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Ether-Containing Lipids of Methanogenic Bacteria

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Summary: Acid-hydrolysis of the phospholipid fraction of Methanobacterium thermoautotrophicum, Methanobacterium formicicum and Methanospirillum hungatii demonstrated the presence of two neutral lipid products. Characterization of these lipids resulted in their identification as dialkyl glyceryl ether and diglycerol tetraethers. The ether-linked alkyl chains were identified as the 20- and 40-carbon branched chains for the diether and tetraether, respectively. M. thermoautotrophicum and M. formicicum were also characterized by the presence of acid-stable phospholipid components.

Interest in methanogenic bacteria, microorganisms capable of producing methane from CO_2 and H_2, has recently increased due to their economic potential for the production of methane and their evolutionary significance. Fox et al. (1) have recently shown that methanogens constitute a distinct phylogenetic group distantly related to other bacteria on the basis of 16S ribosomal RNA. This report describes ether-containing polar lipids in three species of methanogenic bacteria.

MATERIALS AND METHODS

M. thermoautotrophicum (2), M. formicicum (3), and M. hungatii (4), were grown as previously described in 12- or 30-liter fermentors until late exponential growth phase and harvested by continuous flow centrifugation in a N_2 atmosphere.

Extraction of Bacteria: Extraction of M. thermoautotrophicum with $CHCl_3$: CH_3OH (2:1, v/v) resulted in low levels of lipid phorphorus (3-5 μmoles/gm dry weight of cells) recovered. The amount of lipid phosphorus extracted (13-17 μmoles/gm) was significantly greater when cells were extracted according to the method of Bligh and Dyer (5). Further increases in the amount of lipid phosphorus (28-32 μmoles/gm) extracted could be achieved by lysis of the cells at 10,000 psi followed by the Bligh and Dyer extraction procedure.

Thin-layer chromatography (TLC): Thin layer plates prepared with silica gel G (0.4 mm thick) were activated at 100° and used within 30 min. Development of TLC plates was in the following solvent systems unless otherwise specified: (i) solvent A, petroleum ether-diethyl ether-glacial acetic acid (60:40:1, v/v) for separation of glycerol ethers; (ii) solvent B, petroleum ether-diethyl ether-glacial acetic acid (70:30:1, v/v) for separation of acetate derivatives

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of glycerol ethers or alcohols; (iii) solvent C, chloroform-methanol-5N NH_4OH (65:30:5, v/v) for separation of phospholipids; and (iv) solvent D, heptane for separation of alkyl monoiodides and alkyl diiodides on silica gel H plates (0.25 mm). Lipids were detected by either Rhodamine 6G (0.005%, wt/v) and examination by ultraviolet light or by charring with 50% H_2SO_4 at 180°. For preparative purposes, neutral lipids were recovered from TLC plates by extraction of the silica gel with diethyl ether.

Degradative procedures: Hydrolysis was performed in Teflon-lined screw-cap tubes. Acid hydrolysis was carried out at 120° in 4 N HCl for 18-24 h. The lipid products were extracted three times with $CHCl_3$ and analyzed by TLC. Glycerol ethers were hydrolyzed in 55% hydriodic acid at 120° for 18 h to release alkyl iodides and recovered according to Kates et al. (6). The alkyl iodides were either converted to alkyl acetates followed by conversion to the corresponding alcohols (6) or reduced to the corresponding alkanes (7).

Analytical methods: Partial acetylation of intact glycerol ethers was done according to Langworthy (8). Lipid phosphorus was measured by the method of Bartlett as quoted in (9). Infrared spectra were obtained with a Beckman infrared spectrometer, model 33 with samples spread as films between NaCl discs. Vicinal glycols were determined by the periodate-Schiff spray reagent (10). Estimation of the relative concentration of glycerol ethers was obtained by scanning the TLC plates with an Ortec densitometer, model 4310, and the areas determined by triangulation.

Gas-Liquid Chromatography (GLC): GLC analysis were carried out on a Varian aerograph model #1200 equipped with a flame ionization detector. A 3 mm by 1.52 m long stainless steel column packed with 3% OV-17 on 80-100 mesh Gas Chrom Q (Applied Science Laboratories) was used for the separation of alkanes. Nitrogen flow rate was 25 cm^3/min. Column temperatures were 150° and 275° for alkanes derived from diethers and tetraethers, respectively. Alkanes from diethers were also analyzed on 10% Apiezon L under conditions previously described (11) except that the column temperature was 197° and the nitrogen flow rate was 85 cm^3/min.

RESULTS

The phospholipids of the three organisms after Bligh and Dyer extraction of whole cells were obtained by elution of a silicic acid column (2 x 10 cm) with methanol after removal of neutral lipids with $CHCl_3$ and glycolipids with acetone. Alkaline or acid hydrolysis of the phospholipid fraction resulted in trace amounts of fatty acids (less than 0.05 μmole/μmole lipid phosphorus). The fatty acids consisted mainly of 16- and 18-carbon fatty acids, with small amounts of branched chain fatty acids. TLC analysis of the lipid products derived by acid hydrolysis in solvent system A showed two major neutral lipids (A and B) and a phosphate-positive lipid (C) (Fig. 1). Table 1 shows the relative percent composition of each of the neutral lipid products. In all three organisms, Lipid B was the major component. Unless otherwise stated, the results found were the same for the three organisms.

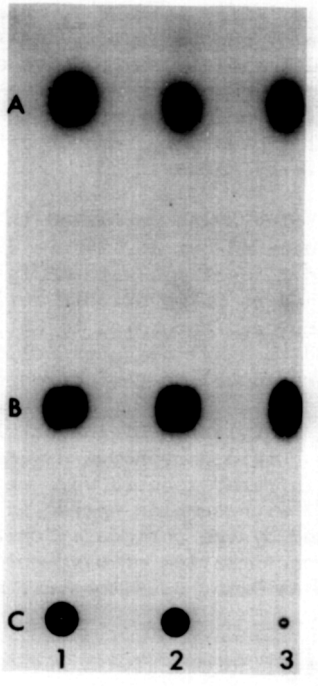

Figure 1: Thin-layer chromatograph of the lipid products derived by acid hydrolysis of the phospholipid fraction from M. thermoautotrophicum (1), M. formicicum (2), and M. hungatii (3). Lipids were visualized by charring with 50% H_2SO_4 at 180°. See text for identification of components.

Table 1. Percent composition of the neutral lipid products obtained from acid-hydrolysis of the phospholipid fraction.

Lipid	M. thermoautotrophicum	M. formicicum	M. hungatii
A(Diether)	26	11	14
B(Tetraether)	74	89	86

Lipid A: Lipid A was purified by TLC in solvent system A. The infrared spectrum of this lipid demonstrated hydroxyl absorption at 3450 cm^{-1}, alkyl bonds at 2950-2869, 1460 and 1375 cm^{-1}, ether (C-O-C) at 1105 cm^{-1} and primary hydroxyl at 1045 cm^{-1} (Fig. 2A). Partial acetylation of lipid A (R_f 0.40) followed by

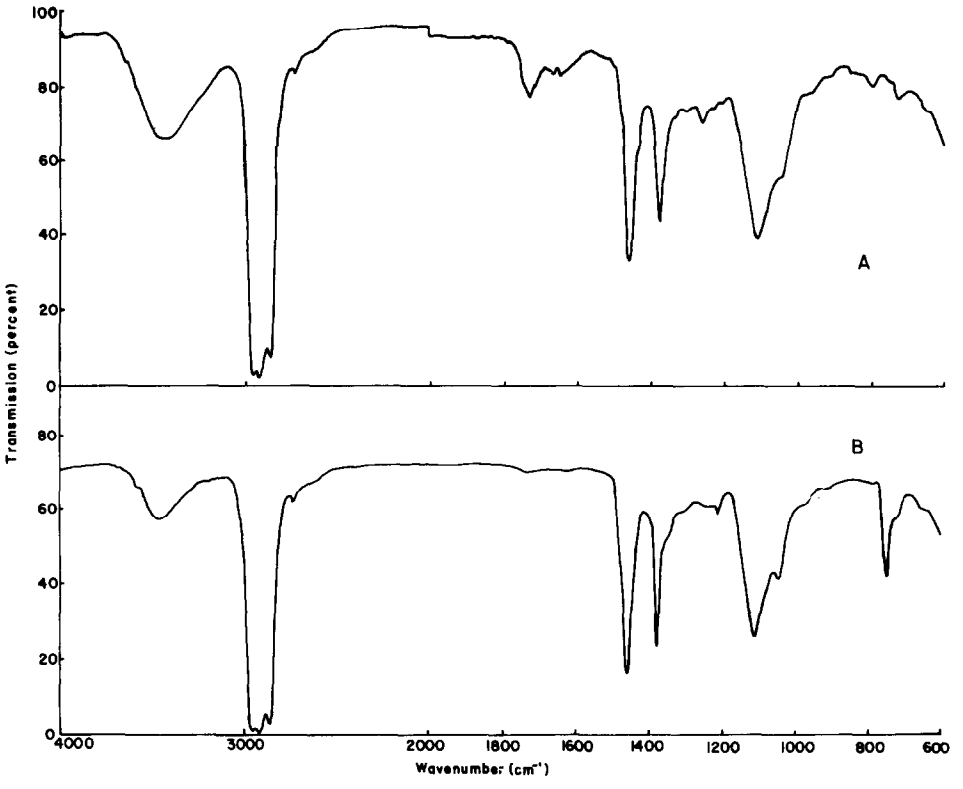

Figure 2: Infrared spectra of Lipid A (A) and Lipid B (B).

TLC analysis in solvent system B resulted in one acetylation product (R_f 0.73) indicating the presence of one free hydroxyl group.

Analysis by TLC of the alkyl iodides released by hydriodic acid hydrolysis demonstrated one component which co-chromatographed with standard hexadecyl iodide (R_f 0.58) in solvent system D (7). GLC analysis, after conversion of the alkyl iodide to the corresponding alkane, demonstrated one component which had an equivalent chain length of 17.7 and 17.6 on 10% Apiezon L and 3% OV-17 columns, respectively. The alkane had a retention time identical to 3,7,11,15-tetramethylhexadecane (phytane; Analabs, Inc.) Lipid A was tentatively identified as a diether with 3,7,11,15-tetramethylhexadecyl groups, similar to that found for the diether of Halobacterium cutirubrum (6).

Lipid B: The infrared spectra of purified lipid B was essentially identical

to that found for lipid A (Fig. 2B). Lipid B was tentatively identified as a diglycerol tetraether - two glycerols joined by two ether-linked alkyl chains.

Lipid B co-chromatographed with the tetraether isolated from Thermoplasma acidophilum (8) in solvent system A and/in chloroform-diethyl ether (9:1, v/v). Partial acetylation of lipid B (R_f 0.14) followed by TLC analysis in solvent system B resulted in two products (R_f 0.27and 0.54) indicating the presence of two hydroxyl groups which were not vicinal since the unacetylated lipid was periodate-Schiff negative.

Since the alkyl chains would be linked at each end by an ether bond, further evidence for a tetraether structure was derived by analysis of the alkyl chains. The alkyl iodides derived by hydriodic acid hydrolysis followed by TLC analysis in solvent system D demonstrated a single component (R_f 0.51), identical to the alkyl diiodide derived from T. acidophilum tetraether. The alkyl diiodide was converted to the alkyl diacetate followed by alkaline hydrolysis resulting in the corresponding dialcohol. Partial acetylation of this dialcohol (R_f 0.13) followed by TLC analysis in solvent system B resulted in two products (R_f 0.25 and 0.62), indicating the presence of 2 hydroxyl groups.

GLC analysis, after conversion of the alkyl diiodide to the alkane, demonstrated one component which had an equivalent chain length of 36.6 on a 3% OV-17 column. The alkane had a retention time identical to the major alkane species derived from T. acidophilum tetraether (8) which is a 40-carbon isopranoid alkyl chain. The acyclic ($C_{40}H_{82}$) or monocyclic ($C_{40}H_{80}$) identity of the alkyl chain remains unresolved.

Lipid C: Phosphate-positive lipids were found at the origin in solvent system A after acid hydrolysis of the phospholipid fraction from M. thermoautotrophicum and M. formicicum while none was observed from M. hungatii. To determine the percentage of the crude phospholipid fraction which was acid-stable, a sample from each of the three organisms was hydrolyzed with 4 N HCl at 125° for 96 h. The samples were dried in vacuo and the amount of orthophosphate released by acid hydrolysis determined. 1-aminoethylphosphonic acid, used as a control,

did not result in the release of orthophosphate. While 20% and 23% of the phospholipid phosphorus from M. thermoautotrophicum and M. formicicum, respectively, was acid-stable, no acid-stable phosphorus was found in M. hungatii. Essentially all the acid-stable phosphorus was found in the organic fraction after $CHCl_3$ extraction of duplicate samples. TLC analysis in solvent system C demonstrated the presence of at least 3 acid-stable phospholipids. These data suggests that part of the phospholipid fraction from M. thermoautotrophicum and M. formicicum occurred as a phosphonic acid rather than a phosphate ester.

Hydriodic acid hydrolysis of this acid-stable phospholipid fraction followed by TLC analysis in solvent system D of the resulting alkyl iodides demonstrated the presence of both alkyl monoiodides (30%) and alkyl diiodides (70%) in M. thermoautotrophicum and M. formicicum. This indicates that 70% of the acid-stable phospholipid fraction consisted of the tetraether form while 30% was the diether form.

DISCUSSION

Dialkyl glyceryl ethers have been identified in extreme halophilic bacteria (12) while tetraether-containing lipids have been found in T. acidophilum and Sulfolobus acidocaldarius (8), thermophiles capable of growth at a pH of 2. It has been suggested that these lipids afford structural stability to the bacterial membranes in the presence of extreme environmental conditions. The methanogenic bacteria contain both diether- and tetraether-containing lipids. M. thermoautotrophicum has an optimal growth temperature of 67° but requires a neutral pH (7.2-7.4) for growth as do the mesophiles, M. formicicum and M. hungatii. It may be that these lipids were necessary for survival when these organisms grew in an extremely reduced atmosphere (1).

M. thermoautotrophicum and M. formicicum, gram-positive organisms, were also characterized by the presence of acid-stable phospholipids while in M. hungatii, a gram-negative organism, these phospholipids were absent. Further evidence for a distinction between M. hungatii and the other two organisms was found by Fox et al. (1) on the basis of the average linkage clustering of 16S

ribosomal RNA. Further work is in progress to elucidate the structure of each of the individual phospholipids.

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