

Ether Polar Lipids of Methanogenic Bacteria: Structures, Comparative Aspects, and Biosyntheses

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INTRODUCTION	164
NOMENCLATURE OF ARCHAEAL ETHER LIPIDS	166
STRUCTURES OF POLAR LIPIDS FROM VARIOUS METHANOGENS	166
<i>Methanobacteriaceae</i>	166
<i>Methanobacterium thermoautotrophicum</i>	166
<i>Methanobrevibacter arboriphilicus</i>	167
<i>Methanococcaceae</i>	167
<i>Methanococcus voltae</i>	167
<i>Methanococcus jannaschii</i>	167
<i>Methanomicrobiaceae</i>	168
<i>Methanospirillum hungatei</i>	168
<i>Methanosarcinaceae</i>	168
<i>Methanotherix soehngenii</i>	168
<i>Methanosarcina barkeri</i>	169
POLAR LIPIDS AS A CHEMOTAXONOMIC MARKER	171
Thin-Layer Chromatography Patterns	171
Distribution of Component Parts of Polar Lipids among Methanogens	172
COMPARISON OF COMPONENTS OF POLAR LIPIDS OF METHANOGENS WITH THOSE OF OTHER GROUPS OF ARCHAEA	173
APPLICATION OF LIPID ANALYSIS TO THE ECOLOGICAL STUDY OF METHANOGENS	174
Estimation of Methanogen Groups Present in an Ecosystem	174
Quantification of Methanogenic Cells by Lipid Core Analysis	174
SPECIAL METHODS OF METHANOGEN LIPID ANALYSIS	174
Acid Extraction	175
Acetolysis and Acid Methanolysis for Complete Removal of Polar Head Groups	175
Mild-Acid Methanolysis for Preparation of Hydroxyarchaeols	176
Boron Trichloride Cleavage of the Ether Bond for Preparation of Glycerophosphoesters	176
Hydrogen Fluoride Cleavage of Phosphodiester	176
BIOSYNTHESIS OF ETHER POLAR LIPIDS	176
SPECULATION ABOUT THE SIGNIFICANCE AND EVOLUTIONARY ORIGIN OF ETHER LIPIDS IN ARCHAEA	177
CONCLUDING REMARKS	179
ACKNOWLEDGMENT	180
REFERENCES	180

INTRODUCTION

Lipids of methanogenic bacteria were first analyzed in 1978 by Tornabene et al. (100) and Makula and Singer (70). They found that the lipids of methanogenic bacteria consisted of di- and tetraethers of glycerol and isoprenoid alcohols. These types of ether lipids had been previously identified in the extreme halophiles (*Halobacterium* species [50]) and thermoacidophiles (*Sulfolobus* and *Thermoplasma* species [17, 64]) in the 1960s and the 1970s, respectively. Since then, the characteristic glycerol ether lipids became one of the most remarkable features that distinguish members of the domain *Archaea* (archaeobacteria) from those of the domains *Bacteria* (eubacteria) and *Eucarya* (eukaryotes) (105). The complete structural determination of ether polar

lipids of a methanogen (*Methanospirillum hungatei*) was first described by Kushwaha et al. in 1981 (63). In the mid-1980s collaborations between lipid biochemists and methanogen bacteriologists were formed in a few laboratories. The outcome was a significant advance in methanogen lipid biochemistry by Ferrante et al. (28-33), Morii et al. (74-76), and Nishihara et al. (81-89), who identified nearly 40 novel lipids from seven species of methanogenic bacteria. These were as unique as other compounds from methanogens (coenzymes of methanogenesis [25] or pseudomurein [59]).

Many specific characteristics have been discovered for methanogen lipids, in contrast to the structures of ether lipids of the extreme halophiles and the sulfur-dependent thermophiles. The studies of methanogen lipids in the past several years have been stimulated by the isolation, description, and taxonomic classification of new and unique methanogens. In most recent examples, analyses of lipids from a new methanogen species have led to the discovery of new

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TABLE 1. Validated genera of methanogenic bacteria

Order	Family	Genus	No. of species	
<i>Methanobacteriales</i>	<i>Methanobacteriaceae</i>	<i>Methanobacterium</i>	12	
		<i>Methanobrevibacter</i>	3	
	Undefined	<i>Methanothermus</i>	2	
		<i>Methanosphaera</i>	2	
<i>Methanococcales</i>	<i>Methanococcaceae</i>	<i>Methanococcus</i>	7	
<i>Methanomicrobiales</i>	<i>Methanomicrobiaceae</i>	<i>Methanomicrobium</i>	1	
		<i>Methanolacinia</i>	1	
		<i>Methanospirillum</i>	1	
		<i>Methanogenium</i>	4	
		<i>Methanoculleus</i>	4	
	<i>Methanocorpusculaceae</i>	<i>Methanocorpusculum</i>	5	
		<i>Methanoplanaceae</i>	<i>Methanoplanus</i>	2
			<i>Methanosarcina</i>	5
	<i>Methanosarcinaceae</i>	<i>Methanococcoides</i>	1	
		<i>Methanobus</i>	3	
		<i>Methanotherix</i>	2	
		<i>Methanohalophilus</i>	4	
		<i>Methanohalobium</i>	1	
	Undefined	Undefined	<i>Halomethanococcus</i>	1
	Undefined	Undefined	<i>Methanopyrus</i>	1

lipids. Methanogenic bacteria are relatively diverse in phylogeny, morphology, and cell surface structures despite having a single energy-acquiring metabolism, i.e., methanogenesis. The structures of methanogenic lipids not only are more diverse than the above-mentioned phenotypes but also parallel the taxonomy of methanogens. It is therefore necessary to briefly outline the taxonomy before reviewing methanogen lipids.

During the last 10 years, a wide variety of methanogenic members of the *Archaea* have been isolated and characterized. Of 62 species of methanogenic bacteria validated in the *International Journal of Systematic Bacteriology* (as of January 1993), 49 were isolated after 1980. Methanogens are mesophilic or thermophilic strict anaerobes that are found together with anaerobic (eu)bacteria or anaerobic protozoa. All methanogenic bacteria form methane, which is produced from a rather restricted list of substrates ($H_2 + CO_2$, formate, acetate, methanol, methylamines, secondary alcohols + CO_2 , primary alcohols + CO_2 , and methyl sulfides). However, they show a great diversity in their cell morphology and the chemical nature of their cell surface components.

All methanogen genera that have been validated to date are shown in Table 1. Methanogens are classified into three orders (3, 7): *Methanobacteriales*, *Methanomicrobiales*, and *Methanococcales*. Cells of members of the *Methanobacteriales* are long or short rods with rigid pseudomurein cell walls, and most of them show a positive reaction to Gram staining (8). They utilize $H_2 + CO_2$ as methanogenic substrates, with the one exception of *Methanosphaera stadtmanae* (72), which uses only methanol + H_2 . Members of the order *Methanococcales* and the family *Methanomicrobiaceae* produce methane from $H_2 + CO_2$ and have a proteinaceous cell surface structure (9, 104). Most species of the former family have been isolated from marine environments. Some of the methanogens of the families *Methanobacteriaceae*, *Methanococcaceae*, and *Methanomicrobiaceae* can also produce methane from formate. *Methanopyrus kandleri* (optimum temperature, 98°C; a member of an undefined

order [62]), *Methanothermus* spp. (optimum temperature, 83°C [97]), and *Methanococcus jannaschii* (45) are the most extremely thermophilic methanogens. Members of the order *Methanomicrobiales* show various morphologies: irregular cocci, spirilla, filaments, irregular flattened plates, and packets (9, 10, 96, 109). The family *Methanosarcinaceae* contains all the acetoclastic and methylotrophic methanogens. Some of them can also utilize $H_2 + CO_2$ but not formate as methanogenic substrates. The cell surface matrix of *Methanosarcina* is composed of methanochondroitin (61), a non-sulfated acidic heteropolysaccharide. *Methanotherix* (*Methanoseta*) cells are filamentous and produce methane only from acetate, an important intermediate in anaerobic digestion. Three genera of halophilic methanogens (*Methanohalophilus*, *Methanohalobium*, and *Halomethanococcus*, with six species) belong to the family *Methanosarcinaceae*.

Two kingdoms (*Crenarchaeota* and *Euryarchaeota* [105]) of the domain *Archaea* (in which there are three major phenotypes: extreme thermophiles, methanogens, and extreme halophiles) are phylogenetically distantly related and show a closer relatedness to each other than to members of the domains *Bacteria* and *Eucarya* (105). All members of the *Archaea* contain common glycerol ether lipids. There are four fundamental and common differences between the ester lipids from the *Bacteria* and *Eucarya* and the ether lipids from the *Archaea*. The first is the nature of the linkage between the glycerol and hydrocarbon chains (ester and ether). The second is the nature of hydrocarbon chains themselves (straight fatty acyl chains of the *Bacteria* and *Eucarya* in contrast to highly methyl-branched saturated isoprenyl chains of the *Archaea*). The third is the stereochemical structure of the di-*O*-radyl glycerol moiety (*sn*-1,2-di-*O*-radyl glycerol for the *Bacteria* and *Eucarya* and *sn*-2,3-di-*O*-radyl glycerol for the *Archaea*). The final difference is the presence of the tetraether type of lipid in members of the *Archaea*. Recent phylogenetic studies have revealed a closer relatedness of *Archaea* to *Eucarya* than to *Bacteria* (44, 105), although there are some examples of specific relations of *Bacteria* and *Eucarya* at the molecular level (41, 113).

This fact raises the question of the origin of the archaeal ether lipids, that is, when and how ester lipids replaced ether lipids or vice versa as cell membrane constituents. This question assumes that the domains *Archaea*, *Eucarya*, and *Bacteria* have a common ancestor and that organisms with ester lipids evolved from organisms with ether lipids or vice versa. It is also possible that the primeval membrane was made of protein (111). A prerequisite for discussing the question of the origin of ether lipids is to have a perspective of the overall structures of archaeal lipids and to consider them in a comparative way. Because methanogen lipids are structurally diverse, it is important to have an overview of the range of structural diversity of all lipids. This paper focuses on recent advances in the studies on methanogen lipids. Several excellent reviews concerning archaeal lipids have been published (19, 50, 65, 66). The first aim of this review is to summarize the more recent findings of novel and unique structures of lipids from methanogens. The second is to present comparative aspects of lipids among methanogens and among archaeal groups. Lastly, we briefly discuss the biosynthesis of methanogen lipids.

NOMENCLATURE OF ARCHAEOAL ETHER LIPIDS

Archaeal ether lipids lacked systematic names and had only lengthy names as analogs of ester lipids (for example, an ether analog of phosphatidylserine) or confusing laboratory designations (for example, DGT, DGD, and PL1). Furthermore, it is more confusing to use the word "diether" as the name of the compound representing 2,3-di-*O*-phytanyl glycerol diether since it is properly used to refer to only the presence of two ether linkages in a compound and does not specify the structure of groups on both sides of the ether linkages. Therefore, Nishihara et al. (87) proposed a new nomenclature for archaeal lipids in 1987. The nomenclature is briefly explained in the following paragraph and will be used throughout this paper.

The 2,3-di-*O*-isopranyl *sn*-glycerol diether and ditetraterpenediyl glycerol tetraether (ether bonds are located at the *sn*-2 and *sn*-3 positions of glycerols) are defined as archaeol and caldarchaeol, respectively. Archaeoic acid and caldarchaeoic acid are monophosphate esters of archaeol and caldarchaeol, respectively. By condensing archaeoic acid or caldarchaeoic acid with an alcohol (serine, ethanolamine, inositol, etc.), a phosphodiester is formed. These lipids are named as derivatives of archaeoic acid or caldarchaeoic acid, for instance, archaeoic acidylserine. Glycoside derivatives of archaeol, caldarchaeol, and the tetraether type of phospholipids are called glycosyl archaeol, glycosyl caldarchaeol, and glycosyl caldarchaeoic acid-X (X is serine, etc.), respectively. Polar lipids with archaeol or caldarchaeol as the core lipid are sometimes called the diether type of polar lipids or the tetraether type of polar lipids, which is abbreviated as diether or tetraether polar lipids.

STRUCTURES OF POLAR LIPIDS FROM VARIOUS METHANOGENS

Polar lipids of methanogens consist of a nonpolar part made up of archaeol or caldarchaeol (core lipids) and polar head groups such as an organic phosphate ester or sugar residue. The core lipid structures are archaeol and caldarchaeol in most cases. In addition, three derivatives of archaeol have been discovered (Fig. 1). Macrocytic archaeol (Fig. 1c) was found in *Methanococcus jannaschii*, and two isomers of hydroxyarchaeols (Fig. 1d and e) were

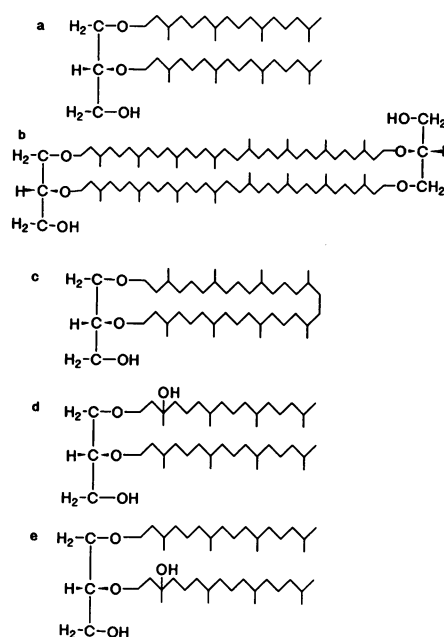


FIG. 1. Variations in the structures of core lipids of methanogenic bacteria. (a) Archaeol; (b) caldarchaeol; (c) macrocytic archaeol; (d) *sn*-3-hydroxyarchaeol; (e) *sn*-2-hydroxyarchaeol.

detected in many members of the families *Methanococcaceae* and *Methanosarcinaceae* (see below). Polar lipids that were found in methanogens and whose structures have been determined are discussed in this section.

Methanobacteriaceae

Methanobacterium thermoautotrophicum. *Methanobacterium thermoautotrophicum* is one of the most thoroughly studied methanogens in terms of both methanogenesis and other biochemical aspects. It was selected as the organism of choice for mass culturing of a methanogen for lipid studies, since the appearance of contaminants in a large fermenter was not a problem when an inorganic medium and high temperature were used.

The caldarchaeol core was predominant in the total lipid of *Methanobacterium thermoautotrophicum* ΔH (75 to 83 mol%). The polar lipids of this organism were separated into 23 or more spots by two-dimensional thin-layer chromatography (TLC) (Fig. 2) (82). On TLC there are at least five pairs of spots with slightly different mobilities. These spots were identified as di- (lower R_f values) and tetraether types of polar lipids with the same polar head group (Table 2). The spots that make up a pair are designated by the same spot designations with the suffix a or b (for example, PNL1a and PNL1b). The structures of 15 lipids from *Methanobacterium thermoautotrophicum* ΔH have been determined; these lipids account for 91 mol% of the total polar lipids in this species (82, 84, 87, 88). Thirteen of the 15 lipids are bare archaeol, bare caldarchaeol, two glycolipids [gentiobiosyl (β -Glc(1-6)- β -Glc) archaeol and gentiobiosyl caldarchaeol], six phospholipids, and three phosphoglycolipids (Fig. 3). The two other phospholipids are archaeoic and caldarchaeoic acids (84). The polar head groups of 11 lipids are simply inositol, serine, ethanolamine, or gentiobiosyl groups. These phospholipids and phosphoglycolipids are easily classified into three groups on the basis of phosphoric

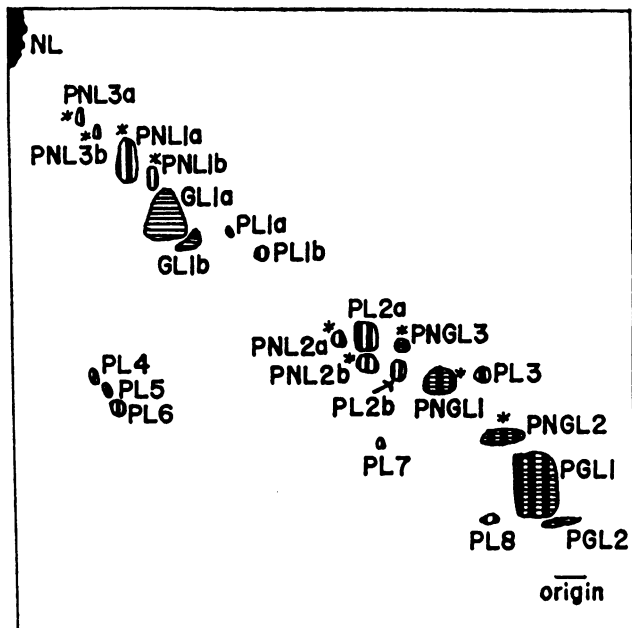


FIG. 2. Two-dimensional TLC of the total lipid of *Methanobacterium thermoautotrophicum*. TLC was developed first in the vertical direction with a solvent of chloroform-methanol-7 M aqueous ammonia (60:35:8) and next in the horizontal direction with a solvent of chloroform-methanol-acetic acid-water (80:30:15:5). The identities of major polar lipids designated in this figure are shown in Table 2. NL, neutral (nonpolar) lipids. Symbols: ○, acid molybdate positive; ●, α -naphthol positive; *, ninhydrin positive. Other spots have not been identified. Reproduced from reference 82 with permission of the Japanese Biochemical Society.

ester groups (serine, ethanolamine, and inositol). Each of the three groups of lipids contains a diether type of phospholipid, a tetraether type of phospholipid, and a tetraether type of phosphoglycolipid. When archaeol, caldarchaeol, and two glycolipids are added to these groups, each group is seen to contain seven lipids (four tetraether lipids and three diether lipids). It appears that the tetraether lipids are structurally constructed from two molecules of diether lipids, that is, archaeol, gentiobiosyl archaeol, and archaetidyl-X (X is serine, ethanolamine, or inositol). Thus, Nishihara et al. (88) proposed that the seven lipids are united in "a heptad of

TABLE 2. Previous designations and proposed names of ether lipids identified in *Methanobacterium thermoautotrophicum*^a

Previous designation	Proposed name
PNL1a	Caldarchaetidylethanolamine
PNL1b	Archaeetidylethanolamine
PNL2a	Caldarchaetidylserine
PNL2b	Archaeetidylserine
PL2a	Caldarchaetidyl- <i>myo</i> -inositol
PL2b	Archaeetidyl- <i>myo</i> -inositol
PL4	Archaeidic acid
PL5	Caldarchaetidic acid
PNGLI	Gentiobiosyl caldarchaetidylethanolamine
PNGL2	Gentiobiosyl caldarchaetidylserine
PGL1	Gentiobiosyl caldarchaetidyl- <i>myo</i> -inositol
GL1a	Gentiobiosyl caldarchaeol
GL1b	Gentiobiosyl archaeol

^a Data from references 82, 84, 87, and 88. See Figure 2 for the previous designations.

lipids." For example, the serine heptad contains archaeol, caldarchaeol, archaetidylserine, caldarchaetidylserine, gentiobiosyl archaeol, gentiobiosyl caldarchaeol, and gentiobiosyl caldarchaetidylserine. The major lipids of *Methanobacterium thermoautotrophicum* Δ H were therefore grouped into three heptads. This regularity is the most remarkable characteristic of the lipid structures. The regularity is summarized as follows: (i) the same kind of polar head group found in diether lipids is also present in tetraether lipids and vice versa; (ii) one polar head group found in tetraether lipid has the same stereochemical structure as that of the corresponding diether lipid; (iii) two polar head groups on each of two glycerol residues of tetraether lipids are not the same, but one is a glycosyl residue and the other is a phosphoric ester. Gentiobiosyl caldarchaetidylinositol is consistently the most abundant lipid.

Methanobrevibacter arboriphilicus. A complete inositol heptad was also found in *Methanobrevibacter arboriphilicus* A2 (75). Gentiobiosyl caldarchaetidylinositol was found to be the predominant polar lipid in several members of the *Methanobacteriaceae* and was designated the signature lipid of this family (75). Archaetidylserine was found in the above two organisms and other members of the *Methanobacteriaceae* as the first-identified primary amino group-containing (ninhydrin-positive) ether phospholipid in the domain *Archaea* (58, 74, 76).

Methanococcaceae

Methanococcus voltae. The lipids of *Methanococcus voltae* were the first analyzed in the family *Methanococcaceae* (Fig. 4) (31). *Methanococcus voltae* contains an unusually high proportion (63% of total polar lipids) of a glycolipid (gentiobiosyl archaeol [Fig. 4b]). The other glycolipid identified was monoglucosyl archaeol. A novel phospholipid, archaetidyl-*N*-acetylglucosamine (Fig. 4a), was also found. The most distinctive feature of this lipid is the phosphoglycosidic group, which is linked directly to archaeol. This kind of linkage has not been found in other organisms. The caldarchaeol core was not detected. The presence of ninhydrin-positive lipids (one of which is presumably nonacetylated archaetidylglucosamine) was suggested by Ferrante et al. (31).

Methanococcus jannaschii. *Methanococcus jannaschii* is one of the extremely thermophilic methanogens, which grows at temperatures up to 86°C (45); it was isolated from the deep-sea hydrothermal vent in the East Pacific Rise (depth, 2,600 m). The lipids of this bacterium are very interesting from the point of view of its extreme thermophilicity. Besides the usual archaeol and caldarchaeol, a unique glycerol ether core lipid was identified in this organism. It was identified as macrocyclic archaeol, in which a 40-carbon bifunctional 1,32-biphytanediyl (3,7,11,15,18,22,26,30-octamethyldotriacontamethylene) group was etherified at the *sn*-2 and *sn*-3 positions of glycerol, forming a 36-member macrocyclic diether compound (Fig. 1c) (14). The composition of core lipids varies with the growth temperature. At 45°C (close to the lowest temperature for growth of this bacterium), archaeol accounts for 80% of the total core. Caldarchaeol and macrocyclic archaeol increase up to 40% each at 75°C (near-optimal temperature [95]). The structures of four polar lipids with the macrocyclic archaeol as the core lipid have been determined (33) and are presented in Fig. 5. These polar groups are 6-(aminoethylphospho)- β -D-glucopyranose, β -D-glucopyranose, gentiobiose, and phosphoethanolamine. The first three lipids (glycolipids)

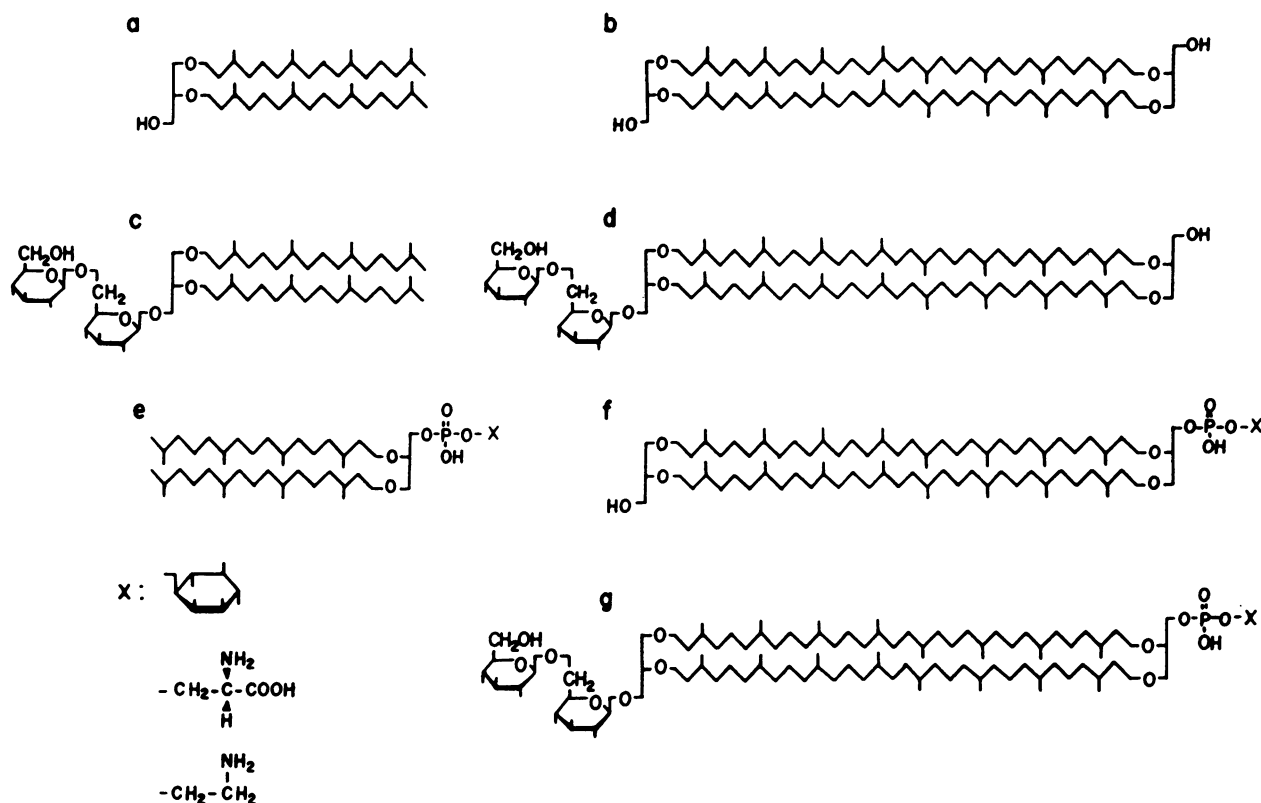


FIG. 3. Heptads of *Methanobacterium thermoautotrophicum* lipids. (a) Archaeol; (b) caldarchaeol; (c) gentiobiosyl archaeol; (d) gentiobiosyl caldarchaeol; (e) archaetidyl-X; (f) caldarchaetidyl-X; (g) gentiobiosyl caldarchaetidyl-X. X is either inositol, serine, or ethanolamine. Reproduced from reference 88 with permission of the American Chemical Society (copyright 1989).

contain macrocyclic archaeol linked through glycosidic linkages, and the fourth is a phospholipid containing a phosphodiester linkage. 6-(Aminoethylphospho)glucosyl archaeol (Fig. 5a) has a glycosyl archaeol structure typical of archaeal glycolipids; however, it is unique because the glucose residue is phosphorylated. The more common archaetidylethanolamine is also present. The five lipids make up 82% of the total lipids, and of these, 6-(aminoethylphospho)- β -D-glucopyranosyl macrocyclic archaeol accounts for 38% of the total. In this bacterium, 68% of the polar lipids contained carbohydrate, as in *Methanococcus voltae*.

Methanomicrobiaceae

The structures of the lipids of three members of the *Methanomicrobiales* (*Methanospirillum hungatei* GP1, *Methanotherix soehngenii* [*Methanosaeta concilii*] GP6, and *Methanosarcina barkeri* MS) have been elucidated.

Methanospirillum hungatei. Kushwaha et al. (63) reported the structures of polar lipids from *M. hungatei* GP1 in 1981 as the first methanogen polar lipids. These lipids consist of two phosphoglycolipids (both tetraether type) and four glycolipids (two tetraether and two diether types). The glycosyl residues of the six lipids comprise only two species: β -glucopyranosyl(1-2) β -galactofuranosyl and β -galactofuranosyl(1-6) β -galactofuranosyl. The occurrence of galactofuranose residues in glycolipids is uncommon. The phosphate ester group of the phosphoglycolipids is *sn*-glycerol 3-phosphate (Fig. 6). In 1987 Ferrante et al. (32) identified two novel aminopolyol residues in phospholipids as *N,N*-dimethylaminopentane-1,2,3-triol and *N,N,N*-trimethylaminopentane-

1,2,3-triol by nuclear magnetic resonance (NMR) and mass-spectral analyses. These groups were bound to archaeol through phosphodiester linkages to make archaetidyl(*N,N*-dimethylamino)pentane-1,2,3-triol and archaetidyl(*N,N,N*-trimethylamino)pentane-1,2,3-triol, respectively (Fig. 7a and b). The presence of archaetidyl-1'-*sn*-glycerol reported in 1981 (63) was not confirmed (32). Archaetidyl(*N,N,N*-trimethylamino)pentane-1,2,3-triol is the only Dragendorff reagent (reactive to quaternary ammonium group)-positive lipid found in members of the *Archaea*. Archaetidyl(*N,N*-dimethylamino)pentane-1,2,3-triol is partially positive to the Dragendorff reagent. Polar head groups found in other families of methanogens (inositol, ethanolamine, and serine) were not detected in *Methanospirillum hungatei*. Two-dimensional TLC of the total lipid of *Methanospirillum hungatei* revealed 15 or more spots, several of which were Dragendorff positive and almost as polar as the tetraether type of phosphoglycolipids (57). Considering the occurrence of two kinds of glycosyl groups and two kinds of aminopentane-1,2,3-triol residues in the bacterium, it is not unlikely that a few more aminopentane-1,2,3-triol lipids with a caldarchaeol core are present, which suggests a possibility of aminopentane-1,2,3-triol heptads. Moreover, Kushwaha et al. (63) detected a trace amount of archaetidyl-3'-*sn*-glycerol which had the same stereochemical structure as the glycerophosphate moiety of the two phosphoglycolipids.

Methanosarcinaceae

Methanotherix soehngenii. A new acid-labile glycerol ether core lipid (Fig. 1d) (30) was identified in *M. soehngenii* GP6

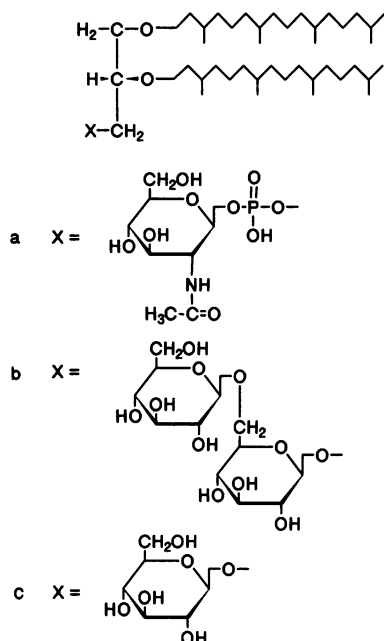


FIG. 4. Major lipids of *Methanococcus voltae*. (a) Archaetidyl-*N*-acetylglucosamine; (b) gentiobiosyl archaeol; (c) monoglucosyl archaeol.

(also known as *Methanotherx concilii* and *Methanosaeta concilii*) (for a discussion of the nomenclature of this organism, see references 6 and 91). Usually, strong-acid methanolysis (2.5% HCl-methanol at 65°C for 5 h [31] or 5% HCl-methanol at 100°C for 3 h [82]) is used to prepare glycerol ether core lipids by splitting off the polar head groups; however, the acid-labile core lipid of this bacterium was degraded to various compounds including monoalkylglycerol under these strong methanolytic conditions. The cause of the lability of the lipid is the presence of a hydroxyl group bound at the 3' position of the phytanyl chain linked to the *sn*-3 position of the core glycerol moiety. Archaeol containing 3'-hydroxyphytanyl chain is easily hydrolyzed in the presence of H^+ to yield monoalkylglycerol. Therefore, Ferrante et al. (30) developed mild-acid methanolysis (0.18% HCl-methanol at 50°C for 24 h) for the preparation of intact hydroxyarchaeol. Because these conditions are not sufficient to break all the phosphodiester bonds of phospholipids, a significant amount of polar lipids remained unhydrolyzed. The hydroxyarchaeol core made up 30% of the total.

The structures of eight polar lipids of *Methanotherx soehngeni* were determined (Fig. 8 and 9) (28, 29). The major lipids were two diglycosylated lipids (Fig. 8d and 9d), which made up 59% of the polar lipids, and archaetidylinositol (which made up 24%). Glycolipids of this organism contain three kinds of hexose residues: glucose, galactose, and mannose.

The stereochemical structure of phospho-*myo*-inositol of archaetidylinositol from *Methanotherx soehngeni* was concluded to be *myo*-L-inositol 1-phosphate (see Fig. 4 of reference 29) on the basis of the value of $[\alpha]_D = -10.0^\circ$ (pH 2.0), which was compared with that of phosphoinositol prepared from soybean phosphatidylinositol (92). Although the structural formula shown by Pizer and Ballou (92) shows 1L-*myo*-inositol 1-phosphate (named according to the IUPAC-IUB Recommendations of Nomenclature of Cyclitols [43]), these authors presented only the value of $[\alpha]_D$ and

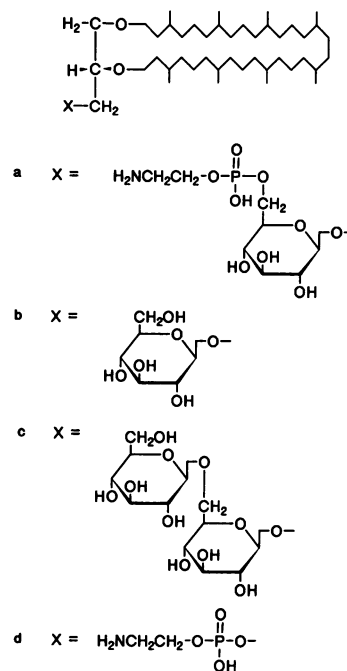


FIG. 5. Macrocyclic archaeol-containing polar lipids of *Methanococcus jannaschii*. (a) 6-(Aminoethylphospho)glucosyl macrocyclic archaeol; (b) glucosyl macrocyclic archaeol; (c) gentiobiosyl macrocyclic archaeol; (d) macrocyclic archaetidylethanolamine.

did not determine the absolute configuration of soybean phospho-*myo*-inositol. The L configuration depicted in their paper (92) therefore had no experimental basis at that time. The correct structure was established by the same laboratory in 1960 to be 1D-*myo*-inositol 1-phosphate (4). Thus levorotatory phospho-*myo*-inositol is 1D-*myo*-inositol 1-phosphate, which is identical to that from *Methanobacterium thermoautotrophicum* (Fig. 3). It should be noted that 1D-*myo*-inositol 1-phosphate, named according to the IUPAC-IUB nomenclature (43), is designated L-*myo*-inositol 1-phosphate in the Fletcher-Anderson-Lardy nomenclature system (34).

Methanosarcina barkeri. De Rosa et al. (20) reported that the lipid core of *Methanosarcina barkeri* DSM800 (MS) consists of diphytanyltetritol, caldarchaeol with various numbers of cyclopentane rings, glycerol-archaeol with hydrocarbon chains with 20 and 25 carbons, and typical archaeol. This description has been corrected independently by Sprott et al. (94) and by Nishihara and Koga (85), who detected only the usual archaeol and hydroxyarchaeol as the core lipids of the organism. The hydroxyarchaeol of *Methanosarcina barkeri* resembles hydroxyarchaeol from *Methanotherx soehngeni* but differs in the position of glycerol on which the hydroxyphytanyl group is linked. The 3'-hydroxyphytanyl chain is etherified at the *sn*-2 position of glycerol in *Methanosarcina barkeri* (Fig. 1e) and at the *sn*-3 position in *Methanotherx soehngeni* (Fig. 1d). This hydroxyarchaeol is also acid labile. The main product of acid methanolysis is 3-monophytanyl *sn*-glycerol, which shows a positive response to the periodate-Schiff reagent as a result of the presence of the vicinal hydroxyl groups (85), in contrast to *Methanotherx* hydroxyarchaeol. The lower mobility and the staining response of the degradation products on TLC resemble the behavior of diphytanyltetritol, archaeol with C_{20} and C_{25} chains, or caldarchaeol. The identification of these

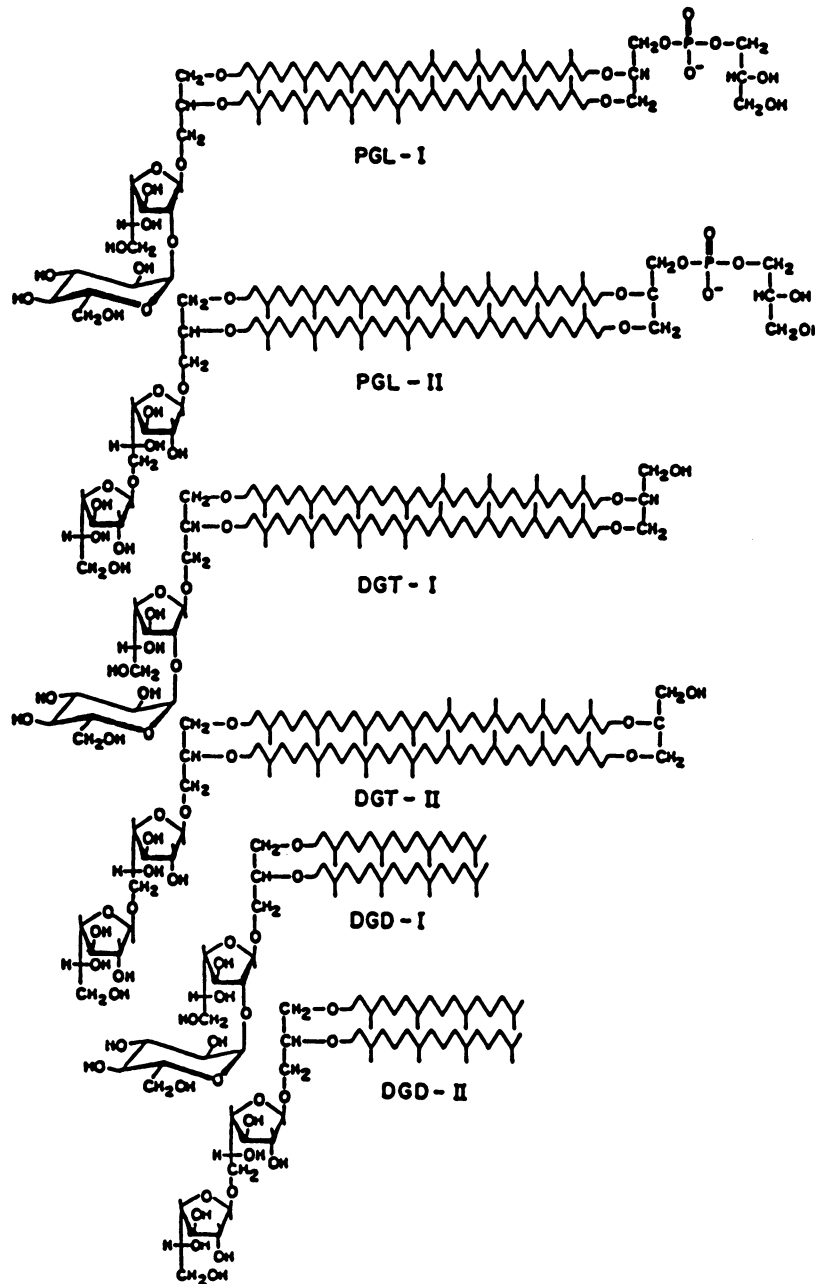


FIG. 6. Lipids of *Methanospirillum hungatei*. PGL-I, glucopyranosylgalactofuranosyl caldarchaetidylglycerol; PGL-II, digalactofuranosyl caldarchaetidylglycerol; DGT-I, glucopyranosylgalactofuranosyl caldarchaeol; DGT-II, digalactofuranosyl caldarchaeol; DGD-I, glucopyranosylgalactofuranosyl archaeol; DGD-II, digalactofuranosyl archaeol. Reproduced from reference 63 with permission of Elsevier Science Publishers and M. Kates.

compounds based solely on TLC mobilities may lead to misidentification, as pointed out by Ferrante et al. (30). Nishihara et al. (85) reported that hydrocarbon chains other than the 20-carbon chain were less than 1/600 (the lowest limit of detection), if any, of the phytanyl chain. The reason for the discrepancy between the results of De Rosa et al. (20) and Nishihara et al. (85) or Sprott et al. (94) in the occurrence of caldarchaeol cores with cyclopentane rings is not known. The molecular ratio of archaeol to hydroxyarchaeol in *Methanosarcina barkeri* is 2:3 (85).

Recently, five polar lipids of *Methanosarcina barkeri* MS

have been identified by Nishihara and Koga (85). The hydroxyarchaeol-containing phospholipids hydroxyarchaetidylserine and hydroxyarchaetidylinositol (Fig. 10) were identified, as were the usual archaeol-containing phospholipids with the same polar head groups, archaetidylserine and archaetidylinositol. Because the hydroxyarchaeol-containing lipid and the usual archaeol-containing lipid with the same polar group showed similar but slightly different mobilities on TLC, they looked like paired lipids on TLC. The fifth lipid identified in this organism was the most abundant and unusual. The lipid contained archaeol as the core lipid

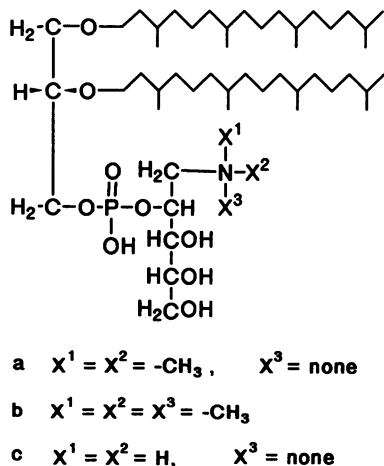


FIG. 7. Aminopentaketol-containing lipids of *Methanospirillum hungatei*. (a) Archaeetidyl(*N,N*-dimethyl)aminopentaketol; (b) archaeetidyl(*N,N,N*-trimethyl)aminopentaketol; (c) archaeetidylaminopentaketol.

and phospho-*myo*-inositol and unacetylated glucosamine as polar groups. The detailed stereostructure of the glucosaminylinositol was 6-(2'-amino-2'-deoxy- α -glucopyranosyl)-1D-*myo*-inositol 1-phosphate (Fig. 11) (89). It is interesting that this structure is the same as the common part of the glycosylated phosphatidylinositol anchor of eukaryotic membrane-bound proteins. It may be assumed that this lipid is the first "hybrid lipid" based on the fact that the glucosaminylinositol moiety has been found only in eucaryal membrane anchor and the glycerol ether core portion is

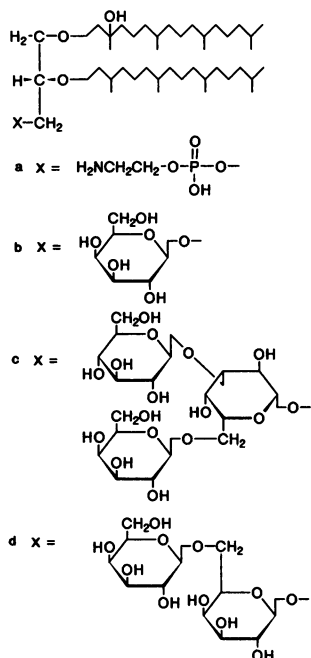


FIG. 8. Hydroxyarchaeol-containing polar lipids of *Methanotheroxobacter*. (a) Hydroxyarchaeetidylethanolamine; (b) galactosyl hydroxyarchaeol; (c) glucosyl(galactosyl)galactosyl hydroxyarchaeol; (d) digalactosyl hydroxyarchaeol.

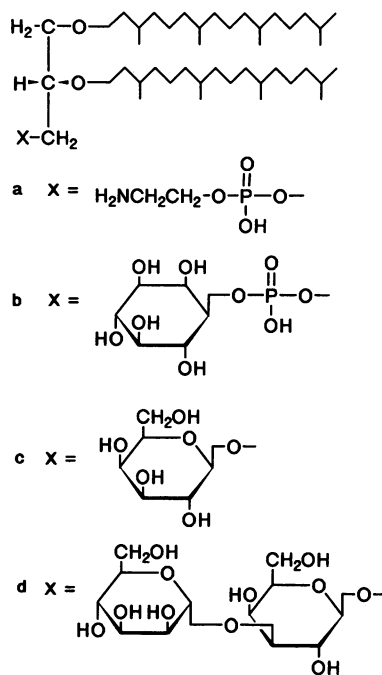


FIG. 9. Archaeol-containing polar lipids of *Methanotheroxobacter*. (a) Archaeetidylethanolamine; (b) archaeetidyl-*myo*-inositol; (c) galactosyl archaeol; (d) mannosylgalactosyl archaeol.

specific to the domain *Archaea*. Archaeetidylethanolamine and hydroxyarchaeetidylethanolamine were also found in *Methanosarcina barkeri* (86).

POLAR LIPIDS AS A CHEMOTAXONOMIC MARKER

Thin-Layer Chromatography Patterns

As described above, the structures and compositions of the polar lipids of methanogens are divergent and complex. When the total lipid of a methanogenic bacterial species was analyzed on two-dimensional TLC, 5 to 25 spots, depending on the methanogen species, were detected. This number is much larger than that in members of the domain *Bacteria*; for example, usually only four or five spots are detected on

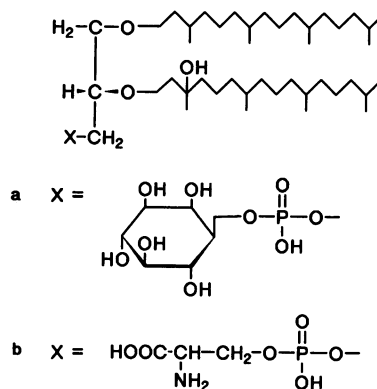


FIG. 10. Hydroxyarchaeol-containing polar lipids of *Methanosarcina barkeri*. (a) Hydroxyarchaeetidyl-*myo*-inositol; (b) hydroxyarchaeetidylserine.

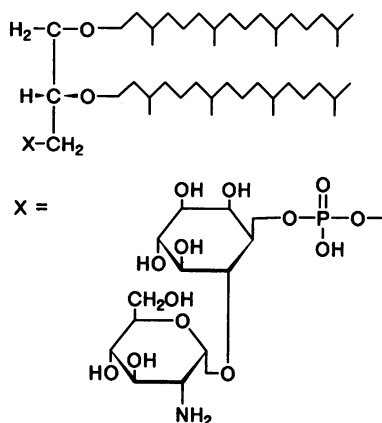


FIG. 11. Structure of glucosaminyl archaetidyl-myoinositol.

TLC of *Escherichia coli* lipids (2). The complexity of the lipid composition of methanogens is caused not only by the diversity of polar head groups but also by the occurrence of two or more kinds of core lipids such as the combination of archaeol, caldarchaeol, hydroxyarchaeol, and macrocyclic archaeol as well as the occurrence of the tetraether type of phosphoglycolipids that have two kinds of polar groups on one molecule. Grant et al. (39) first reported the TLC patterns of various methanogens with taxonomic implications. Nevertheless, because none of the spots was structurally identified and no mobility marker was cochromatographed in their experiments, it was hard for other workers to compare patterns from other methanogens with theirs. Because of the variability of mobility on TLC, depending on the kinds and activation conditions of TLC plates and even the humidity of the laboratory, spots with slightly different mobilities were difficult to distinguish on separate TLC plates. Koga et al. (58) tried a method of overcoming the inherent ambiguity of TLC mobility by using ³²P-labeled archaetidylserine and ³²P-labeled archaetidylethanolamine as internal standards in two-dimensional TLC. Complete superimposition of a radioactive spot and a chemically visualized spot confirmed the identity of the respective spots.

Koga et al. (58) reported the following distribution of polar lipids among methanogens as determined by TLC of total lipids with radioactive internal standards. The ninhydrin-positive amino-group-containing polar lipids were found in all the methanogens analyzed so far. Archaetidylserine was detected in members of the *Methanobacteriaceae* and *Methanococcus* and *Methanosarcina* species. Among the members of the *Methanobacteriaceae*, *Methanobacterium* species contained archaetidylethanolamine but *Methanobrevibacter* species did not. A characteristic tetraether phosphoglycolipid, gentiobiosyl caldarchaetidylinositol, was specifically found in members of the *Methanobacteriaceae* (58, 75). Another characteristic phospholipid, designated PX, was specific to members of the *Methanomicrobiaceae* (58). Recently, PX was tentatively identified as unmethylated archaetidylaminopentane-tetrol (Fig. 7c) (57). The comparison of TLC patterns of total lipids had been carried out before a number of unique polar lipids were identified. However, the TLC patterns suggested the significance of polar lipids in methanogen taxonomy and have therefore been recommended when describing new taxa of methanogenic bacteria (11).

Distribution of Component Parts of Polar Lipids among Methanogens

Although radioactive internal standards of known lipids were useful for surveys of the distribution of lipids among methanogens, a TLC pattern of the total lipid is less informative about lipid structure. Only the relative locations of lipid spots on a TLC plate could be compared with another chromatogram of total lipid from another methanogen or a chromatogram reported by other workers. Although the best way to show the relationship between lipids is to compare their complete structures, the determination of the complete structure of a lipid is a laborious and time-consuming effort that is not suitable for routine taxonomic work.

A method that gives more information about lipid structure than TLC and is less time-consuming than complete structure determination is the analysis of the component parts of polar lipids (analyses of polar lipid-constituting moieties) in the total lipid such as the presence or absence of polar head groups, monosaccharide units, core portions, and hydrocarbon chains. This approach provides important information concerning the lipid chemistry of a specific methanogen, although it disregards the arrangement of the moieties. To examine this approach, we have been analyzing lipid component parts from approximately 18 species of methanogens from 12 genera belonging to 5 families (56). The result is shown in Table 3.

Archaeol was present in all species of methanogens examined, so this core lipid cannot be used for taxonomic purposes. Caldarchaeol core lipid was found in the families *Methanobacteriaceae*, thermophilic *Methanococcaceae*, and *Methanomicrobiaceae* but not in *Methanosarcina* and mesophilic *Methanococcus* species. Hydroxyarchaeol core lipids were present in *Methanosarcina*, *Methanotherix*, and mesophilic *Methanococcus* species.

Inositol was a constituent of the polar head groups of the phospholipids from the families *Methanobacteriaceae* and *Methanosarcinaceae* but was not detected in *Methanococcus* species or the family *Methanomicrobiaceae*. Phosphoethanolamine was the marker of the genus *Methanobacterium* in the family *Methanobacteriaceae*; that is, ethanolamine is present in *Methanobacterium* spp. but not in *Methanobrevibacter arboriphilicus*. This is confirmed by TLC profiles of total lipids of other *Methanobrevibacter* species (58). Di- and trimethylaminopentane-tetrols were recognized only in species of the family *Methanomicrobiaceae*. As mentioned above, unmethylated aminopentane-tetrol was also found as a unique lipid, PX, in a few methanogens of the family *Methanomicrobiaceae*, including *Methanospirillum hungatei*, *Methanolacinia paynteri*, and *Methanogenium cariaci* (58). Therefore aminopentane-tetrols, irrespective of methylation, seem to be a specific marker of the family *Methanomicrobiaceae*.

Glucose was found as a sugar moiety of glycolipids in all the methanogenic species studied so far and was the sole sugar component of glycolipids in many methanogens. Galactose occurs in the glycolipid of members of the *Methanomicrobiaceae* in addition to glucose. Mannose is a constituent of glycolipids in only *Methanotherix soehngenii*. Therefore, the glycolipid-sugar composition may be a discriminating marker of the family *Methanomicrobiaceae* and the genus *Methanotherix*. *N*-Acetylglucosamine or nonacetylated glucosamine was found in some species, but its chemotaxonomic implication is not clear. We are in the process of analyzing lipid components of several additional methanogens. The results of this study will extend the information

TABLE 3. Distribution of component parts of polar lipids in methanogens

Species	Presence of ^{a,b} :											Reference(s)
	Core lipid				Sugar			Polar head group				
	ArOH	CAOH	c-Ar	hy-Ar	Glc	Gal	Man	EtN	Ser	Ino	APT	
<i>Methanobacterium formicicum</i>	+	+	-	-	+	-	-	+	+	+	-	56
<i>Methanobacterium thermoautotrophicum</i>	+	+	-	-	+	-	-	+	+	+	-	87, 88
<i>Methanobacterium wolfei</i>	+	+	-	-	+	-	-	+	+	+	-	56
<i>Methanobrevibacter arboriphilicus</i>	+	+	-	-	+	-	-	-	+	+	-	75, 76
<i>Methanothermobacter fervidus</i>	+	+	-	-	+	-	-	-	-	+	-	56
<i>Methanosphaera stadtmanae</i>	+	+	-	+	+	-	-	-	+	+	-	56
<i>Methanococcus vannielii</i>	+	-	-	+	+	-	-	-	+	-	-	56
<i>Methanococcus voltae</i>	+	-	-	+	+	-	-	-	+	-	-	31, 56
<i>Methanococcus thermolithotrophicus</i>	+	+	-	+	+	-	-	-	+	-	-	56
<i>Methanococcus jannaschii</i>	+	+	+	-	+	-	-	+	+	-	-	14, 33, 56
<i>Methanolacinia paynteri</i>	+	+	-	-	+	+	-	-	-	-	+	56
<i>Methanospirillum hungatei</i>	+	+	-	-	+	+	-	-	-	-	+	32, 56, 63
<i>Methanogenium cariaci</i>	+	+	-	-	+	+	-	(-)	(-)	-	(+)	56, 58
<i>Methanosarcina barkeri</i>	+	-	-	+	+	-	-	+	+	+	-	56, 85, 86, 94
<i>Methanobolus tindarius</i>	+	-	-	+	+	-	-	+	+	+	-	56
<i>Methanohalophilus mahii</i>	+	-	-	+	+	-	-	+	-	+	-	56
<i>Methanothermobacter soehngenii</i>	+	-	-	+	+	+	+	+	(+)	+	-	28-30, 90
<i>Methanothermobacter thermophila</i>	+	+	-	-	-	+	-	+	-	+	-	56

^a Abbreviations: ArOH, archaeol; CAOH, caldarchaeol; c-Ar, macrocyclic archaeol; hy-Ar, hydroxyarchaeol; Glc, glucose; Gal, galactose; Man, mannose; EtN, ethanolamine; Ser, serine; Ino, inositol; APT, aminopentane-2-tetrol.

^b Symbols: +, present; -, absent. Symbols in parentheses indicate the results from TLC profiles.

base for lipid component analysis and help determine its significance as a chemotaxonomic marker.

COMPARISON OF COMPONENTS OF POLAR LIPIDS OF METHANOGENS WITH THOSE OF OTHER GROUPS OF ARCHAEA

The structural features of the lipids of three groups of the domain *Archaea* are presented in Table 4. When compared with methanogen lipids, the polar lipids from extremely halophilic and from sulfur-dependent members of the *Archaea* have features characteristic of each group. Extreme

TABLE 4. Comparison of lipid structure among three major groups of the domain *Archaea*

Component	Presence in ^a :		
	Methanogens	Sulfur-dependent thermophiles	Extreme halophiles
Core lipid			
Archaeol	+	+	+
Hydroxyarchaeol	+	-	-
Macrocyclic archaeol	+	-	-
C ₂₅ chain	-	-	+
Caldarchaeol	+	+	+
Cyclopentane ring	-	+	-
Polar head group			
Inositol	+	+	-
Glycerol	+	-	+
Amino compound	+	-	-
Major sugar	Glc	Glc, Gal	Glc, Gal, Man, sulfated sugar

^a Symbols: +, present; -, absent.

halophiles contain archaeol as the sole core lipid. Most sulfur-dependent members of the *Archaea* have caldarchaeol with a trace amount of archaeol, except *Thermococcus celer*, which has only an archaeol core (24). The core lipids of a number of groups of methanogens are usually both archaeol and caldarchaeol. Variations of core lipids of the three groups are also characteristic. A C₂₅-sesterterpanyl chain-containing archaeol (23) is characteristic of alkaliphilic extreme halophiles. Caldarchaeols of *Sulfolobus solfataricus* contain various numbers of cyclopentane rings in their hydrocarbon chains (22). In this bacterium, one of the two glycerol moieties of some caldarchaeol molecules is replaced by a nine-carbon polyol, nonitol (16). The variation of core lipids in methanogens is restricted to the archaeols as described above (Fig. 1). The caldarchaeol cores of the methanogens identified so far all have the standard structure.

In contrast to methanogens, all the phosphate-containing polar head groups of extreme halophiles contain a glycerol moiety (archaeotidylglycerol, archaeotidylglycerophosphate, and archaeotidylglycerosulfate [50]). The only phosphodiester group of lipids identified thus far in sulfur-dependent members of the *Archaea* is phospho-*myo*-inositol (21, 24, 67, 68, 99, 102). *myo*-Inositol and glycerol are found in some but not all groups of methanogens, whereas inositol is not found in extreme halophiles. The presence of a glycerol moiety as a polar head group also has not been reported in sulfur-dependent thermophiles. *Methanospirillum hungatei* is the only methanogen that has a nonalkylated glycerol moiety in its polar lipids. It was shown, on the basis of the sequence analyses of 16S rRNA (107) and 23S rRNA (12), that *Methanospirillum hungatei* has a closer relationship than the other methanogens to the extreme halophiles. These facts suggest that the occurrence of glycerol in the polar lipids in *Methanospirillum hungatei* is not an accidental phenomenon

but implies a phylogenetic relatedness. One of the most characteristic features of polar lipids of methanogenic bacteria is the occurrence of a variety of phosphate-linked head groups (see above). The simplicity of phosphate-containing polar groups in halobacteria and sulfur-dependent thermophiles may be compensated by the complexity of their sugar groups. As to the sugar moieties of glycolipids, extreme halophiles have mono-, di-, tri-, and tetrasaccharide residues composed of glucose, mannose, galactose, and sulfated galactose (52). At least seven kinds of sugar residues composed of glucose, galactose, sulfated glucose, and phosphoglucose have been identified in sulfur-dependent thermophiles (19, 67, 68, 99). Most methanogens contain mono- or diglucose residues as glycosyl groups of glycolipids. At present, a few species of methanogenic bacteria are known to contain sugars other than glucose in their glycolipids. In conclusion, membrane lipid variation in methanogenic bacteria seems to occur among phosphodiester-bonded, water-soluble alcoholic residues, whereas in extremely halophilic bacteria and sulfur-dependent thermophilic members of the *Archaea* it occurs in the sugar residues of glycolipids.

APPLICATION OF LIPID ANALYSIS TO THE ECOLOGICAL STUDY OF METHANOGENS

Estimation of Methanogen Groups Present in an Ecosystem

Methanogens in natural ecosystems cohabit with many kinds of organisms of the domain *Bacteria* and sometimes with protozoa through interspecies hydrogen transfer. Qualitative and quantitative analysis of methanogen species and biomass are an important aspect of microbial ecology. In pure culture, for example, *Methanosarcina* and *Methanotherix* species can be readily differentiated by microscopic observation. For samples from consortia collected from natural environments, immunological methods (69) and gene amplification methods such as the polymerase chain reaction (37) are two main strategies for the identification of methanogen species. Another method entails the direct analyses of total lipid extracted from a natural sample to estimate methanogen biomass (15) and the composition of families of methanogenic bacteria that constitute the ecosystem (56, 81).

Analysis of component parts of ether lipids could also be a tool for estimating the groups of methanogens in a sample taken from the natural environment. After the total lipid extracted directly from such a sample is treated with mild acid and alkali to degrade acyl esters and alkenyl ether (plasmalogen) lipids, respectively, the resultant lipid is analyzed for its components as described above. For example, Nishihara et al. (81) concluded that a sludge sample from an anaerobic digester contained *Methanotherix* species on the basis of the total lipid analysis of the sample, in which they found mannose and galactose as lipid-sugar moieties and hydroxyarchaeol as a core lipid. In another sludge they detected glucose as the major lipid-sugar moiety; ethanolamine, serine, and inositol as polar head groups; and hydroxyarchaeol as a core lipid. These data indicated the predominance of *Methanosarcina* species in the sludge (81). If inositol and caldarchaeol are not detected in the lipid fraction of a sample, it is highly likely that members of the *Methanobacteriaceae* are absent.

Quantification of Methanogenic Cells by Lipid Core Analysis

Ether core lipids are specific to the domain *Archaea* and are highly stable to chemical reactions that degrade diacyl

and plasmalogen lipids. In most methanogens, which do not have the outer membranes or special wall lipids, the cytoplasmic membrane is the sole membrane structure made of lipids in the cells, and the cell size and surface area per dry-cell weight are almost constant. On this basis, a high-pressure liquid chromatography (HPLC) method was developed to determine methanogen biomass in natural environments (15). Total lipid was subjected to acetolysis and acid methanolysis to convert polar lipids completely into core lipids. During this treatment, diacyl and plasmalogen lipids were degraded to glycerol, fatty acids, and fatty aldehydes, which could be easily separated from ether core lipids. A UV-absorbing group, dinitrobenzoyl, was introduced into the hydroxyl groups of the resultant core lipids for sensitive detection on HPLC. Dinitrobenzoyl-archaeol and dinitrobenzoyl-caldarchaeol were well resolved and measured in a short time with HPLC equipped with a UV detector. The total amount of core lipids (archaeol plus caldarchaeol) detected by UV absorption on HPLC was proportional to the dry-cell weight of methanogens independent of species for *Methanobacterium thermoautotrophicum* or *Methanospirillum hungatei*. The lowest limit of detection was 2.5 μg of cells (approximately 3×10^6 cells). A similar method was reported by White and coworkers (71, 80) and was based on HPLC detection of the refractive index of core lipids prepared from the phospholipid fraction rather than from total lipid after treatment by acid methanolysis alone. Demizu et al. (15) pointed out that the phospholipid content in total lipid varied from species to species of methanogens and that a significant amount of phospholipids could not be methanolized. The method of Demizu et al. therefore improved the accuracy of the above-mentioned estimation of methanogenic cells. However, this new method, as well as the method of White et al., does not detect hydroxyarchaeols, because the strong-acid treatment used to prepare core lipids causes their degradation. Since hydroxyarchaeols are not only the predominant constituents of *Methanosarcina* and *Methanotherix* species but are also found in several *Methanococcus* species (56, 94), a further improved method in which hydroxyarchaeols as well as archaeol and caldarchaeol can be measured during one assay should be developed.

Applications of lipid analysis to ecological studies of methanogens still remain in the developmental stage.

SPECIAL METHODS OF METHANOGEN LIPID ANALYSIS

Various methods for analysis of archaeal ether lipids have been established by Kates. Each polar lipid from the extreme halophiles and thermoacidophilic members of the *Archaea* contains polyols (glycerol or inositol) or sugars as polar head groups. On the other hand, several novel structures are present in methanogens, for example, acid-labile hydroxyarchaeol cores, serine, or ethanolamine that is poorly released from the ether lipids by the usual acid methanolysis. Some new analytical methods were therefore required not only for the elucidation of ether lipid structure but also for chemotaxonomic and ecological purposes. Considering the special significance of new analytical methods that were developed for the unique methanogen lipids, the new methods are briefly reviewed here. Important but conventional or routine analytical methods are not included here.

TABLE 5. Lipid extraction from cells of the family *Methanobacteriaceae*

Reference	Cells ^a	Growth phase	Extraction solvent	Total lipid/cell (%)	ArOH/CAOH ^b (molar ratio)
70	Intact	Late log	Neutral	2.0 ^c	41:59
	Disrupted	Late log	Neutral	3.9 ^c	
82	Intact	5 days	Neutral	0.98	35:36
	Intact	5 days	Acidic	5.6	10:83
	Disrupted	5 days	Neutral	5.3	9:83
75	Intact ^d	Late log	Neutral	3.0	52:48
	Disrupted ^d	Late log	Acidic	5.8	19:81
40	Intact ^e		Neutral	0.026 ^f	65:35
	Intact ^e		Acidic	0.038 ^f	45:55
60	Intact	2 days	Neutral	1.6	41:59
	Intact	4 days	Neutral		80:20
	Intact	6 days	Neutral		89:11

^a *Methanobacterium thermoautotrophicum* except when otherwise noted.

^b ArOH, archaeol; CAOH, caldarchaeol.

^c Phospholipid in dry cell calculated from data in reference 70 for lipid-phosphorus, assuming that the average molecular weight is 1,300.

^d *Methanobrevibacter arboriphilicus*.

^e *Methanobacterium formicicum*.

^f Calculated from data in reference 40, assuming that the molecular weights of diether and tetraether polar lipids are 850 and 1,700, respectively.

Acid Extraction

Extraction of *Methanobacterium thermoautotrophicum* by standard neutral Bligh-and-Dyer solvent mixture (5) resulted in a low level of lipid (for example, 1.6% on a dry-cell weight basis) (40, 60, 70, 100). Acidification of the solvent with trichloroacetic acid was found to be effective for maximum yield of lipid (5.6% of dry-cell weight) from this bacterium (82). Cell disruption was also effective for high-yield lipid extraction in *Methanobacterium thermoautotrophicum* and *Methanobrevibacter arboriphilicus* (70, 75, 82). It should be noted that the acidic solvent increased not only the total lipid yield but also the recovery of tetraether lipids. Therefore, neutral extraction resulted in incomplete extraction of tetraether polar lipids, which remained in the residual fraction containing macromolecular materials. The extraction yields reported by various authors are summarized in Table 5. The results of Makula et al. (70) and Nishihara et al. (82) are almost consistent. Hedrick et al. (40) reexamined the effectiveness of the acid-extraction method of Nishihara et al. (82) by using *Methanobacterium formicicum*. They also obtained twice as much tetraether lipid by acid extraction as by neutral extraction, although their reported amount of lipid per gram (dry-cell weight) was extremely small (1/40 to 1/100, if there is no error in their calculation) compared with values obtained under similar conditions by other authors. They used the usual Bligh-and-Dyer method for the analysis of bacterial and archaeal lipids in one extract to avoid any destructive effect of the acidic conditions on cyclopropane fatty acids. After Bligh-and-Dyer extraction, they recovered caldarchaeol, which accounted for 75% of total core lipids, in the residual fraction by acid methanolysis. This implied that the neutral Bligh-and-Dyer extraction could extract only 25% of the total lipids from this methanogen. Kramer and Sauer (60) reported the core lipid composition of *Methanobacterium thermoautotrophicum* cells collected at various growth phases. They found that the archaeol-to-caldarchaeol ratio increased remarkably from the 2-day culture to the 6-day culture (Table 5). It is, however, important to reexamine whether the

growth phase affects the extraction efficiency of tetraether lipids when the lipid is extracted from intact cells with a neutral solvent, because the extraction yield of total lipid reported by Kramer and Sauer was considerably lower (1.6%) than that reported by Nishihara et al. (82). In general, attention should be given to extraction yield and especially to fractional extraction during examinations of the core lipid composition of cells under various growth conditions or in different growth phases.

Acetolysis and Acid Methanolysis for Complete Removal of Polar Head Groups

Makula and Singer (70) suggested the presence of phosphonolipids (lipids containing a P—C bond) in *Methanobacterium* species because the acid methanolysis-resistant phosphorus-containing lipids were retained at the origin in TLC. The difficulty in achieving complete hydrolysis of archaetidylserine was also described by Morii et al. (76), who found that archaetidylserine did not contain the phosphonate (P—C) group. The reason for the acid stability of some types of ether phospholipids has been discussed by Nishihara et al. (87). They concluded that the polar head group lacking free hydroxyl groups (serine or ethanolamine) in ether phospholipid could not be split off from the core residues, because ether bonds are not readily methanolized. Because the polar lipids containing these types of polar residues accounted for a significant part of the total lipids, the presence of unmethanolizable lipids caused significant error in the quantitative preparation of ether core lipids.

Morii et al. (76) used acetolysis for the complete removal of phosphoserine from archaetidylserine. The acetyl group(s) added to archaeol or caldarchaeol during acetolysis and the glycoside groups of glycolipids were hydrolyzed by subsequent acid methanolysis. This procedure permitted a quantitative preparation of ether core lipids from all kinds of polar lipids when used in conjunction with the acidic-extraction method, and it gave more correct values for the core lipid composition. In the tetraether type of phosphoglycolipid, phosphoric ester and glycosyl groups are attached to

the different glycerol moieties. In the structural determination of a phosphoglycolipid, the selective liberation of either group has particular significance because acetolysis does not cleave a glycosidic bond. HCl methanolysis (removal of the glycosyl bond) or acetolysis and subsequent mild-alkali methanolysis (removal of the phosphate ester) permit the selective liberation of either polar group (87). These procedures constitute the most powerful tools for the determination of the structure of tetraether phosphoglycolipids.

Mild-Acid Methanolysis for Preparation of Hydroxyarchaeols

Because of the acid lability of hydroxyarchaeol-containing lipids, they were converted to monoalkylglycerol by using the usual (strong) methanolytic conditions under which the polar head groups of the polyol-containing ether lipids are split off to produce the archaeol or caldarchaeol core. Ferrante et al. (30) found that mild-acid methanolysis (0.18% HCl-methanol at 50°C for 24 h) was sufficient to split off phosphodiester head groups while keeping the hydroxyarchaeol core intact. This mild-acid methanolysis was readily used for the preparation of *Methanothrix sn-3*-hydroxyarchaeol and *Methanosarcina sn-2*-hydroxyarchaeol (85). Because hydroxyarchaeols are not restricted to these two genera (56, 94), this method has potential significance for methanogen lipid biochemistry. HF is also used to prepare unmodified hydroxyarchaeols (94). A very characteristic signal of the ¹H-NMR spectrum at 1.24 ppm, attributed to the C-17' methyl group (bound at C-3' of the phytanyl chain) in the aliphatic region, was found to be specific to hydroxyarchaeols and could be used as a marker of this kind of core lipid (94).

Boron Trichloride Cleavage of the Ether Bond for Preparation of Glycerophosphoesters

BCl₃ treatment was first introduced by Gerrard and Lappert (36) to cleave ether bonds and applied to the ether bonds between glycerol and phytanyl groups of archaeol prepared from *Halobacterium* phospholipids (54). Because the phosphodiester groups had been removed by acid methanolysis in this case, the effect of BCl₃ on the phosphodiester bond was not known. Nishihara and Koga (83) found that BCl₃ cleaved ether bonds while keeping phosphodiester bonds intact. BCl₃ cleavage converted the ether phospholipids into the corresponding glycerophosphodiester with a nearly 100% yield. Glycerophosphodiester were analyzed by cellulose-TLC, which provided information on the polar head groups of ether lipids. This method is as significant as mild-alkali methanolysis as a tool for the structural analysis of diacyl-lipids. Although HI can also cleave ether bonds, it is not adequate to prepare glycerophosphoesters because HI treatment also degrades phosphodiester bonds. Unfortunately, the BCl₃ method did not yield glyceryl glycosides from the ether glycolipids since BCl₃ degrades glycosidic bonds.

Hydrogen Fluoride Cleavage of Phosphodiester

When glycerophosphoesters prepared by BCl₃ treatment were hydrolyzed with HCl at 100°C, stereoisomers such as L-serine were racemized. To identify the stereochemical configuration of a polar group, HF cleavage (0°C for 24 h) of the intact lipid is useful. Although the yield of serine from archaetidylserine by HF cleavage was 60 to 70% and a

variable amount of NH₃ was produced, the liberated serine was not racemized (74). HF cleavage is also used to prepare intact hydroxyarchaeols from ethanolamine- or serine-containing phospholipids which are not methanolized by mild-acid methanolysis.

BIOSYNTHESIS OF ETHER POLAR LIPIDS

The interesting points of biosynthesis of ether lipids are (i) the synthesis of the phytanyl chain, (ii) the formation of ether bonds, (iii) the formation of the unusual 2,3-di-*O*-radyl *sn*-glycerol structure, and (iv) the head-to-head condensation for the formation of tetraether lipids. These points correspond to the uniqueness of archaeal ether lipid structure. Studies of lipid biosynthesis that focus on methanogens are very fragmentary at present. The following therefore includes results obtained from experiments on other kinds of organisms of the *Archaea*. Recently, in vivo studies of the incorporation of radioactive or stable isotopes into archaeal lipids followed by chemical or NMR analyses were carried out, and two in vitro experiments have been reported. The major results of these studies are summarized as follows.

(i) *Methanospirillum hungatei* (26) and *Methanothrix soehngenii* (27) cells incorporated [1-¹³C]acetate or [2-¹³C]acetate into the isoprenoid hydrocarbon chains of their lipids in a position-specific manner. That is, every second carbon along the chain and branch methyl groups is labeled by the methyl group of acetate ([2-¹³C]acetate) and the remaining carbons are labeled by the carboxyl carbon of acetate ([1-¹³C]acetate). This labeling pattern is consistent with the typical condensation of three acetate molecules to form one mevalonate molecule. However, Ekiel et al. (26) pointed out that caution should be exercised in reaching conclusions about mevalonate biosynthesis because of the presence of a nonstandard pathway to this compound in halobacteria. Tachibana et al. (98) detected prenyl transferase activity in the cell extract of *Methanobacterium thermoformicum* (recently reclassified as *Methanobacterium thermoautotrophicum* [101]).

(ii) Formation of an unusual 2,3-di-*O*-radyl *sn*-glycerol structure seems to proceed through different mechanisms in *Halobacterium* and *Sulfolobus* or *Methanobacterium* species. On the basis of the fact that [1,3-³H]glycerol but not [2-³H]glycerol was incorporated into the glycerol moiety of the *Halobacterium* lipid without loss of radioactivity, dehydrogenation at the 2 position (oxidation of the 2-hydroxyl group to a 2-keto group) was postulated to be involved in lipid biosynthesis (53). To investigate the behavior of individual hydrogen atoms of the glycerol molecule and the prochirality of glycerol, Kakinuma et al. (47) fed [*sn*-1,1-²H₂]glycerol or [*sn*-3,3-²H₂]glycerol separately to a culture of *Halobacterium halobium* and determined the position on the glycerol moiety of archaeol into which ²H was incorporated by using ¹H-NMR and ²H-NMR. The results showed that *sn*-1-²H of glycerol appeared at the *sn*-3 position of archaeol-glycerol and that *sn*-3-²H of glycerol appeared at the *sn*-1 position, indicating that an inversion between the *sn*-1 and *sn*-3 positions occurred during the biosynthesis of ether lipids. Their data confirmed the results of Kates et al. (53), however, suggesting that the participation of dihydroxyacetone (a symmetric molecule) was eliminated. Similar types of experiments with *Sulfolobus acidocaldarius* showed no loss of ²H at the *sn*-2 position of glycerol and no inversion during ether lipid synthesis (46). This is in contrast to the situation with *Halobacterium* species, in which the glycerol moiety was inverted at the *sn*-2 position after

phosphorylation at the α position. Zhang et al. (110) showed that the cell extract of *Methanobacterium thermoautotrophicum* catalyzed the synthesis of 3-geranylgeranyl-*sn*-glycerol 1-phosphate from *sn*-glycerol 1-phosphate and geranylgeranyl PP_i. This result suggests that inversion at the *sn*-2 position either is not involved in the synthesis of ether lipids of *Methanobacterium thermoautotrophicum* or takes place only before alkylation. Their experiment, however, has not determined how *sn*-glycerol 1-phosphate is synthesized (direct phosphorylation of glycerol at *sn*-1 position, inversion of *sn*-glycerol 3-phosphate, or other mechanisms) in *Methanobacterium* species. Because this bacterium incorporated exogenous glycerol into its polar lipids very poorly, experiments similar to those carried out with *Halobacterium* or *Sulfolobus* species were difficult.

(iii) [1(3)-¹⁸O]glycerol was incorporated into ether lipid (48), indicating that the nucleophilic attack by the glycerol-oxygen atom to the C-1 carbon atom of the alkyl donor leads to ether bond formation.

(iv) It was suggested that the hydrocarbon chain had not been saturated at the time when the ether bond formed (73). This suggestion was based on results obtained by Moldoveanu and Kates (73) that ether lipids extracted from a culture of *Halobacterium cutirubrum* pulse-labeled with a radioactive precursor were readily degraded by acid methanolysis and that compounds derived from the phytol group or the geranylgeranyl group were produced.

(v) Geranylgeraniol was incorporated in vivo into *Methanospirillum hungatei* polar lipids with higher efficiency than phytol was (93). Therefore, the former is a more likely precursor than the latter is.

On the basis of the above observations, an outline of ether lipid biosynthesis was derived. The hydrocarbon chain is supplied as diterpenyl alcohol (probably geranylgeraniol, in its PP_i ester form) synthesized by the mevalonate pathway. The ether bond is formed through nucleophilic attack by *sn*-glycerol 1-phosphate (in *Methanobacterium* or *Sulfolobus* species) or dihydroxyacetonephosphate or *sn*-glycerol 3-phosphate (in *Halobacterium* species) on a putative unsaturated hydrocarbon precursor, geranylgeranyl PP_i. The hydroxyl group at the *sn*-2 position of the glycerol-phosphate moiety had to be inverted either before or after ether bond formation at the α -hydroxyl group in *Halobacterium* species. Glycerol kinase and glycerophosphate dehydrogenase of *Halobacterium* species were stereospecific for *sn*-3-phosphate (103). Figure 12 illustrates a tentative biosynthetic pathway for ether polar lipids that is based mainly on the halobacterial pathway proposed by Kakinuma et al. (48). The main route is probably adopted only by halobacteria. In Fig. 12, an alternate route to synthesis of pre-archaeidic acid in methanogens is also illustrated. However, the other parts of the biosynthesis of methanogen lipids is not known at all. Moldoveanu et al. (73) showed that a polar lipid-like, phosphate-free precursor that was the common precursor of various polar lipids formed before archaeidic acid formed. The results obtained by Kakinuma et al. (46–48) do not indicate any precursors with or without a phosphate group. Their pathway contains no phosphate-free lipidous precursor. It remains to be seen whether one can construct a plausible pathway that is consistent with both the data of Moldoveanu et al. (73) and those of Kakinuma et al. (46–48).

Little is known about the biosynthetic pathway of various polar lipids from archaeol, archaeidic acid, or its derivatives. Only in vivo incorporation kinetic studies have suggested metabolic pathways of polar lipids. Ferrante et al. (32) monitored the incorporation of [¹⁴C]mevalonic acid into polar

lipids in *Methanospirillum hungatei* and showed that there was a distinct possibility that archaetidyl(dimethylamino)pentanetetrol was a biosynthetic precursor of archaetidyl(trimethylamino)pentanetetrol. An unpublished pulse-chase experiment with ³²P_i and *Methanobacterium thermoautotrophicum* suggested that archaetidylethanolamine was derived from archaetidylserine (57). This pathway is similar to phosphatidylethanolamine synthesis in *E. coli* (49). On the basis of structural relationships (lipids shown in Fig. 8b to d and those shown in Fig. 9c and d), Ferrante et al. hypothesized two possible biosynthetic schemes of glycolipids (28).

The primary problem in tetraether lipid biosynthesis is whether the head-to-head condensation of the alkyl groups occurs between two molecules of free archaeols or between diether lipids that have already been substituted by polar head groups. On the basis of the examination of the structural regularities in the tetraether core lipids, Langworthy (65) and De Rosa and Gambacorta (18) inferred that two molecules of archaeol were condensed before polar head groups became attached to the archaeol, although Langworthy (65) circumspcctly did not exclude the possibility that condensation occurred between diether lipids already substituted with polar head groups. On the other hand, Kushwaha et al. (63) considered, on the basis of the structures of polar diether and tetraether lipids of *Methanospirillum hungatei*, that biosynthesis of tetraether polar lipids might occur by head-to-head condensation of diether polar lipids. Three heptads of polar lipids of *Methanobacterium thermoautotrophicum* (88) and one heptad of inositol lipids of *Methanobrevibacter arboriphilicus* (75) provided another line of evidence for the latter speculation. Incorporation kinetics and pulse-chase experiments (88) also supported the mechanism of condensation of diether polar lipids. When growing cells of *M. thermoautotrophicum* were pulse-labeled with ³²P_i, there was a significant lag between the rapid incorporation of radioactivity into the diether phospholipids and the incorporation of label into the corresponding tetraether polar lipids. In a pulse-chase experiment with ³²P_i, rapid turnover of the diether polar lipids was observed. At the same time, radioactivity was incorporated into the tetraether polar lipids. Poulter et al. (93) reported that archaeol added to the culture medium of *Methanospirillum hungatei* was incorporated into the nonpolar lipid fraction but not converted to the diether or to the tetraether polar lipids. The exact precursors and the chemical mechanism of tetraether biosynthesis remain to be elucidated.

SPECULATION ABOUT THE SIGNIFICANCE AND EVOLUTIONARY ORIGIN OF ETHER LIPIDS IN ARCHAEA

The question of the significance, origin, and evolution of archaeal ether lipids as mentioned in the Introduction can be answered only speculatively at present, because "there is a great deal of interplay between many forces in the determination of what lipids shall be present in a given cell at a given time" (79). Here we briefly discuss this problem, considering mainly the biosynthetic mechanisms and the distribution of ether lipids.

Zillig et al. (112) have proposed that the domain *Eucarya* originated by a fusion event based on the chimeric nature of eucaryal RNA polymerases and some other molecules. In this context, the eucaryal ester lipids are speculated to be acquired from members of the *Bacteria*. This hypothesis can account for the sharing of ester lipids by the *Bacteria* and *Eucarya*, although the origin of ester lipids in the *Bacteria*

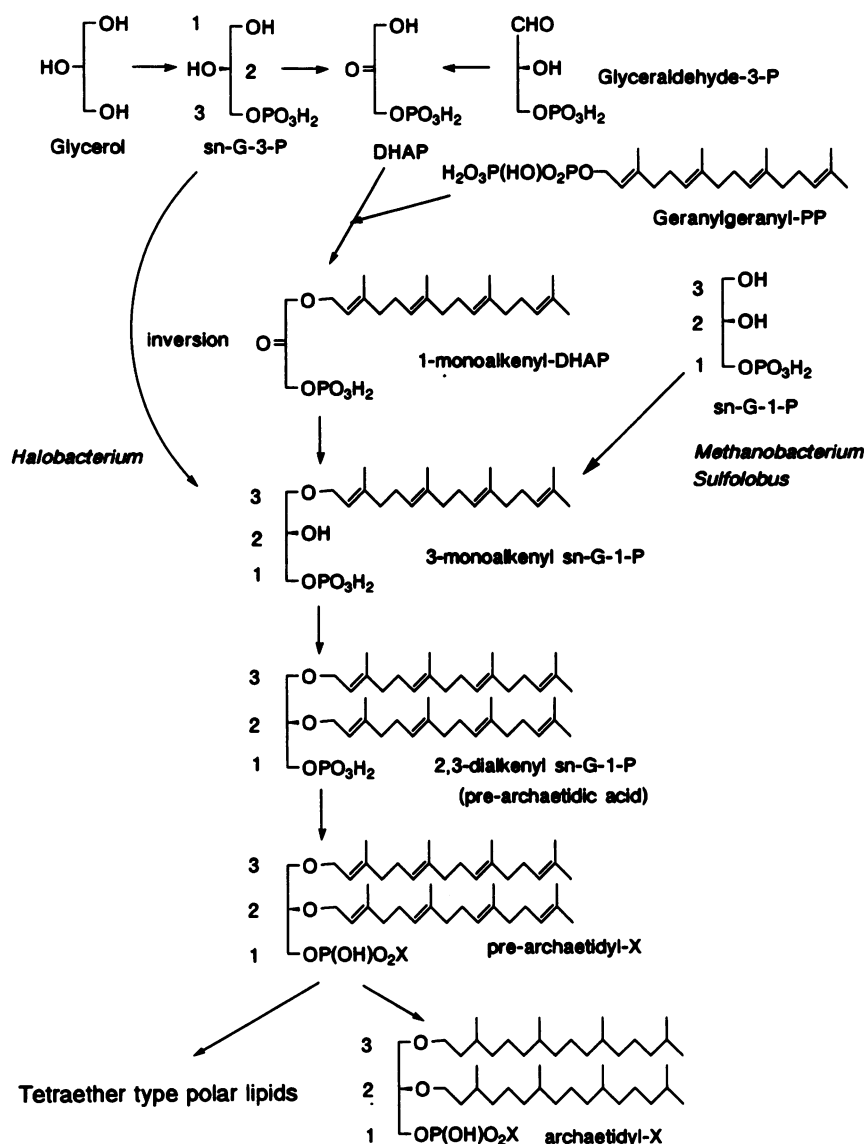


FIG. 12. Plausible biosynthetic pathway of archaeal ether lipids. The pathway from glycerol through 2,3-dialkenyl *sn*-G-1-P (pre-archaeidic acid) is deduced mainly from the data on *Halobacterium halobium* (46–48). A different route of ether lipid biosynthesis is also proposed for *Methanobacterium* and *Sulfolobus* species, as shown in this figure. The numbers 1, 2, and 3 attached near the glycerol skeleton carbons indicate the *sn* numbers of carbon atoms of the glycerophosphate moiety. *sn*-G-3-P, *sn*-glycerol 3-phosphate; *sn*-G-1-P, *sn*-glycerol 1-phosphate; geranylgeranyl-PP, geranylgeranyl pyrophosphate; pre-archaeidic acid and pre-archaeidyl-X are the unsaturated precursors of archaeidic acid and archaeidyl-X, respectively. The word “alkenyl” means simply unsaturated hydrocarbon group, which does not specify the number of double bonds on the chain.

and ether lipids in the *Archaea* is still in question. Later, Zillig (111) postulated that the primeval membrane was probably neither of isoprenyl ether nor of the fatty acid ester type but was rather, for example, made of protein. If this is the case, the question raised in the Introduction still remains to be answered in a modified form: when and how did the lipid membrane replace the protein membrane after the bifurcation of the *Archaea* and *Bacteria*?

Among the four differences in structure between the ether lipids of the *Archaea* and the glycerol-fatty acid ester lipids of the *Bacteria* and *Eucarya*, the most significant points are the nature of the ether and ester linkages and the nature of the hydrocarbon chains (high methyl branching without unsaturation in the *Archaea* and almost straight chains with

unsaturation in the *Bacteria* and *Eucarya*). Assuming that the most significant role of polar lipids is to constitute cell membranes, some fractions of straight-chain fatty acids must have *cis* unsaturation to give membranes their proper physicochemical properties, since membranes composed of only saturated fatty acid chains are too rigid to play a proper role in vital cells at moderate temperatures. On the other hand, highly methyl-branched, saturated isoprenoid hydrocarbon chains are bulky and have a phase transition temperature low enough for cells to function properly (51). Unsaturated fatty acids are synthesized in most aerobic organisms, from bacteria to mammals, by desaturation of saturated fatty acids in the presence of molecular oxygen (35). Biosynthesis of isoprenoid does not require molecular oxygen (38, 77, 78).

It is hypothesized that during the early stages of the origin or evolution of life under an anoxic atmosphere before the generation of atmospheric oxygen on Earth, isoprenoid hydrocarbon chains prevailed as the lipid components of biomembranes. Unsaturated fatty acids synthesized through the nonoxidative mechanism found in many microorganisms such as *E. coli* are also assumed to be hydrophobic components of biomembranes during the anaerobic stage of life on Earth. Isoprenoid carboxylic acids rarely occur but are detected as highly restricted metabolites (78). It is conceivable that under anoxic conditions isoprenoid alcohols were readily reduced to saturated isoprenoid alcohols rather than oxidized to isoprenoid carboxylic acids. As a result, isoprenoid alcohol could produce ether with glycerol during polar-lipid formation. This is the hypothetical reason for the anaerobic origin of ether lipids. Fatty acids might more easily modulate their physicochemical properties by desaturation than isoprenoids would on changes in the environmental growth conditions. Organisms with fatty acids therefore acquired a greater capability to adapt to diverse and changeable environments. After the appearance of a significant amount of molecular oxygen in the atmosphere, such organisms became prevalent on Earth, gaining the adaptability of fatty acid ester lipids.

The fact that tetraether lipids were found first in the thermoacidophiles *Sulfolobus* and *Thermoplasma* species has led many people to conclude that tetraether lipids evolved in those thermoacidophiles to adapt to the environment of high temperature and low pH. The monolayer structure of membranes made of tetraether lipids was believed to give suitable rigidity to the membrane at high temperature. Woese, however, called attention to the fact that the ether lipids are not simply an adaptation to extreme environments (106). Nes and Nes (79) also pointed out that the absence of an association between the presence of unusual lipids and the nature of the environment presumably reflects a familial rather than an adaptive relationship. Now we have more examples of ether core lipid composition of organisms grown under various conditions. According to a list of core lipid composition, the tetraether lipid structure is not restricted to thermophiles but is also found in some mesophilic methanogens (*Methanobrevibacter arboriphilicus* [75] and *Methanospirillum hungatei* [63]). On the other hand, two extremely thermophilic members of the *Archaea* (*Methanopyrus kandleri* and *Thermococcus celer*) contain only an archaeol core (24, 62). These facts strongly indicate that the occurrence or the evolution of tetraether lipids is not related to an adaptation to high temperature or low pH and that qualitative but not quantitative core lipid composition might be determined rather by a genetic or phylogenetic relationship. Methanogens that have ether lipids generally share living conditions in their habitat with members of the *Bacteria* that have ester lipids. Furthermore, not all the thermophilic microorganisms have ether type lipids. Thermophilic members of the *Bacteria* such as *Thermus* spp. have ester lipids (79). Recently it has been assumed that the ancestor of recent members of the *Archaea* and *Bacteria* most probably was an extreme thermophile (1) since *Thermotoga maritima* has been isolated and placed on the deepest branch of the bacterial line (1, 42). Although *Thermotoga* spp. and sulfur-dependent thermophilic members of the *Archaea* share the habitat, their lipids are not at all similar; the former bacteria do not contain diether or tetraether lipids but do contain unique long-chain dicarboxylic fatty acids. Moreover, the same long-chain dicarboxylic fatty acid $[\text{HOOC}(\text{CH}_2)_{13}(\text{CHCH}_3)_2(\text{CH}_2)_{13}\text{COOH}]$ is not

specific to *Thermotoga* spp. but occurs also in a mesophilic rumen bacterium, *Butyrivibrio* sp. strain S-2 (13, 55). This fact again indicates the unrelatedness of lipid constitution and environmental adaptation. These simple examinations of lipid distribution seem to show that the conditions in the ancestral habitat do not determine whether an organism has ester or ether lipids in its membrane.

The above consideration does not necessarily mean that ether lipids do not have any advantages over ester lipids in survival of the organism at elevated temperatures. Certain physicochemical properties of membranes made of isoprenoid ether lipids might give an advantage to archaeal organisms. For example, a chemically synthesized model lipid with phytanyl chains which mimicked archaeal tetraether lipids shows a greater ability than ester lipids and diether lipids to retain low- and high-molecular-weight compounds inside the vesicles at high temperatures (108). If it is assumed that a low rate of leakage is an important property of biological membranes, it may be concluded that isoprenoid tetraether lipids have a thermophilic or thermostable property that allows thermophilic organisms to grow at elevated temperatures. However, because of a variety of habitat conditions (high-salt, high-temperature, or anoxic conditions) of the *Archaea*, a variety of metabolisms, and many other kinds of presumed properties of ether lipid membranes, it is difficult to attribute a single common physiological function to an ether lipid property.

CONCLUDING REMARKS

Complete structures of lipids from seven species of methanogens have been elucidated during the past 10 years. Although the 7 species make up a minor fraction of the 62 species of methanogens, they represent four of seven families. Because to date the composition of polar lipids is uniform among all species belonging to a genus, lipids of almost 31 species may be presumed from the identified lipids of 7 species. This does not exclude the possibility that lipids with new and unique structures will not be discovered in the future. Lipids from methanogens that inhabit extreme environments or have unique metabolisms are attracting special interest; these include *Methanosphaera stadtmanae*, *Methanothermobacter fervidus*, *Methanococcus thermolithotrophicus*, *Methanohalophilus mahii*, *Methanococcus igneus*, *Methanopyrus kandleri*, and some members of the order *Methanomicrobiales* for which lipid studies have not been reported or are very limited.

Methanogen lipids were characterized by their diversity in phosphate-containing polar head groups and core lipids and, in turn, could be used in studies of the chemotaxonomy of methanogens. The uniqueness of ether lipids of methanogens was also exploited in a method to determine the methanogenic biomass in natural samples. In no other bacteria are the lipids more useful in chemotaxonomy and ecological surveys than in methanogens.

Biosynthetic studies of methanogen lipids have revealed significant progress in recent years, but the methods have remained, as a whole, at the in vivo experimental level. We hope to be able to develop cell-free systems of ether lipid biosynthesis and to elucidate enzymatic mechanisms of novel reactions such as ether bond formation and head-to-head condensation of isoprenoid hydrocarbon chains of diether polar lipids.

The significance and the origin and evolution of ether lipids in the *Archaea* is only speculatively discussed. A clue to resolving this problem may be obtained by metabolic

studies of archaeal ether lipids and gene analysis of enzymes involved in their metabolism.

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