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10. Any ^{35}S incorporated into fulvic acids is almost certainly reduced from $^{35}SO_4^{2-}$ to $^{35}S^{2-}$ first and then incorporated as the sulfide, as Nissenbaum and Kaplan (*8*) have demonstrated for the introduction of sulfur into humic acids. Thus, even if some of the ^{35}S label in the "pyrite fraction" is not pyrite, it is reduced sulfur and must be considered when measuring sulfate reduction. For routine sulfate reduction measurements, there is no need to distinguish between pyrite sulfur and fulvic acid sulfur.
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14. D. T. Rickard's finding [*ibid.* **275**, 636 (1975)] that pyrite can be formed rapidly through the direct reaction of aqueous ferrous ions and polysulfide ions strengthens my use of this theoretically calculated solubility product for pyrite if saturation with solid orthorhombic sulfur is assumed; elemental sulfur is abundant in marsh peat, and so saturation is likely.

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Diphytanyl and Dibiphytanyl Glycerol Ether Lipids of Methanogenic Archaebacteria

Abstract. *The lipids of nine different methanogenic bacterial strains are comprised of diphytanyl glycerol diethers, previously known only in extremely halophilic bacteria, as well as dibiphytanyl diglycerol tetraethers, known formerly only in the extremely thermoacidophilic bacteria* Thermoplasma *and* Sulfolobus. *Of the methanogens examined from four representative taxonomic groups,* Methanobacterium *and* Methanospirillum *contained both types of isopranyl ethers in nearly equal proportions, whereas the coccal forms,* Methanosarcina *and* Methanococcus, *possessed diphytanyl glycerol diethers, but with only a trace of or no dibiphytanyl diglycerol tetraethers. The occurrence of both types of isopranyl glycerol ethers in methanogenic bacteria supports the proposal that they have a close genealogical relationship to the extremely halophilic and thermoacidophilic bacteria.*

On the basis of 16*S* ribosomal RNA sequence analyses, Woese and Fox (*1*) and Fox *et al.* (*2*) proposed that the anaerobic methane-producing bacteria, which utilize hydrogen and carbon dioxide as energy and carbon sources, represent ancestral life-forms, designated as archaebacteria. The extremely halophilic bacteria and the thermoacidophilic organisms *Thermoplasma* and *Sulfolobus* also appeared to be closely related (*3*, *4*). As demonstrated elsewhere (*5*), the isopranyl ether lipids of the thermophilic methanogen *Methanobacterium thermoautotrophicum* are surprisingly similar to those of the halophile *Halobacterium cutirubrum* (*6*) and the thermoacidophile *Thermoplasma* (*7*, *8*) and *Sulfolobus* (*9*). The lipids were shown to contain isopranyl ethers of glycerol or its derivatives and a fraction of C_{20}, C_{25}, and C_{30} acyclic isoprenoid hydrocarbons (*5*). The results supported the concept that methanogens, halophiles, and thermoacidophiles share a common evolutionary episode. However, all of these bacteria whose lipids have been examined were originally isolated from extreme environments, having high temperature, low *p*H, high salinity, or some combination thereof. Because these ether lipids may reflect adaptation to an extreme environment rather than true genealogical relationships, it is necessary to determine whether mesophilic methanogens also possess the same unique composition of lipids. This report describes the basic component structure of the polar lipids from eight mesophilic methanogens relative to those of *M. thermoautotrophicum* and halophilic and thermoacidophilic microorganisms.

Lyophilized cells of *M. thermoautotrophicum, Methanobacterium* M.O.H., *Methanobacterium* strain AZ, *Methanobacterium ruminantium* PS, *Methanobacterium ruminantium* M-1, *Methanospirillum hungatii, Methanococcus vannielii, Methanococcus* strain PS, and *Methanosarcina barkeri* were prepared at the University of Illinois by W. E. Balch and were obtained from R. S. Wolfe. *Methanobacterium thermoautotrophicum* (*10*) and the remaining methanogens (*2*) were grown and harvested as previously described. One gram of lyophilized cells was suspended in a saline divalent solution (*11*) and lipids were extracted by the method of Bligh and Dyer (*12*). The residue was reextracted by an acid Bligh-Dyer procedure by replacing the water with 0.1*M* acetate buffer (*p*H 5.0). The twice-extracted residues were finally extracted a third time by refluxing in boiling methanol and chloroform (2:1) for 1 to 2 hours. Each of the three extracts was weighed and then combined to give total extractable lipids. The total lipids of all methanogens were fractionated on columns of silicic acid (Unisil, 325 mesh), with *n*-hexane, benzene, and finally chloroform to remove nonpolar lipids (20 to 30 percent of total lipids). The remaining polar glycolipids and phospholipids (70 to 80 percent of total lipids) were recovered by eluting with chloroform and methanol (2:1 by volume) and methanol and the eluates were pooled.

All studies described in this report were conducted on the pooled polar lipid fraction. In some instances, polar lipids contained a major component that was not soluble in chloroform. This insoluble material was acetylated and then dissolved in chloroform (*5*). Each of the lipid fractions was hydrolyzed in anhydrous 2.5 percent methanolic HCl and extracted with petroleum ether (*13*). The petroleum ether–soluble products were chromatographed on thin-layer chromatography (TLC) plates made with silica gel G and developed in solvents *n*-hexane, diethyl ether, and acetic acid (80:20:1) or chloroform and diethyl ether (9:1). Components were visualized with phosphate spray (*14*), periodate-Shiff reagent for vicinal glycols (*15*), acid charring, or by exposure to iodine vapors. Components were identified by comparing R_F values to those of both established and known standards. The relative intensities of components were recorded with a Zeineh soft laser densitometer. Components were scraped from the plates and eluted with chloroform and methanol (1:1). Portions of alkyl ether–containing components were further digested by refluxing in 47 percent HI for 12 hours to release alkyl iodides (*5*, *6*). The halides were either reduced to the alkane derivatives by refluxing in acetic acid and zinc (*16*) or converted to the alkyl acetates by refluxing with silver ace-

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tate in acetic acid (*6*). Intact alkyl-glycerol lipids obtained after acid methanolysis and recovered from TLC were analyzed as the trimethylsilyl (TMS) ether derivatives.

Lipid samples were analyzed on a Perkin-Elmer IR-257 spectrophotometer as a thin film in carbon tetrachloride. Lipid hydrolysates and derivatives were analyzed on a Hewlett-Packard F & M 5750 gas-liquid chromatograph (GLC) equipped with flame ionization detectors. Analyses were carried out with 1.8 m by 6 mm stainless steel columns packed with either 5 percent SE-30 on 80/100 Gas-Chrom Q, 1.8 m by 3 mm stainless steel columns packed with 10 percent SP 2330 on 100/120 Chromosorb WAW, or 1.8 m by 6 mm glass columns packed with 5 percent QF 1 + 5 percent OV-17 on 80/100 Gas-Chrom Q. Mass spectra were recorded with a Finnigan 4203 gas chromatograph–mass spectrometer equipped with an Incos data system.

The total lipids extracted from the methanogens were determined on the basis of cell dry weight as 4.4 percent (*Methanobacterium* strain AZ), 2.0 percent (*M. ruminantium* PS), 4.2 percent (*M. ruminantium* M-1), 2.8 percent (*M. thermoautotrophicum*), 3.8 percent (*Methanobacterium* M.O.H.), 3.3 percent (*M. vannielii*), 2.9 percent (*Methanococcus* strain PS), 5.0 percent (*M. hungatii*), and 4.7 percent (*M. barkeri*). The first neutral Bligh-Dyer extraction of the methanogens effectively removed the total cellular lipids with the exception of those from *M. thermoautotrophicum* and *Methanobacterium* strain AZ. The acid reextraction of cell residues was ineffective in removing additional lipids in all cells studied, and refluxing the twice-extracted residues in a mixture of chloroform and methanol provided additional lipid material (0.5 and 1 percent of cell dry weight) only in *Methanobacterium* strain AZ and *M. thermoautotrophicum*. The two latter lipid extracts also differed from those of the other methanogens in that they separated into colorless gummy-appearing materials as well as freely soluble lipids in chloroform. The less soluble lipids in these two organisms were acetylated and solubilized in CCl_4 for comparative spectral analyses. Infrared spectra of the total lipid extracts from all the organisms and acetylated subfractions showed the following groups: OH (3450 cm^{-1}), CH_2 and CH_3 (2930, 2860, 1460, and 730 cm^{-1}), C–CH_3 (1380 cm^{-1}), C–$(CH_3)_2$ (1380 and 1365 cm^{-1}, doublet), ether C–O–C (1110 cm^{-1}), P=O (1260 and 1230 cm^{-1}), and C=O (1730 cm^{-1}). These spectra were indicative of isopranyl glycerol ethers and were virtually identical to those reported for isopranyl ether lipids of *Halobacterium* (*6*), *Thermoplasma* (*7, 8*), *Sulfolobus* (*9*), and *M. thermoautotrophicum* (*5*).

A thin-layer chromatogram containing the petroleum ether–soluble components released by acid methanolysis of the polar lipids is shown in Fig. 1. As many as 12 components could be detected (designated 1 to 12 in Fig. 1). Many were present in only trace quantities. Compounds 4, 8, 9, and 10 cochromatographed with authentic dibiphytanyl diglycerol tetraether (*7*), free fatty acids, diphytanyl glycerol diether (*6*), and fatty acid methyl esters, respectively. These four compounds cochromatographed with authentic standards in both TLC solvents and accounted for approximately 80 percent of the released neutral lipid material. Free vicinal hydroxyl groups were absent in all components on the plate except component 2 as judged by the periodate-Schiff stain.

Components 4 and 9 from each organism were recovered after TLC. The TMS derivatives of component 9 cochromatographed on GLC with an established standard of authentic TMS-diphytanyl glycerol diether obtained from *H. cutirubrum*. In addition, the mass spectral fragmentation patterns of both were identical. Similar analyses of intact component 4 were not possible because of its high molecular weight (1300) (*7*).

Hydriodic acid digests of components 4 and 9 (Fig. 1) yielded the *O*-alkyl hydrocarbon chains of the glycerol ethers, which were recovered as the iodide, acetyl, or alkane derivatives. These derivatives, the sole hydrocarbons from component 9, cochromatographed on GLC with authentic phytanyl iodide ($C_{20}H_{41}I$), phytanyl acetate ($C_{22}H_{44}O_2$), and phytane ($C_{20}H_{42}$), respectively. The alkyl chains of component 4 were also comprised of a single compound. Component 4 cochromatographed with the authentic acyclic 16,16′-biphytanyl C_{40} diol ($C_{40}H_{82}O_2$) from *Thermoplasma* (*7*), as both the diacetate ($C_{44}H_{86}O_4$) and alkane ($C_{40}H_{82}$) derivatives. The acyclic C_{40} was the only hydrocarbon present. No pen-

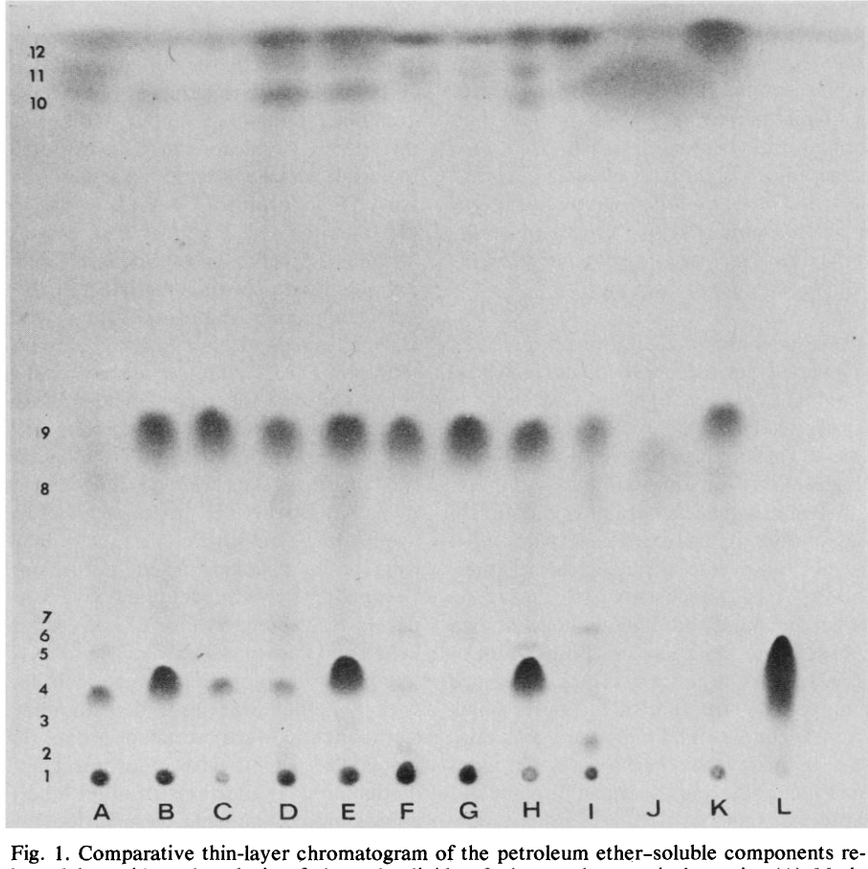
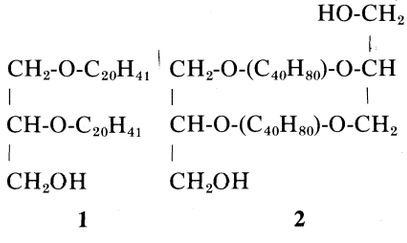


Fig. 1. Comparative thin-layer chromatogram of the petroleum ether–soluble components released by acid methanolysis of the polar lipids of nine methanogenic bacteria. (A) *Methanobacterium* strain AZ; (B) *M. ruminantium* PS; (C) *M. ruminantium* M-1; (D) *M. thermoautotrophicum*; (E) *Methanobacterium* M.O.H.; (F) *M. vannielii*; (G) *Methanococcus* strain PS; (H) *M. hungatii*; (I) *M. barkeri*; (J) standard fatty acid (spot 8) and fatty acid methyl ester (spot 10) mixture; (K) authentic diphytanyl glycerol diether; and (L) authentic dibiphytanyl diglycerol tetraether. The solvent was *n*-hexane, diethyl ether, and acetic acid (80:20:1 by volume).

tacarbon ring containing C_{40} chains, as found in *Thermoplasma* and *Sulfolobus* (*7, 17*), was detected. The GLC retention times of the components relative to phytane were 1.0 (phytane), 2.3 (phytanyl acetate), 5.6 (biphytane), and 13.8 (biphytanyldiacetate) on a 5 percent SE-30 column at 300°C and a flow rate of N_2 of 40 cm^3/min.

The mass spectra of the C_{20} phytane and phytanyl acetate derivatives, as well as the C_{40} biphytane and its diacetate derivative, were identical in all respects to the spectra determined on authentic phytane, biphytane, and their acetate derivatives, confirming the identity of the isopranyl ethers of methanogens. The mass spectra showed expected weak molecular ion peaks at mass-to-charge ratios (*m/e*) 282, 340, 562, and 678 for phytane, phytanyl acetate, biphytane, and biphytanyl diacetate, respectively. For the phytanyl acetate, the peak at *m/e* 281 corresponding to the loss (M − 59) of a single acetate reveals the fragmentation pattern of the acetate derivative of phytol. Similarly, the biphytanyl diacetate showed peaks at *m/e* 618 and *m/e* 559 corresponding to the loss of one (M − 59) and then two (M − 118) acetates, demonstrating unequivocally the biphytanyl diol structure. These data establish the occurrence of phytanyl glycerol diether (structure **1**) and biphytanyl diglycerol tetraether (structure **2**) in the methanogenic bacteria.



1 **2**

Components remaining at the origin were phosphorus stainable, with the exception of *M. hungatii*, in which no phosphorus was detectable. The material amounted to approximately 10 to 30 percent of the acid methanolysates. Small and variable quantities of phytane and biphytane were identified in compounds isolated from the origin.

The only other components identified were 8 and 10 (Fig. 1), which represented about 1 to 10 percent of polar lipid methanolysates. These were fatty acids identified principally as $C_{18:0}$, $C_{18:1}$, $C_{17:0}$, $C_{16:0}$, $C_{15:0}$, and branched chain C_{17} and C_{15} acids.

This survey of the basic lipid structure of nine methanogens shows that hydrophobic lipid components are principally isopranyl glycerol diethers identified as diphytanyl glycerol ethers (structure **1**) and dibiphytanyl diglycerol tetraethers (structure **2**) rather than more typical glycerides or plasmalogens. The distribution between the two isopranyl ethers in the methanolysates of total lipid extracts of the nine methanogens examined is shown in Table 1. The diphytanyl glycerol ethers were prominent in all organisms studied; the dibiphytanyl diglycerol tetraethers also occurred as a major component in all samples, except that a trace was detected in *M. vannielii* and there were no readily detectable amounts in *Methanococcus* strain PS and *M. barkeri*. The ratios of phytanyl derivatives to biphytanyl derivatives in *Methanobacterium* strain AZ and *M. thermoautotrophicum* shown in Fig. 1 are misleading since significant portions of the total lipids would not remain soluble in lipid solvents.

Table 1. Distribution of diphytanyl diethers and dibiphytanyl tetraethers in nine methanogenic bacteria.

Organism	Di-ether (%)	Tetra-ether (%)
Methanobacterium strain AZ	37.5	62.4
Methanobacterium strain M.O.H.	43.5	56.5
Methanobacterium thermoautotrophicum	44.5	55.5
Methanobacterium ruminantium PS	44.7	55.3
Methanobacterium ruminantium M-1	71.8	28.2
Methanospirillum hungatii	40.5	59.5
Methanosarcina barkeri	100.0	0
Methanococcus vannielii	99.9	0.1
Methanococcus strain PS	100.0	0

The methanogen strains studied represent members from each group of the taxonomic tree proposed by Fox *et al.* (*2*). Eight of these methanogens are organisms from temperate environments. The lipids of these organisms are identical to those of the organisms from extreme environments, namely *Halobacterium, Sulfolobus, Thermoplasma*, and *M. thermoautotrophicum*. The primary differences between the lipids of *Halobacterium* and those of *Sulfolobus* and *Thermoplasma* has been the occurrence of phytanyl diethers in the halophiles and biphytanyl tetraethers in the thermoacidophiles. *Methanobacterium* and *Methanospirillum* now represent a link between these two groups, as their lipid contents consist predominantly of both ether lipid forms. The coccal morphological methanogens *Methanococcus* and *Methanosarcina* appear to be more removed from the other mesophiles, as they have predominantly phytanyl ether lipids and only a trace of or no biphytanyl ethers. The cocci thus more closely resemble the halophiles.

Woese and Fox (*1*) and Fox *et al.* (*2*) defined *M. thermoautotrophicum, Sulfolobus*, and *Thermoplasma*, from extreme environments, as archaebacteria on the basis of their 16*S* RNA characterization. Further evidence from lipid studies revealing the unique isopranyl glycerol ethers of these organisms support this proposal. The data reported here establish that the mesophilic methanogens also contain the same ether lipids, which must represent a long evolutionary relationship between methanogens, *Halobacterium, Thermoplasma*, and *Sulfolobus*; this argues against the possibility that these ether lipids reflect environmental adaptation.

Makula and Singer (*18*) have reported the tentative identification of phytanyl and biphytanyl ethers in three methanogens by chromatographing digests of phospholipid extracts. There are no disagreements between their preliminary data and the data reported here.

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