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Phytanyl-Glycerol Ethers and Squalenes in the Archaebacterium *Methanobacterium Thermoautotrophicum*

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Summary. The lipids of a thermophilic chemolithotroph, *Methanobacterium thermoautotrophicum*, have been analyzed by chromatographic techniques and identified by infrared spectrometry and combined gas chromatography-mass spectrometry. Of the total chloroform soluble lipids 79% and 21% are polar and non-polar lipids, respectively. The major components of the polar lipids are dialkyl ethers of glycerol or its derivatives. The nature of the glycerol ether alkyl groups was found to be that of the saturated tetraisoprenoid hydrocarbon phytane. The non-polar lipids of the chloroform soluble fraction consist principally of three series of C_{20}, C_{25} and C_{30} acyclic isoprenoid hydrocarbons, the major components being squalene and a continuous range of hydrosqualene derivatives, from dihydrosqualene up to and including decahydrosqualene. These data establish that *M. thermoautotrophicum* contains predominantly non-saponifiable lipids as do *Halobacterium, Halococcus, Sulfolobus* and *Thermoplasma*. In particular, the composition of the chloroform soluble lipids of *M. thermoautotrophicum* is quite similar to that of *Halobacterium cutirubrum*. The results strongly support the recent proposal, based on 16S rRNA sequence homologies, that the extreme halophiles and methanogens share a common ancestor. In addition, it is pointed out that the occurrence of phytane and related polyisoprenoid compounds in ancient sediments can no longer be considered unequivocally as indicative of past photosynthetic activity. Finally, speculations are made concerning the possible role of and evolutionary significance of the presence of squalene and hydrosqualenes in these organisms. To our knowledge this is the first report of squalene and hydrosqualenes in a strictly anaerobic microorganism.

Key words: Ether lipids – Glycerol ethers – Squalene – Hydrosqualenes – Isoprenoids – Phytane – Archaebacteria – Methanogens

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Introduction

Analyses of the 16S ribosomal RNA (rRNA) of 10 species of methanogenic bacteria have shown that these organisms are only distantly related to typical bacteria (Fox et al., 1977), and that they should be considered in the light of additional evidence as forming part of a separate line of evolution, or distinct phylogenetic group, which has been tentatively designated as Archaebacteria (Woese and Fox, 1977). Subsequent 16S rRNA characterizations have indicated that the archaebacterial line should also include the halophilic bacteria (Magrum et al., 1978). From other studies it is known that these halophilic organisms have a unique photoconversion system based on bacteriorhodopsin (Osterhelt and Stoeckenius, 1971), and that they possess an unusual membrane made of ether derivatives of glycerol and phytanol (Kates, 1972), and contain substantial amounts of squalenes (Tornabene et al., 1969).

The finding that the halophiles are specifically related to the methanogens appears surprising, not only because the two types of organisms are nutritionally different, one being chemoorganotrophic and the other chemolithotrophic, but also because the halophiles possess a sophisticated cytochrome system which interacts with molecular oxygen (Cheah, 1969; Lanyi, 1968, 1969) whereas the methanogens are strict anaerobes.

It is for these reasons that it would be highly desirable to obtain other lines of evidence that would reinforce the 16S rRNA affinities of these two groups of organisms; for instance, determining if the unique features of the halophilic membrane lipid composition are also shared by the methanogens. We have just done this, and the preliminary evidence shows that *Methanobacterium thermoautotrophicum* has a very similar lipid composition to that of *Halobacterium cutirubrum* analyzed by Kates et al. (1965), and Tornabene et al. (1969). Namely, it contains the phytanyl-glycerol ethers, squalenes, and other membranogenic compounds, as the halophile, with compositional variations which are probably related to the expected biological diversity of the two organisms.

Methods

Lyophilized cell culture samples of *Methanobacterium thermoautotrophicum* were prepared at the University of Illinois and analyzed at Colorado State University and the University of Houston. They were grown and harvested by the method of Zeikus and Wolfe (1972). Two grams of lyophilized cells were suspended in saline, divalent solution (Tornabene, 1973) and extracted by the method of Bligh-Dyer (1959). The residue was reextracted with methanol-chloroform-0.1 M acetate buffer (pH 5.0) as described by Bertsch et al. (1969). These combined extracts were the total lipid extract. One half of the total lipids was fractionated on a column of silicic acid (Unisil, 325 mesh) with hexane, benzene, chloroform, chloroform-methanol, 2:1, and methanol. The eluates were monitored by thin-layer chromatography (TLC) in solvents hexane-benzene, 9:1; chloroform-ethyl ether, 9:1; and chloroform-acetone-methanol-acetic acid water, 50:20:10:10:5. Compounds were visualized by exposure to iodine and by acid charring. The eluates were also chromatographed on silicic acid impregnated paper with diisobutyl ketone, acetic acid and water, 50:40:5, and the compounds were visualized with three different reagents: rhodamine, ninhydrin and the periodate-Shift reagent, as described

by Marinetti (1962). Total lipids and lipid fractions were subjected to mild alkaline hydrolysis (Tornabene and Ogg, 1971), alkaline and acid methanolysis (Kates, 1964), and hydroiodic acid hydrolysis (Kates et al., 1965). The lipid and water hydrolysates were studied by silicic acid and cellulose TLC and paper chromatography (Tornabene, 1973).

The total lipids, fractionated lipids and hydrolysates were analyzed for phosphorus (Allen, 1940) and total carbohydrates (Dubois et al., 1956). Infrared spectra were taken on thin films or solutions of the compounds in carbon tetrachloride with an IR-257 Perkin-Elmer spectrophotometer. Samples were analyzed on a F&M 5750 Gas Liquid Chromatograph (GLC), equipped with dual flame detectors. Analyses were carried out with a 93 m x 0.75 cm stainless steel capillary column coated with 5% OV-17, 1.8 m x 3 mm glass column packed with 5% QFI + 5% OV-17 on 80/100 gas chrom Q and a 10 m x 0.2 mm glass capillary column coated with OV-101. All chromatograms were recorded with a Columbia Model CS1-208 automatic digital integrator or a Zeineh Soft laser scanning densitometer. Mass spectra were recorded with a LKB 9000 gas chromatograph-mass spectrometer combination. The chromatographic section of the instrument had previously been replaced with a Perkin-Elmer Model 9000 gas chromatograph.

Results

The total lipids extracted by the above described methods amounted to 2.7% of the cell dry weight, about two thirds of which (1.8%) was chloroform soluble. The analyses we are reporting here are of these chloroform soluble lipids. The remaining lipid extract had the appearance of colorless gummy material presumably made by higher molecular weight, less soluble lipid components. Its composition will be reported in a separate paper. The chloroform soluble lipids contained 1.65% phosphorus and 7.6% carbohydrates and related substances as determined colorimetrically. Infrared spectra of the total lipids showed a broad band at 3300 cm^{-1} for hydrogen-bonded OH groups, strong CH_2 and CH_3 absorption bands at 2940, 2860, and 1465 cm^{-1}, a doublet at 1380-1370 cm^{-1} indicative of *gem*-dimethyl and C-CH_3 groups, strong free and bonded P=O bands at 1260 and 1230 cm^{-1}, respectively, a very strong ether band at 1105 cm^{-1}, and a fairly strong ester C=0 band at 1730 cm^{-1}. The total lipids consisted of approximately 21% neutral lipids and 79% polar lipids as determined by chromatographic procedures and densitometry.

A significant fraction of the neutral lipids was identified as squalene (S), dihydrosqualene (S-2), tetrahydrosqualene (S-4), hexahydrosqualene (S-6), octahydrosqualene (S-8) and decahydrosqualene (S-10) by TLC and/or GC-MS (Table 1). The S, S-2, S-4, S-6 were present in the ratio of 1.0:1.1:0.4: < 0.05. S-8 and S-10 were found in smaller but significant concentrations. The gas chromatographic evidence indicated only a trace of S-12. Some of the more abundant components and their ratios are comparable to those found (S., S-2, and S-4) in *Halobacterium cutirubrum* (Tornabene et al., 1969).

Mass spectra of squalene (S) and the hydrosqualenes (see Tornabene et al., 1969), showed that component S was identical with squalene and that the hydrosqualenes differed from squalene only in their degree of unsaturation. Both standard squalene ($C_{30}H_{50}$) and component S gave parent molecular ions corresponding to M-410. S-2,

Table 1. Neutral lipid content of *M. thermoautotrophicum*

Squalene (S) and hydrosqualenes	C-25 Isoprenoid	C-20 Isoprenoid
C-30 : 6 (S)	C-25 : 3	C-20 : 2 (5 isomers)
C-30 : 5 (S-2)	C-25 : 2	C-20 : 1
C-30 : 4 (S-4)	C-25 : 1	
C-30 : 3 (S-6)		
C-30 : 2 (S-8)		
C-30 : 1 (S-10)		
C-30 : 0 (S-12)		

All the above compounds were identified by gas chromatography and mass spectrometry (see text for details), except for C-30 : 0 (S-12), squalane, which was only identified by gas chromatography because of its lower concentration. The isoprenoids are indicated by the notation C-n, where n is the number of carbons of the isoprenoid, followed up by the number of double bonds. In the case of the hydrosqualenes, they are abbreviated with the letter S followed by the number of hydrogens added to squalene, S-12 being the totally hydrogenated squalene (squalane). We have not used the earlier equivalent notation using a subscript (S_n) in order to avoid possible confusion with oligomers of squalene. The hydrocarbons listed as phytene and phytadienes (C_{20}), and the unsaturated C_{25} polyisoprenoids were the major ones of these two series of compounds detected. It is possible that other polyisoprenoid compounds exist in smaller amounts among the non-polar lipids of *M. thermoautotrophicum*

S-4, S-6, S-8 and S-10, however, had parent molecular ions at M-412, M-414, M-416, M-418 and M-420, respectively. The mass spectra of both compound S and standard squalene showed a base peak at m/e 81, relatively intense doublets at m/e 136 and 137, and fragments at m/e values 341, 367 and 395, corresponding to M-69, M-43 and M-15, respectively. The mass spectra of the hydrosqualenes S-2, S-4 and S-6 showed a fragmentation pattern similar to that of standard squalene, except that the M-113, M-85, M-69, M-43, M-15, and parent M ions were higher by two, four and six mass units, respectively. This indicates that compound S-2 has the same structural characteristics as squalene (2, 6, 10, 15, 19, 23-hexamethyl tetracosa-2, 6, 10, 14, 18, 22 hexaene), but with one double bond less. Similarly, components S-4 and S-6 have two double bonds and four double bonds less, respectively, since their mass spectra had the M-113, M-85, M-69, M-43, M-15 and M ions described above but with values 4 and 6 mass units higher than in S. The mass spectrum of S-8, with a parent M ion at 418, showed a M-15 and M-85 peak. Only trace amounts of the decahydrosqualene S-10 was detected. The substance was tentatively identified by its parents M ion at 420, and by its expected retention time value.

The other neutral lipids (Table 1) with less than 5% relative percentage concentration consisted of isoprenoid C-25 monoenes, dienes and trienes, various structural isomers of isoprenoid C-20 dienes and a series of straight chain n-alkanes. The remaining neutral lipids will be described elsewhere. They were tentatively identified as mono- and di-phytanyl glycerol ether analogs, and a mixture of hydrocarbons and pigments.

The polar lipids consisted of nine discernible compounds on paper and thin-layer chromatograms. Three of the components comprised 87% of the polar lipids. Analyses of lipid soluble and water soluble fractions from methanolic-HCL and methanolic-NaOH hydrolyses of the total lipids revealed that 90% of the lipids were nonsaponifiable. The saponifiable lipids consisted predominantly of a fraction which was tentatively identified as glycolipid. The following fatty acids were identified: C-18:0, C-18:1, C-17:0, C-16:0 and C-15:0. The infrared spectra of the nonsaponifiable oily lipids from methanolic-HCL hydrolysis of the polar lipid fraction 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-$(CH_3)_2$ (1380-1365 cm^{-1} doublet), ether C-O-C (1110 cm^{-1}) and primary alcoholic C-O (1045 cm^{-1}). This spectrum was indicative of long chain isopranyl glycerol diethers and identical to that reported by Kates et al., (1965) for methanolic-HCL hydrolyzed *H. cutirubrum* lipids.

Cleavage of the diether with hydroiodic acid yielding a yellowish oily alkyl iodide gave only one major peak on GLC with a retention time value identical to the established standard phytanyl iodide derived from *H. citirubrum* (Kates et al., 1965). The mass spectrum showed an expected weak molecular ion peak at m/e 408 and strong peaks at M-126 and M-128. The fragmentation pattern was similar to that of phytane. Catalytic hydrogenation of the alkyl iodide yielded a substance with a retention time value identical to phytane. The identity of phytane was verified by mass spectral analysis, thus confirming the structure of the alkyl iodide as 3, 7, 11, 15-tetramethylhexadecane-1-iodide. Other studies will report on the exact nature of the intact individual lipid components.

The preliminary results from our studies on the chloroform soluble lipids of *M. thermoautotrophicum* show that the major lipid components are long chain isoprenoids and isopranyl glycerol diethers. The major portion of the neutral lipids is comprised of S, S-2, S-4, while phytanyl is the saturated and branched alkyl structure of the glycerol diethers. These data establish that *M. thermoautotrophicum* also contains predominantly non-saponifiable lipids as do *Halobacterium, Halococcus, Sulfolobus* and *Thermoplasma* (Kates, 1972; Langworthy et al., 1972, 1974, 1977).

The properties of the chloroform soluble lipids of *M. thermoautotrophicum*, and the nature of the di-O-phytanyl glycerol ethers and squalenes is similar to that of the compounds identified previously in *H. cutirubrum* (Kates et al., 1965; Tornabene et al., 1969). The variation in the polyisoprenoid structures that we have so far observed between the chloroform soluble lipids of *M. thermoautotrophicum* and *H. cutirubrum* are mainly limited to the extent of hydrogenation of the double bonds present in these branched hydrocarbons.

On the other hand, the long chain isopranyl structures of *Sulfolobus* and *Thermoplasma* have been identified as high molecular weight tetraethers made by combination of 2 moles of glycerol with 2 moles of a C_{40}-isoprane-1,32-diol (Langworthy, 1977). Although they are different, the latter compounds structurally resemble a covalently linked dimer of the di-O-phytanyl glycerol ethers from *Halobacterium* and *Methanobacterium*. The chloroform insoluble fraction of the *M. thermoautotrophicum* was subsequently solubilized in a benzene-pyridine-acetic anhydride mixture. Spectrophotometric and colorimetric analyses provided evidence that the fraction consists predominantly of long chain isopranyl glycerol ethers that are linked to phosphorus

and a sugar moeity. The exact identity of the component of this fraction will be described in another paper.

The identification of the chloroform-soluble glycolipid fraction in *M. thermoautotrophicum* is consistent with the presence of glycolipids in both the acidothermophilic (Langworthy et al., 1972, 1974) and halophilic (Kates et al., 1967) bacteria. In *Sulfolobus* the glycolipids comprised 68% of the total lipids (Langworthy et al., 1974).

Discussion

While the present work was underway further 16S rRNA characterizations identified *Themoplasma* and *Sulfolobus* as archaebacteria too, (Woese, personal communication). In both of these genera, as indicated above, there is evidence for the presence of polyisoprenoid ethers (Langworthy et al., 1972, 1974). The composite of this evidence, taken together with other available information concerning the various individual genera, is clarifying the nature of the archaebacteria.

It has been inferred (Woese and Fox, 1978) that several common features can be taken as characteristic of this new kingdom. In addition to their highly specialized ecology these are (a) the composition of the lipids which are predominantly derivatives of non-saponifiable long-chain alkyl glycerol ethers (Kates et al., 1965; Tornabene et al., 1969; Kates, 1972; Langworthy et al., 1972, 1974), (b) the absence of a peptidoglycan cell wall (Brock et al., 1972; Darland et al., 1970; Stoeckenius and Rowen, 1967; Kandler and Hippe, 1977) and (c) characteristic RNA's associated with the translation apparatus (Fox et al., 1977; Magrum et al., 1978; and Woese, personal communication). The new data presented here concerning the lipids of a typical thermophilic methanogen extend the information concerning that criterion and thus support not only the proposed relationship between the halophiles and the methanogens but the larger concept indicated above as well. Moreover, the finding of squalene in both the methanogens and the halophiles suggests that this too may be characteristic of the archaebacteria. Hence, *Thermoplasma* and *Sulfolobus* need to be reexamined for the presence of squalene.

It is important to realize that all of the archaebacterial strains whose lipids have been examined were originally isolated from extreme environments, e.g., high temperature, low pH, high salinity or some combination thereof. It is necessary to consider that the unusual ether linkages may reflect a straightforward environmental adaptation rather than true genealogical relationships. This seems unlikely as the acidothermophile *Bacillus acidocaldarius* (Langworthy et al., 1976) has ester linked glycolipids. Detailed characterization of the lipids of a mesophilic methanogen will be necessary to fully resolve this issue. If, as expected, the mesophilic methanogen is also found to have ether linked lipids, environmental adaptation must still be considered as a factor in early archaebacterial evolution. Certainly the fact that so many archaebacterial strains are found in extreme environments suggests that the lipids may have originally evolved in such environments.

The realization that there may be a close evolutionary link between the halophiles and the methanogens is at first quite startling and difficult to explain. Certain observations concerning squalene and its derivatives may shed some light on these matters. In the case of the halophiles, only S, S-2 and S-4 are found (Tornabene et al., 1969). When these organisms are grown under varying oxygen tensions the proportion of

these materials is seen to shift dramatically (Tornabene, 1978). In particular, high oxygen tensions give high S and low S-4 whereas low oxygen levels give low S and high S-4. Even under extremely low oxygen tensions S, S-2 and S-4 are the only squalenes observed. *M. thermoautotrophicum*, an anaerobe, exhibited a wider range of derivatives extending from S through S-12 (squalene).

The role of squalenes in *M. thermoautotrophicum* is not known. In view of the above observations it may be speculated that one of the roles of squalene is to act as a hydrogen sink, accepting and donating hydrogens in a reversible manner. This may allow the methanogen to control its internal reduction potential (eH) and any hydrogen transfer processes occurring in the membrane, by interconverting the various squalene derivatives from S to S-12 and vice versa. In the halophile (*H. cutirubrum*) the apparent inability to convert squalene into the full range of hydrogenated squalene derivatives may be related to the fact that the growth of this organism is restricted to aerobic environments, although it may be able to survive under extremely low concentrations of oxygen (Tornabene, 1978). Evolutionarily, the formation of squalene might represent a protective mechanism against an increasingly aerobic environment. The onset of aerobic conditions may thus have provided in isoprenoid producing organisms the driving force for the eventual appearance of sterols and other oxygen initiated cyclic isoprenoid derivatives.

K. Bloch (1976) emphasized the importance of this evolutionary transition when he pointed out that "oxygen is obligatory for the biological synthesis of the sterol structure at least as we know it today," and that "any evolution of the sterol pathway (under anoxidic conditions) must have ended with squalene." Although the occurrence of squalene in anaerobic bacteria had not yet been reported, he indicated that it had not been looked for systematically, implying that it may be eventually found. Indeed the present work is a confirmation of his suspicion and to our knowledge is the first identification of squalene in a strict anaerobic organism.

The observations presented here have also major implications to biogechemical evolution. For instance, the presence of phytane and related polyisoprenoid compounds in ancient sediments or petroleum can no longer be considered unequivocally as indicative of past photosynthetic activity (Oro et al., 1965; Didyk et al., 1978), since such compounds could have been derived from methanogens, halobacteria (Anderson et al., 1977) and related non-photosynthetic organisms.

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