugar moiety of the MPL by MS, NMR, GLC–MS of acetylated butyl glycoside, and optical rotation data.[45] Thus the MPL was identified as β-L-gulosylcaldarchaetidylglycerol. Gulose and an L-form sugar are rare in nature, but a few examples in bacteria and eukaryotes are cited in Swain's paper.[45] Hydroxyarchaetidylglycerol and hydroxyarchaetidylinositol were detected by MS of roughly fractionated polar lipid as minor phospholipids in the same organism.[45] This was the first report of the occurrence of hydroxylated phospholipids in other than methanogenic archaea. It is not known whether the hydroxyarchaeol is an α- or β-isomer. Because the polar head group of *Thermoplas-*

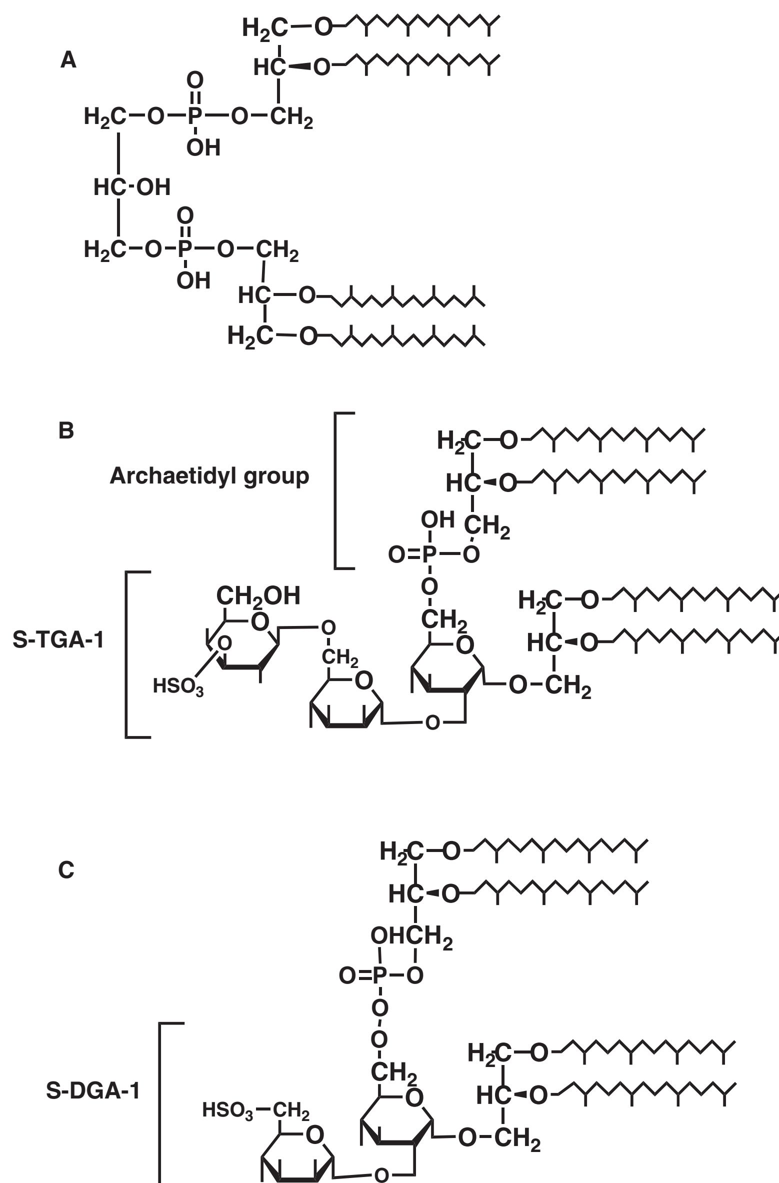

Fig. 8. New Lipids from Extremely Halophylic Archaea.

A, ether-type analog of cardiolipin, *viz.*, bisarchaetidylglycerol;[42] B, archaetidylated S-TGA-1, *viz.*, 3″-sulfoGal*p*-β1″,6′Man*p*-α1′,2-(6-archaetidyl)-Glc*p*-α1-archaeol;[42] C, archaetidylated S-DGA-1, *viz.*, 6′-sulfoMan*p*-α1′,2-(6-archaetidyl)-Glc*p*-α1-archaeol.[43]

ma lipids had been thought to be only glycerol, inositol as the polar head group of *Thermoplasma* lipids is a new finding. Gulosyl caldarchaetidylglycerol was also present in another species of the genus *Thermoplasma* (*T. volcanium*).[45]

Uda *et al.*[46,47] identified six minor glycolipids in *T. acidophilum*. Although most tetraether type bipolar lipids carry glycosyl groups in one end and a phosphate ester at another end, they found unusual glycolipids in which glycosyl groups are bound at both ends of caldarchaeol. The sugar moieties bound at the both ends of caldarchaeol are gulose/gulose (GL-2a), gulose/glucose (GL-2b), and glucose/glucose (GL-2c). The other two lipids are gulosyl caldarchaeol and glucosyl caldarchaeol. They also identified a minor phosphoglycolipid, GPL-K, which is α-D-glucosyl caldarchaetidylglycerol. This is an isomer of GPL-A (MPL or gulosyl caldarchaetidylglycerol). In this archaeon, it is interesting that L-gulose is in a β-anomer and D-glucose in an α-anomer. It should be noted that the orientations of the hydroxyl group at the 1 position and the hydrogen at the 5 position of sugars with D-α- and L-β-configurations are the same. Shimada *et al.*[48] comprehensively analyzed the structures of 13 polar lipids separated by HPLC with an evaporative light-scattering detecter. Of the 13 lipids, 7 had not been previously reported. All these new lipids are composed of a caldarchaeol core, one or two gulose and one to three mannose as sugar moieties, and phosphoglycerol as a phosphodiester-linked polar group in some phosphoglycolipids. In addition, a small amount

of diether-type archaetidylglycerol was detected. *Ferroplasma acidiphilum*, a mesophilic relative to *T. acidophilum*, produced β-D-glucopyranosyl caldarchaetidylglycerol,[49] the anomer of *T. acidophilum* minor lipid (GPL-K) found by Uda *et al.*[47]

Polar lipids of Methanothermus fervidus, Pyrococcus furiosus, and Sulfolobus acidocaldarius

Sprott *et al.*[33] reported polar lipid constituents in four hyperthermophilic archaea mainly using negative-ion FAB-MS. They identified the following polar lipids in *M. fervidus* total lipids by FAB-MS: acetyl-di-hexosyl caldarchaetidylinositol, di-hexosyl caldarchaetidylinositol, acetylhexosyl caldarchaetidylinositol, hexosyl caldarchaetidylinositol, caldarchaetidylinositol, caldarchaetidic acid, acetyl-di-hexosyl archaeol, di-hexosyl archaeol, phosphorylated *N*-acetylhexosyl archaeol, archaetidylinositol, hexosyl archaeol, archaetidylethanolamine, and archaetidylglycerol, of which the phosphoglycolipids with acetylated sugar were new lipids. Removal and analysis of polar groups showed the presence of glucose, *N*-acetylglucosamine, and inositol.

In *P. furiosus*, dihexosylcaldarchaetidylinositol, dihexosylcaldarchaetidylglycerol, monohexosylcaldarchaetidylinositol, caldarchaetidylinositol, dihexosylarchaeol, phosphorylated acetylhexosylarchaeol, archaetidylinositol or phosphorylated hexosylarchaeol, and archaetidylglycerol were identified as the most probable structures from m/z of their molecular ions. Identification of individual lipids with only FAB-MS data of unfractionated total lipid is accompanied by certain ambiguities; *viz.*, archaetidylinositol and phosphorylated hexosylarchaeol cannot be distinguished, kinds of hexose cannot be determined, structural and stereochemical isomers cannot be distinguished, and it is not possible to distinguish whether a m/z signal represents an intact molecule of a lipid or a fragmentation product of a lipid of higher mass. Nevertheless, FAB-MS was a useful method to detect individual lipids in total lipid extracted from archaeal cells without purification, and it should, therefore, be of use for ecological or environmental analysis or taxonomic purposes. For determination of complete structure, other instrumental and chemical methods must be combined.

By the same methods, diether polar lipids of *Sulfolobus acidocaldarius* were identified: dihexosyl archaeol, hydroxyarchaetidylinositol, archaetidylinositol, hydroxyarchaetidylglycerol, archaetidylglycerol, and archaetidylethanolamine. These lipids were similar to the lipids found in *Methanosarcina barkeri*. Hydroxyarchaeol as a core and glycerol and ethanolamine as phosphodiester-linked polar head groups were first found in *Sulfolobus* sp. It is expected that the occurrence of these lipids will be confirmed by application of other methods to the isolated lipids. Sugai *et al.* identified a new major neutral glycolipid (GL-1a) as β-glucosylcalditoglycerocaldarchaeol with two or three cyclopentane rings on the hydrocarbon chains.[50]

III. Taxonomic and Ecological (environmental) Aspects

Ether lipids as a chemotaxonomic marker

The taxonomic significance of the polar lipid composition of archaea was suggested as early as the mid 1980s for methanogenic[51,52] and extremely halophilic[53] archaea. This was based on comparisons of TLC patterns of total polar lipid of each archaeon. Koga *et al.*[54] developed lipid component parts analysis for taxonomical purposes to incorporate more structural information on lipids maintaining a less time-consuming simplicity. The method has been discussed in a previous review.[7] Koga *et al.* confirmed the effectiveness of the method and showed the correlation of polar lipid composition with 16S rRNA phylogeny in methanogens.[8] The latest version of *Bergey's Manual of Systematic Bacteriology*[55] incorporated much lipid information in the description of each taxon. Lipid component parts analysis was applied to identification of a few newly isolated methanogens.[56,57]

Ether lipids in environmental research

Lipid analysis of environmental samples yielded one of the base of the hypothesis of possible anaerobic methane oxidizing archaea. Hinrichs *et al.*[58] found archaeol and β-hydroxyarchaeol in lipids extracted from marine sediment samples taken from methane seepage (in offshore northern California). These archaea-specific lipid biomarkers were extremely depleted in ^{13}C. Because biologically produced methane is highly depleted in ^{13}C, it was assumed that the ^{13}C-depleted lipids are produced by new archaea that anaerobically metabolize ^{13}C-depleted methane. A parallel gene survey of 16S rRNA indicated the predominance of new archaeal groups that are peripherally related to the methanogenic orders *Methanomicrobiales* and *Methanosarcinales*. These genes were not found in control sediment samples. Because hydroxyarchaeol is found predominantly in organisms of the order *Methanosarcinales*, the results of the analysis of lipid biomarkers and those of 16S rRNA phylogenetic analysis are consistent. It was shown by the fluorescent *in situ* hybridization (FISH) method that cells of one of the *Methanosarcinales*-related groups of archaea (ANME-2) were present, forming close consortia with sulfate-reducing bacteria.[59] Although no methane-oxidizing anaerobic archaea has been isolated, anaerobic methane oxidation is thought to be a globally significant process of the carbon cycle and to contribute greatly to reduce greenhouse gas flow from the ocean. Lipid analysis made an important contribution in elucidating the microorganisms involved in this significant process.

In the ecological application of archaea-specific lipid analysis, large progress was made by identification of a variety of new hydrocarbons derived from archaeal core lipids or core lipids themselves, as described in the previous section. These findings strongly suggest new

archaea species producing these lipids in marine environments at not high temperatures and ubiquitous distribution of archaea on Earth in accordance with 16S rRNA ecology.[60,61]

HPLC analysis of archaeal core lipids was applied to quantitation of cell masses of methanogenic archaea in given environmental samples, such as anaerobic sewage sludge,[62] paddy field soil,[63] and marine sediments of Tokyo Bay,[64] and information was obtained. This method is inevitably accompanied by the weakness that it cannot discriminate whether detected core lipids were derived from living cells, or were accumulated lipid released from dead cells.

IV. Physiological Significance of Archaeal Lipids—What Is a Heat-Tolerant Lipid Membrane?

In spite of the variety of structures of archaeal lipids, as described above, four characteristics unique and common to archaeal lipids have been identified. (1) Hydrocarbon chains are bound at the *sn*-2 and 3 positions of the glycerol moiety in archaeal lipids, while bacterial lipids have *sn*-1, 2-radyl chains. These are mirror image structures of each other. (2) Hydrocarbon chains are bonded to the glycerol moiety exclusively by ether linkages in archaeal polar lipids in contrast with bacterial polar lipids, most of which have ester linkages between fatty acids and a glycerol moiety. (3) Hydrocarbon chains of polar lipids are highly methyl-branched isoprenoid in archaea, while bacterial counterparts are mostly straight-chain fatty acids. (4) A significant number of archaea species contain bipolar lipids with a tetraether core that span through a membrane. Of these four characteristics, the most exclusive feature of archaeal lipids in phylogenetic and evolutionary significance is the mirror-image structure of core lipids.[65–68] The enantiomeric structure of the lipid backbone appears, however, to be insignificant among physicochemical properties of lipid membrane of archaea, because enantiomers are equivalent in physicochemical properties except for chiral properties. Therefore, the other three characteristics will be discussed in relation to physiological functions of archaeal membranes compared with bacterial lipid membranes. The primary and fundamental physiological function of polar lipids is to form a cell membrane, which encloses a cell and makes a permeability barrier of various essential solutes for life. Therefore, the physiological significance of archaeal polar lipids should be discussed from this point of view.

One of the essential general features required for lipid membrane to fulfil biological functions is that it is kept in the liquid crystalline phase. In relation to this point, the first characteristic physicochemical property of archaeal lipid membranes is their phase transition temperature, which is far lower than that of fatty acyl ester lipids. Differential scanning calorimetry has shown that the phase transition point of polar tetraether type lipids from *Thermoplasam acidophilum* is between -20 and $-15\,^{\circ}$C.[69] Yamauchi *et al.* also reported lower than $-20\,^{\circ}$C for the phase transition temperature of diphytanyl PC (diether type) liposomes.[70] These temperatures are far lower than those of the membranes of ordinary fatty acyl ester phospholipids, for example, $41.5\,^{\circ}$C for dipalmitoyl PC and $55\,^{\circ}$C for distearoyl PC (both diester-type) liposomes.[70] The phase transition temperatures of fatty acyl ester lipid membranes are different depending on their chain length, number of double bonds, and the position of methyl branching. Therefore, archaeol- and caldarchaeol-based polar lipid membranes of archaea can be assumed to be in the liquid crystalline phase at the temperature range of 0 and $100\,^{\circ}$C, at which most archaea grow (biological temperature), while liposomes made of fatty acyl diester lipids are in the gel phase or the liquid crystalline phase depending on their fatty acid composition in the same temperature range. Because phase transition properties are less affected by inorganic cations, Blöcher *et al.*[69] concluded that the thermotropic properties of polar tetraether lipids are dependent mainly on the properties of the apolar moiety of the lipid.

The second characteristic property of archaeal lipid membrane is extremely low permeability of solutes. Unilamellar liposomes made of tetraether polar lipids (polar lipid fraction E = PLFE) from *Sulfolobus acidocaldarius* have been shown to be less permeable to protons and carboxyfluolesceine, a model compound of low molecular weight, between 25 and $75\,^{\circ}$C, than ester phospholipids liposomes.[71–73] In addition, the permeability was less temperature-sensitive and the slight monotonic increase indicated a lack of lipid-phase transition in the temperature range examined. In contrast to the PLFE liposomes, liposomes made of ester phospholipids (*viz.*, egg PC and dipalmitoyl PC) showed low permeability at low temperature, but drastically increased as temperature rose.[73] Diphytanoyl PC liposomes showed a similar permeability profile for CF but significantly low permeability for protons than the above fatty acyl ester PC liposomes between 20 to $80\,^{\circ}$C,[73] suggesting that mainly tetraether lipid structure contributes to temperature-insensitive low permeability and that phytanyl chains also contribute particularly to low permeability to proton.

Yamauchi *et al.*[70] reported that liposomes made of dipalmitoyl PC (diester type, transition temperature $41.4\,^{\circ}$C) and dihexadecyl PC (diether type, $45.4\,^{\circ}$C) showed protuberant high CF permeability at their respective transition temperatures, while diphytanyl PC (diether type, $<-20\,^{\circ}$C) showed far lower permeability throughout a temperature range between 10 and $70\,^{\circ}$C and no such protuberant high permeability at any temperature. These results suggest that highly branched isoprenoid chains are a major cause of the low permeability of liposomes.

Because isoprenoid ether lipid membranes are in a

liquid crystalline phase and have low permeability at biological temperatures, archaea live at as low as 1 °C and at as high as 100 °C with the same archaeol and caldarchaeol lipid composition in their membranes. This indicates that an archaeal lipid membrane can meet the two conditions required for a biological membrane, liquid crystalline phase and low permeability, with the same archaeol/caldarcheaol composition in a wide range of growth temperatures. This is the most fundamental characteristic feature of archaeal lipid membranes. In fact, all of the hyperthermophilic *Pyrococcus furiosus* (optimum temperature, 98 °C),[33] moderately thermophilic *Methanothermobacter thermautotrophicus* (65 °C),[74] mesophilic *Methanobacterium formicicum* (37 °C),[54] and *Methanogenium cariaci* (23 °C)[54] have caldarcheaol-based polar lipids with a smaller amount of archaeol-based lipid. Unsaturated archaeol (geranylgeranyl group-containing archaeol) is present in psychrophilic *Methanococcoides burtonii*[37] that can grow at 2 °C as well as in hyperthermophilic *Methanopyrus kandleri* (98 °C).[34] The same ether lipids of archaea can be utilized at both high and low temperatures because of their liquid crystalline phase and low permeability in a wide range of temperatures. Such a lipid can be called a "heat tolerant" lipid. The heat tolerance of membranes is not due to one lipid component. In other words, unsaturation of lipid hydrocarbon does not exist for adaptation to cold environments. It should be noted that geranylgeranyl hydrocarbon has four double bonds, with, however, an all *trans* configuration but not *cis*, as in many fatty acids of bacterial polar lipids.

On the other hand, in order to meet the two conditions of the liquid crystalline phase and low permeability for biological membranes, fatty acyl ester lipid membranes in bacteria should function only at the lowest temperature at which both the liquid crystalline state and the lowest permeability are kept. This can be realized at a temperature just above the phase transition temperature. Therefore, many bacteria with ester lipids control their fatty acid composition to meet these conditions. The mechanism of control varies from species to species. In *Escherichia coli*, unsaturated fatty acids increase at lower growth temperatures. But unsaturation is not the only mechanism to adapt to low temperatures. In *Bacillus* spp., temperature adaptation is regulated by changing iso/anteiso fatty acid composition.[75] In some archaea, control of the property of hydrocarbon chains is carried out by regulation of number of cyclopentane rings (*Sulfoloobus solfataricus*),[76] and by regulation of the ratio of caldarchaeol/cyclic archaeol/archaeol (*Methanocaldcoccus jannschii*).[77] The content of trans-unsaturation of isoprenoid chain has been reported to decrease with higher growth temperatures in *Methanococcoides burtonii.*,[37] but it is not known what influence these changes in isoprenoid chains exerts on the properties of lipid membranes (*e.g.*, phase behavior, permeability, or rigidity). Archaeal lipid membrane is not required to regulate its hydrocarbon composition to meet the two conditions for adaptation to temperature because the two conditions are realized over a wide range of temperatures.

Because ether linkages are stable under a ordinary acid hydrolytic conditions, one generally tends to consider that ether lipids are suitable to the thermophilic life of archaea. This is assumed to be chemical heat tolerance. In other words, chemical heat tolerance is stability at the covalent bond level. If chemical heat stability is required for an organism to survive at extremely high temperatures, the hyperthermophilic archaeon *Methanopyrus kandleri* could not survive in its original habitat, because a significant portion of the lipid of *M. kandleri* cells consists of greatly labile allyl ether lipid.[34] An allyl ether bond is as labile as a fatty acyl ester bond. On the other hand, phospholipids with the same unsaturated isoprenoid are present in cold-tolerant methanoarchaeon *Methanococcoides burtonii* cells.[37] This shows that stable ether bonds are not required for life in hot environments. This is not an exceptional example. The biosynthetic intermediates of ether polar lipids in archaea include geranylgeranyl ether lipids, which are a kind of allyl ethers.[78–81] If a stable saturated ether bond is essential for survival at high temperatures and unstable allyl ether bonds are degraded at those temperatures, the organism cannot survive at high temperatures because a trace amount of the unstable allyl ether intermediates would be degraded and the stable lipid could not be synthesized at high temperatures. It can, therefore, be concluded that stable ether bonds are not a result of adaptation to hot environments. The chemical stability of lipids does not determine the heat tolerance of lipid membranes.

In analogy to heat-resistant protein, chemical heat tolerance corresponds to heat resistance at a primary structure of the protein. But heat resistance is by no means discussed at the level of primary structure. Heat tolerance of protein implies that higher level structures and biological function are maintained at a high temperature. Heat tolerance of lipid membrane should be considered from the point of view of the biological function of membrane which is an assembly of lipids. It is significant whether a biological membrane can function at a high temperature, or whether it loses physiological function before degradation of covalent linkages. The heat tolerance of lipid membranes should be approached from this point of view. Chang[71] stated that "the issue of membrane stability is crucial for thermophiles, as the membrane is considered a primary site for cellular thermal-death". As he explains, membrane properties, such as permeability, can be perturbed significantly before any global breakup of the membrane occurs. It is the breakdown in the membrane function as a permeability barrier that is of importance for the cell's viability.

Concluding Remarks

In the last decade, more and more unique structures of lipids have been identified from various archaea species and environmental samples. The variety of lipids includes unforeseen structures such as H-shaped caldarchaeol, L-gulose, isocaldarchaeol, and hybrid natured hydrocarbons of archaeal and bacterial lipids from unknown organisms. On the other hand, ether lipids analogous to bacterial ester lipids have been found, such as ether type cardiolipin or archaetidylcholine. Other new lipids are minor modifications of known lipids of archaea. As a whole, a range of new ether lipids has expanded the concept of archaeal membrane ether lipids. Archaea and bacteria, which diversified from a common ancestor at the most ancient time in the history of biological evolution, are the most fundamental groups of living organisms. Recently the concept that they were differentiated by a difference in their membrane lipids has been advocated.[65–68] The variety of archaeal lipids is growing, as reviewed here, while knowledge of the bacterial lipids appears to be reaching maturation. It is expected that more and more novel lipids will be found in archaea.

The application and physiological significance of ether lipids have been also discussed. Especially, analysis of environmental lipids combined with analysis of stable isotope content might lead to the discovery of new organisms, for example, anaerobic methane oxidizing archaea. This field will expand in the near future. The application of liposomes of ether lipids to pharmaceutical carriers and protein-lipid interaction in a membrane are further subjects of interest, but were not reviewed in this paper.

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