THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 265, No. 23, Issue of August 15, pp. 13735-13740, 1990
Printed in U.S.A.

Novel, Acid-labile, Hydroxydiether Lipid Cores in Methanogenic Bacteria*

(Received for publication, January 30, 1990)

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Polar ether lipids extracted from 15 methanogenic bacteria, representative of seven genera, were screened by nuclear magnetic resonance and thin layer chromatography for the presence of hydroxyl groups on the C_{20}-phytanyl moieties. Major amounts of hydroxydiether core lipid were confirmed for *Methanosaeta concilii* and discovered in two *Methanosarcina* species, *Methanococcus voltae,* and tentatively in several *Methanobacterium* species. Signals at 1.24 and 1.8–1.9 ppm in ^1H NMR spectra are characteristic of *Methanosaeta concilii* lipids hydroxylated on carbon-3 (*sn*-3 chain). Related signals, which were shifted slightly, appeared in spectra of the polar lipids extracted from both *Methanosarcina* species. Following mild hydrolysis to remove the polar head groups, only two chromatographically distinct core lipids were found in significant amounts in *Methanosarcina barkeri* (and *Methanosarcina mazei*) consisting of 43% 2,3-di-*O*-phytanyl-*sn*-glycerol ($C_{20,20}$-diether) and 57% $C_{20,20}$-hydroxydiether. This latter core lipid differed from the hydroxydiether from *M. concilii* by hydroxylation, on carbon-3, of the phytanyl chain in ether linkage to the *sn*-2 carbon of glycerol. The structural assignment was based on identification of the novel hydroxydiether core and its methylation products by ^1H NMR, ^{13}C NMR, and mass spectroscopy. The hydroxy core lipid degraded to various products during standard methanolic HCl and sulfuric acid procedures, including a methoxy derivative (methanolic HCl) and the 3-mono-*O*-phytanyl-*sn*-glycerol.

Core lipids of methanogenic bacteria were thought initially to consist of only diphytanylglycerol diether (Fig. 1) and dibiphytanyldiglycerol tetraether (1, 2). The diether structure was the 2,3-di-*O*-phytanyl-*sn*-glycerol found originally in *Halobacterium cutirubrum* (3). A macrocyclic glycerol diether was the first variation in a methanogen to this core structure, found in the deep sea thermophile *Methanococcus jannaschii* (4).

Methanosarcina barkeri has been reported to be unusual among the methanogens with respect to the large variety of core lipids which it contains (5, 6). These include the $C_{20,20}$-glycerol diether, $C_{20,20}$-tetritol diether, $C_{20,25}$-glycerol diether, C_{20}-glycerol monoether, and dibiphytanyldiglycerol tetraethers containing 1–3 cyclopentane rings.

Recently we identified, in addition to 2,3-di-*O*-phytanyl-*sn*-glycerol, an unusual core lipid synthesized by *Methanosaeta concilii* (previously *Methanothrix concilii* (7)). This lipid was a $C_{20,20}$-glycerol diether modified by the presence of a hydroxyl group on carbon-3 of specifically the phytanyl chain in ether linkage to the *sn*-3 carbon of glycerol (8). This modification to approximately one-third of the core lipid resulted in an acid lability that could, during acidic hydrolysis to remove the lipid head groups, produce a series of degradation products (8). Two of these products were identified as 2-mono-*O*-phytanyl-*sn*-glycerol and the methylated derivative of hydroxydiether.

Here we report that lipid extracts of *M. barkeri* and *M. mazei* contain signals diagnostic of hydroxydiether lipids. The hydroxydiether lipids in the *Methanosarcina* species differ from those found in *M. concilii* (8) according to the phytanyl chain that contains the hydroxyl group. Further, in unmodified lipid extracts or following mild acid hydrolysis, only two lipid cores are detected in appreciable amounts.

EXPERIMENTAL PROCEDURES

Materials—Silica Gel 60 plates (0.25 mm) were purchased from BDH Chemicals Canada Ltd. (Toronto), and silica Gel G-25 plates (0.25 mm) were from Brinkmann Instruments. J. T. Baker Chemical Co. supplied methyl iodide and silver oxide. No-screen x-ray film was from Kodak. Petroleum ether (b.p. 35–60 °C) and other solvents were purchased from Anachemia (Montreal), and [2-^{14}C]mevalonate dibenzoylethylenediamine salt (50.1 mCi/mmol) was from Du Pont-New England Nuclear. $^{13}CH_3OH$ (99.2 atom %) was a product of MSD Isotopes (Montreal). Core lipid standards were prepared from *Methanospirillum hungatei* and *M. concilii,* as described (2, 8).

Methanogens and Growth—The organisms used in this study and their origins are detailed in Table I. Growth was conducted in closed glass vessels containing media reduced by cysteine-sodium sulfide. Defined medium JM (9), with growth substrates as indicated in Table I, was used for most methanogens. Defined media for *Methanococcus voltae* and *M. jannaschii* were described previously (10, 11). *Methanothermus fervidus* was grown in Balch medium-1 (12), modified to omit acetate, yeast extract, and trypticase and to include (μM) $NiCl_2 \cdot 6H_2O$, 5; $NaSeO_4$, 5; $Na_2MoO_4 \cdot 2H_2O$, 8; and $NaWO_4 \cdot 2H_2O$, 5. *M. mazei* was grown in nondefined Balch medium-3 (12) containing 3% (v/v) methanol, under an N_2 atmosphere. The phytanyl chains of ether lipids of *M. barkeri* were labeled during growth to the early stationary phase by including 10 μCi of [2-^{14}C]mevalonate salt (50.1 mCi/mmol) in 100 ml of medium prior to inoculation.

Lipid Extraction—Cell pastes were frozen at −20 °C and thawed prior to extraction by a neutral Bligh and Dyer procedure (13). *M. barkeri* (20 g, dry weight) was mixed overnight at 23 °C in $CH_3OH/CHCl_3/H_2O$ (250 ml:125 ml:100 ml). Cell debris was removed by centrifugation and similarly extracted twice more. Total lipid was recovered from the combined extracts by partitioning into $CHCl_3$ (13). Cell debris was sometimes reextracted by an acidic Bligh and Dyer modification using trichloroacetic acid (14).

Polar lipids were prepared by acetone precipitation from total lipid extracts (11). Neutral and polar fractions were quantitated by weighing.

Hydrolytic Conditions—Polar lipids were hydrolyzed unless stated otherwise with either 0.18% methanolic HCl (50 °C, 24 h), 2.5% methanolic HCl (70 °C, 2 h), or 48% hydrofluoric acid (0 °C, 16 h).

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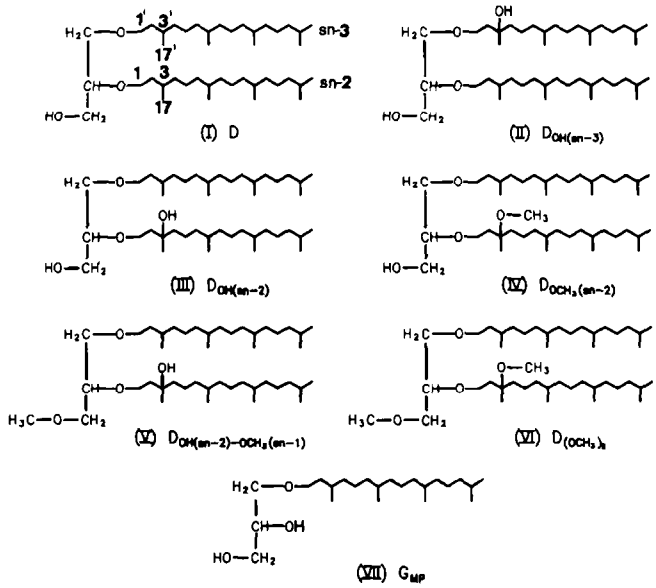

FIG. 1. **Structures of $C_{20,20}$-diether lipids and their methylated derivatives.** *D*, 2,3-di-*O*-phytanyl-*sn*-glycerol; $D_{OH(sn-3)}$ from *M. concilii*, 2-*O*-[3,7,11,15-tetramethyl]hexadecyl-3-*O*-[3′-hydroxy-3′,7′,11′,15′-tetramethyl]hexadecyl-*sn*-glycerol; $D_{OH(sn-2)}$ from *M. barkeri* and *M. mazei*, 2-*O*-[3-hydroxy-3,7,11,15-tetramethyl]hexadecyl-3-*O*-[3′,7′,11′,15′-tetramethyl]hexadecyl-*sn*-glycerol; *IV–VI*, methylated diether derivatives of III; *VII*, 3-mono-*O*-phytanyl-*sn*-glycerol (G_{MP}).

Core lipids were recovered from the hydrolysates by partitioning into petroleum ether or removal of hydrofluoric acid by a nitrogen stream (15). Acid lability of purified core lipid III (Fig. 1) was tested using the sulfuric acid procedure described by Ross *et al* (16).

Purification of Core Lipids—Core lipids of *M. barkeri* were purified by TLC (8) from polar lipids hydrolyzed for 24 or 48 h at 50 °C in 0.18% methanolic HCl. Silica Gel 60 plates were developed using petroleum ether/diethyl ether/acetic acid (50:50:1, v/v).

Acetylation—Core lipids (0.5–1 mg) were acetylated in 2 ml of acetic anhydride and 0.5 ml of pyridine at 100 °C for 2 h.

Methylation—Lipids III (4.1 mg) and IV (2.5 mg) were methylated by refluxing with 5 ml of methyl iodide plus 150 mg of silver oxide (8). Reaction products were purified using the TLC system employed for core lipid separations.

Detection on TLC Plates—Lipid spots were visualized for photography by acid charring (13). ^{14}C-Labeled lipids were detected by autoradiography and quantitated by liquid scintillation counting of the adsorbent removed from the plate. Quenching was corrected by spiking with ^{14}C and recounting.

TLC of Polar Lipids—Polar lipids were separated on Silica Gel G plates using $CHCl_3/CH_3OH/H_3CCOOH/H_2O$ (85:22.5:10:4, v/v) (11).

Physical Measurements—Purified core lipids of *M. barkeri* were subjected to optical rotation measurements in $CHCl_3$ (11). Ammonium-chemical ionization-mass spectrometry was performed on unmodified or acetylated core lipids. Unmodified lipids (approximately 10–15 mg/ml) dissolved in deuterated benzene/methanol (7:2, v/v) or lipid cores in $CDCl_3$ were analyzed at room temperature using Bruker AM500, AM360, or MSL-300 spectrometers with conditions as described (8). ^1H NMR spectra of the lipid cores were determined also in deuterated dimethyl sulfoxide.

RESULTS

Screening for Hydroxylated Core Lipids—In ^{13}C and ^1H NMR spectra of the polar lipids of *M. concilii*, signals appeared which were diagnostic of a 3′-hydroxydiether (D_{OH}[1] $_{(sn-3)}$) (8). Similar signals appeared in spectra of the polar lipids of *M. barkeri* and *M. mazei*. For example, in ^1H spectra the sharp singlet, diagnostic of the C-17 methyl group of a hydroxyphytanyl chain (8), was downfield shifted from 1.24 ppm in extracts of *M. concilii* to 1.27 ppm in extracts from both *Methanosarcina* species. TLC of the core lipids obtained from *M. voltae* and several *M.* species (Table I) revealed a component with mobility identical to $D_{OH(sn-3)}$ found previously in *M. concilii* (8). An inability to detect hydroxydiether lipids in the latter species by NMR was attributed to the minor amounts present.[2]

Extraction and Distribution of Lipids in M. barkeri—Extraction of [^{14}C]mevalonate-labeled cells by a neutral Bligh and Dyer procedure resulted in a lipid recovery of 4% (by weight), with a specific activity of labeling of 186.8 μCi/g of lipid. Of the total lipid extracted, 82% was comprised of acetone-insoluble polar lipids. Reextraction of the cell debris using an acidic Bligh and Dyer procedure resulted in a further recovery of 0.9% of the cell dry weight as acetone and $CHCl_3$-soluble material behaving on a polar TLC system as primarily neutral components ($R_F > 0.9$). Because the specific activity of labeling was low in the acidic Bligh and Dyer extract (0.92 μCi/g), subsequent studies employed only the neutral procedure for recovery of total polar lipids.

Structural Studies on Intact Lipids—^{13}C NMR spectra of the total polar lipids from *M. barkeri* and *M. mazei* were compared with a spectrum of a lipid from *M. concilii* having the *sn*-3 hydroxylated core (Fig. 2). Clearly, both *Methanosarcina* species contain a significant amount of the same core lipid. As found for the lipids in *M. concilii* (8), signals atypical of the phytanyl chains of standard diether were assigned to carbons 1–5 with C-3 being most shifted, therefore supporting the presence of a 3-hydroxylated chain. Because differences in the ^{13}C NMR spectra of the chain region occurred between *Methanosaeta* and both *Methanosarcina* species (see carbon-2, Fig. 2) it was suspected that the 3-hydroxy group occurred on opposite phytanyl chains. Further detailed evidence is given in the following sections.

^{13}C NMR spectra of total polar lipids (Fig. 2) gave doublets for signals of the carbons close to the 3-hydroxyl. In the spectrum for *M. mazei*, doublets already occur following growth in a $^{13}C/^{12}C$ mixture of methanol. For *panel A*, however, the doublets indicate the presence of two closely related structures, the difference between them being either in the core structure or being caused by interactions with heterogeneous head groups. To clarify this point, the core lipids of *M. barkeri* were purified and subjected to structural analysis.

Head groups were removed from the total polar lipids to characterize the core lipids initially by TLC (Fig. 3). Strong methanolic HCl hydrolysis produced at least six lipid components (*lane 2*), one corresponding by R_F value to diether present as a reference core lipid in *M. hungatei* (2) and *M. concilii* (8). In contrast, mild methanolic HCl for 24 h released two principal lipids (*lane 3*), each compared closely with one of the two reference core lipids from *M. concilii* (*lane 6*). The lower R_F value of spot III compares with that of spot II (*lane 6*) and is consistent with the presence of a hydroxyl group on a phytanyl chain (8). Parallel results were found for the core lipids of *M. mazei* (not illustrated). Continued hydrolysis for an additional 24 h resulted in increased amounts of spots IV and VII (*lane 5*). Fast moving spots near the solvent front were observed for *lanes 2* and *5*. Components I, III, IV, and VII were purified.

Core lipid I (Fig. 3) had a molecular rotation of +51 degrees, in keeping with the *sn*-2,3 configuration (2, 3, 8). The molecular ion of acetylated I was 712, or 652 less the acetyl group and ammonia, as expected for diether (structure I). Finally,

[1] The abbreviations used are: D_{OH}, hydroxydiether; D_{OCH_3}, monomethylated product of hydroxydiether.

[2] Detection of 3-hydroxydiether lipids by ^1H NMR would require an estimated abundance of at least 10% of the core lipids, the limiting factor being the amount of various overlapping signals present in the lipid sample. ^{13}C NMR is less sensitive and less rapid.

TABLE I

Screening for 3-hydroxydiethers in polar lipid extracts from methanogenic bacteria

The acetone-insoluble, polar lipids extracted from various methanogens were tested for hydroxydiethers using NMR and TLC methods. Detection of hydroxylated chains by NMR was based on characteristic signals from the C-17 methyl and C-2 methylene groups (^1H spectra) or C-2 to C-5 and C-17 (^{13}C spectra). For detection by TLC, the polar lipids were hydrolyzed with HF to generate core lipids that were then separated on thin-layer plates. Standards were core lipids from *M. concilii* and *M. hungatei*. The inability to detect hydroxy lipid is noted by –; ND, not done.

Methanogen	Source[a]	Growth substrate	Growth temperature	NMR		TLC
				^1H	^{13}C	
			°C			
M. concilii	NRC 2989	Acetate	35	+	+	+
M. barkeri MS	DSM 800	CO_2/H_2	35	+	+	+
M. mazei S6	DSM 2053	MeOH	35	+	+	+
M. voltae	DSM 1537	CO_2/H_2	35	ND	ND	+
Methanobacterium G2R	NRC 2239	CO_2/H_2	35	–	–	+[b]
Methanobacterium bryantii MoHG	DSM 862	CO_2/H_2	35	–	–	+[b]
Methanobacterium bryantii MoH	DSM 863	CO_2/H_2	35	–	–	+[b]
Methanobrevibacter arboriphilus	DSM 1125	CO_2/H_2	35	–	ND	–
Methanobrevibacter smithii	DSM 861	CO_2/H_2	35	–	–	–
M. hungatei GP1	NRC 2214	CO_2/H_2/acetate	35	–	ND	–
M. jannaschii JAL-1	DSM 2661	CO_2/H_2	65	–	–	–
M. fervidus	DSM 2088	CO_2/H_2	70	ND	–	–
Methanobacterium formicicum	DSM 1535	CO_2/H_2	35	–	–	–
Methanobacterium thermoautotrophicum GC1	NRC 2878	CO_2/H_2	62	–	ND	–
*Methanobacterium thermoautotrophicum*ΔH	DSM 1053	CO_2/H_2	62	–	ND	–

[a] NRC, National Research Council of Canada, Ottawa; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen.

[b] Present in trace amount.

the ^{13}C NMR spectrum (Fig. 4*B*) was virtually identical to previous spectra of diether from other methanogens (8, 17, 18).

The ^{13}C NMR spectrum of the new core lipid III is compared with the diether and with the 3′-hydroxydiether ($D_{OH(sn\text{-}3)}$) from *M. concilii* (Fig. 4). Clearly, lipid III is closely related but subtly different from $D_{OH(sn\text{-}3)}$. Lipid III (compared with diether) exhibits all the characteristics for hydroxylation on C-3 of a phytanyl chain, the most diagnostic being large shifts of C-3 (22.9 ppm to 72.8 ppm) and C-17 (19.7 ppm to 26.6 ppm), and smaller shifts of C-1, C-2, C-4, and C-5 as illustrated in Fig. 4. The distortionless enhancement by polarization transfer spectrum, revealing that a C-3 is a quaternary carbon, is shown later. As predicted for a hydroxydiether (8), the molecular weight of III was 668 (686 less ammonia) using dibiphytanyldiglycerol tetraether (1318 less ammonia) as a reference. The molecular rotation of +35.1 degrees compared closely with +39.8 for $D_{OH(sn\text{-}3)}$ (8), supporting an *sn*-2,3 configuration. Furthermore, signals in the 60–80-ppm region of the ^{13}C spectrum provide evidence for two phytanyl chains linked to glycerol.

The C-1 and C-2 resonances of lipid III, compared with standard diether and $D_{OH(sn\text{-}3)}$, indicate hydroxylation of the *sn*-2 phytanyl chain. Hydroxylation of C-3 of the *sn*-3 phytanyl chain results in a characteristic shift of signals for C-1 and C-2 close to the hydroxyl group. In the case of lipid III, signals for these carbons of the *sn*-3 alkyl chain remain similar to standard diether, whereas the signals for C-1 and C-2 of the opposite (*sn*-2) chain shift, as illustrated (Fig. 4).

An ^1H NMR spectrum of III fully supports the proposed structure (see Fig. 1). A strong methyl signal is present at 1.25 ppm (C-17 methyl), and C-2 methylene signals are shifted downfield to 1.7 and 1.95 ppm compared with standard diether, as shown before for $D_{OH(sn\text{-}3)}$ (8). Similarly, the ^1H NMR spectrum of III in dimethyl sulfoxide revealed two hydroxyl groups in the molecule, one belonging to a CH_2OH group in the glycerol moiety (4.44 ppm) and a second giving a very characteristic singlet (3.92 ppm) belonging to an OH group on a quaternary carbon (8).

Structures of Selected Hydrolysis Products—The structures of hydrolysis products IV and VII were determined in order to obtain further support for the structure of III and to evaluate data conflicting with those presented here on the core lipids of *M. barkeri* (5, 6).

Core lipid VII gave a ^{13}C NMR spectrum with only four signals between 60 and 80 ppm, namely, at 70.20 (C-1 of a phytanyl chain), at 72.61 (-CH_2-O-R of glycerol), at 70.41 ppm (-CH-OH of glycerol), and at 64.36 (-CH_2OH of glycerol). Signal multiplicities obtained by distortionless enhancement by polarization transfer supported these assignments. Thus, there is only one C-1 signal indicating one phytanyl chain, and the chemical shift value of the *sn*-2 carbon of glycerol indicates that the hydroxyl group on this carbon is free. Further support comes from the aliphatic region of the spectrum; in particular, the signal of the C-2 of the *sn*-2 chain is missing. From this evidence and its origin from III discussed below, it follows that the structure of VII is 3-mono-*O*-phytanyl-*sn*-glycerol.

The stability of the purified 3-hydroxydiether (III) to various acids and conditions is shown in Fig. 5. Strong methanolic HCl hydrolysis degraded the hydroxydiether to VII (minor) and IV (major), plus several spots with greater mobilities (*lane 3*). These latter spots may include the known methanolysis degradation products of phytyl derivatives such as methoxyphytol and phytene (19), but these components with higher mobility have yet to be characterized in detail. The sulfuric acid procedure used by Trincone *et al.* (6) and recommended by Ross *et al.* (16) resulted in a complete loss of the hydroxydiether and formation of primarily the monophytanyl ether (*lane 5*). In contrast, the hydroxydiether structure appeared unaffected by HF (*lane 4*).

The isomeric form of the monophytanylglycerol produced by strong acid methanolysis of the hydroxydiether is characteristic of the chain (*sn*-2 or *sn*-3) that contains the 3-OH group. Specifically, the hydroxydiether III (from *Methanosarcina* species) containing a 3-OH on the *sn*-2 chain yields 3-

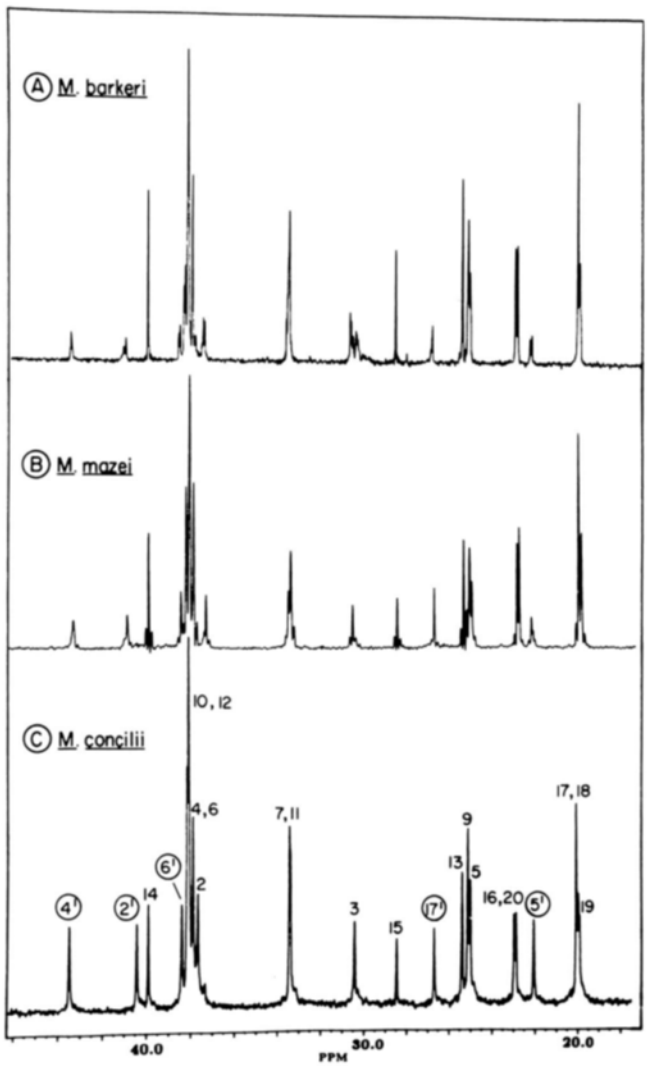

FIG. 2. **^{13}C NMR spectra of total polar lipids extracted from *M. barkeri* (*A*) and *M. mazei* (*B*).** Purified digalactosylhydroxydiether ($GalGalD_{OH}$) from *M. concilii* (22) is shown as a reference (*C*). Signal assignments (8) are shown as *carbon numbers primed* to indicate origin from the *sn*-3 chain. In *panel B, M. mazei* was grown on $^{13}CH_3OH/^{12}CH_3OH$ (1:3.5, v/v). Polar lipids for *A* and *B* were prepared by acetone precipitation from the total lipid extracted from frozen-thawed cells (see "Experimental Procedures"). Spectra were recorded using benzene-d_6/methanol-d_4 (7:2, v/v).

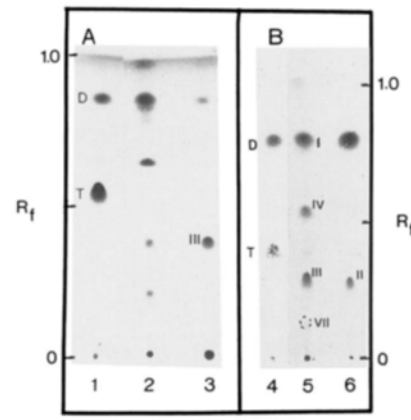

FIG. 3. **Separation by TLC of the lipids released from the polar lipids of *M. barkeri* by strong and mild acidic hydrolysis.** Strong hydrolysis was at 70 °C in 2.5% methanolic HCl; weak hydrolysis was at 50 °C in 0.18% methanolic HCl. *Panel A: lane 1,* strong hydrolysis of *M. hungatei* polar lipids for 2 h; *lane 2,* strong hydrolysis of *M. barkeri* polar lipids for 2 h; *lane 3,* mild hydrolysis of *M. barkeri* polar lipids for 24 h. *Panel B: lane 4,* strong hydrolysis of *M. hungatei* polar lipids for 2 h; *lane 5,* mild hydrolysis of *M. barkeri* polar lipids for 48 h; *lane 6,* mild hydrolysis of *M. concilii* polar lipids for 24 h. Lipid reference standards are indicated on the figure according to abbreviations shown in Fig. 1 (*D,* diether; *T,* tetraether). Plates were developed with petroleum ether/diethyl ether/acetic acid (50:50:1, v/v). *Spots* were located by acid charring.

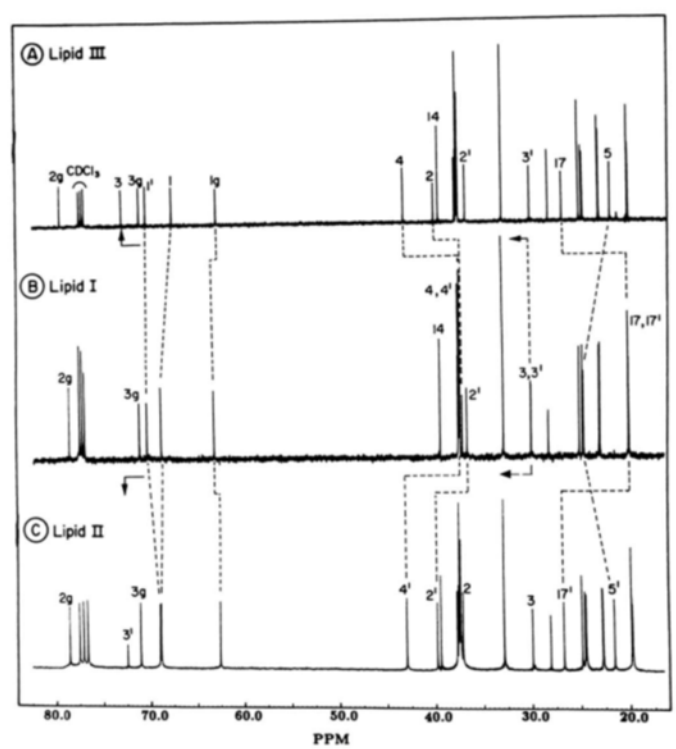

FIG. 4. **^{13}C NMR comparison between purified hydroxydiethers from *M. barkeri* and *M. concilii*, and diether.** Lipids were isolated from TLC plates following hydrolysis of total polar lipids in 0.18% methanolic HCl for 24 h at 50 °C (see Fig. 3). Purity of the core lipids was confirmed by TLC. Spectra were recorded using $CDCL_3$ as solvent. *Panel A,* $D_{OH(sn\text{-}2)}$ from *M. barkeri; panel B,* diether from *M. barkeri; panel C,* $D_{OH(sn\text{-}3)}$ from *M. concilii.* Signal shifts are illustrated; g, glycerol.

mono-*O*-phytanylglycerol (VII) whereas II (from *M. concilii*) with a 3′-OH on the *sn*-3 chain yielded the expected 2-mono-*O*-phytanyl-*sn*-glycerol (8).

Core lipid IV was formed upon methanolic HCl hydrolysis of either the total polar lipids (Fig. 3) or the purified 3-hydroxydiether (Fig. 5). Purified IV (from either source) has all the signals characteristic of the hydroxydiether although some are shifted (Fig. 6, *A* and *B*). The key feature is the additional signal of a methyl group with a chemical shift (48.79 ppm) characteristic of the methylated 3-hydroxy group (8). Signals of C-17 methyl, C-2, and C-4 are shifted upfield and of C-3 downfield (all on the *sn*-2 chain) compared with the spectrum of $D_{OH(sn\text{-}2)}$, very similar to observations made for the methylated hydroxydiether of *M. concilii* (8). The molecular rotation for IV was +48.8 degrees. Therefore, compound IV was identified as the monomethylated product of the hydroxydiether ($D_{OCH_3(sn\text{-}2)}$).

Hydrolysis of the polar lipids from *M. voltae* by strong methanolic HCl generated a major compound identical to lipid IV by TLC, thus supporting the presumptive evidence for a hydroxydiether in this methanogen.

Methylation Products of $D_{OH(sn\text{-}2)}$ and $D_{OCH_3(sn\text{-}2)}$—Methylation using CH_3I/Ag_2O of III indicated two methylation sites, the first to methylate yielding the new compound V rather

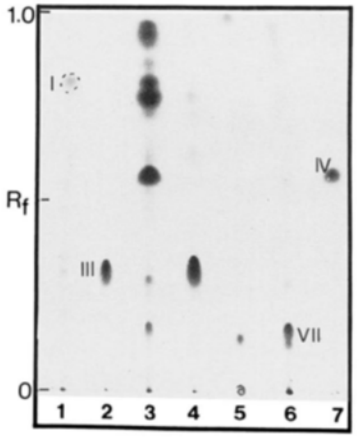

FIG. 5. **Acid lability of purified 3-hydroxydiether $D_{OH(sn\text{-}2)}$ from *M. barkeri*.** Lipid standards were prepared by preparative TLC of total polar lipids hydrolyzed in 0.18% methanolic HCl at 50 °C for 24 h. Plates were developed with petroleum ether/diethyl ether/acetic acid (50:50:1, v/v). *Lane 1,* diether; *lane 2,* hydroxydiether; *lane 3,* hydroxydiether treated for 4 h at 70 °C with 2.5% methanolic HCl; *lane 4,* hydroxydiether treated 16 h at 0 °C in 48% HF; *lane 5,* hydroxydiether treated 16 h at 60 °C with H_2SO_4; *lane 6,* VII (3-mono-*O*-phytanyl-*sn*-glycerol); *lane 7,* core lipid IV ($D_{OCH_3(sn\text{-}2)}$).

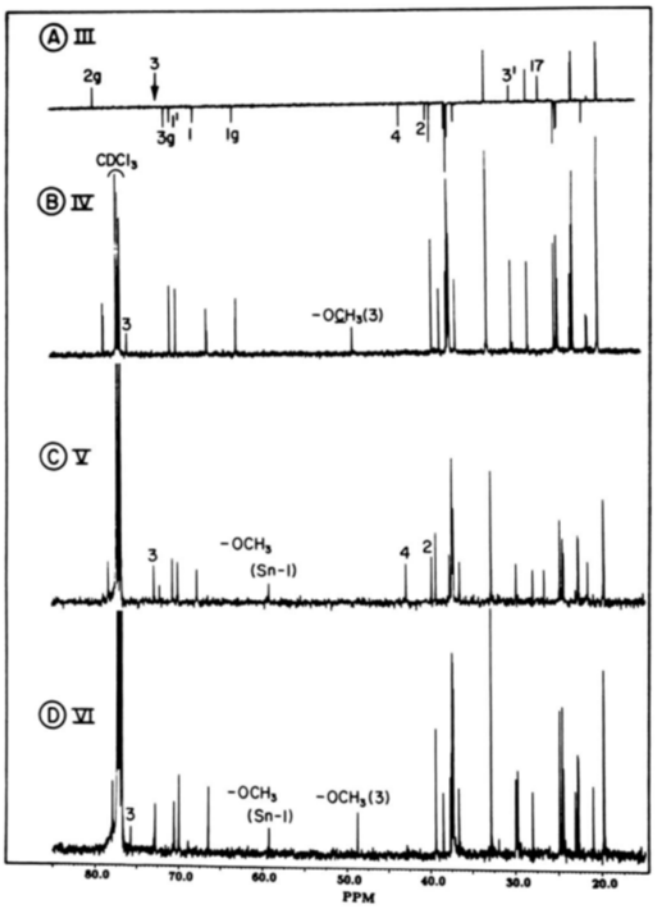

FIG. 6. **^{13}C NMR spectra of purified core lipids III and IV of *M. barkeri* and their methylated products.** Purified core lipids and methylation products were analyzed by TLC as in Fig. 7. *Panel A,* distortionless enhancement by polarization transfer spectrum and signal assignments for lipid III; *panel B,* lipid IV; *panel C,* 5-h methylated product of III; *panel D,* 29-h methylated product of IV.

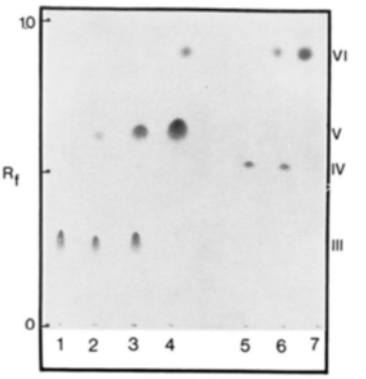

FIG. 7. **Thin-layer chromatogram illustrating methylation rates for core lipids $D_{OH(sn\text{-}2)}$ (*III*) and $D_{OCH_3(sn\text{-}2)}$ (*IV*) of *M. barkeri*.** Purified core lipids III and IV (see Fig. 1) were methylated by refluxing in CH_3I with Ag_2O as catalyst. Samples were removed for TLC analysis at various times. *Lane 1,* core lipid III; *lanes 2–4,* core lipid III methylated for 2, 5, and 29 h, respectively; *lane 5,* core lipid IV; *lanes 6* and *7,* core lipid IV methylated for 5 and 29 h, respectively.

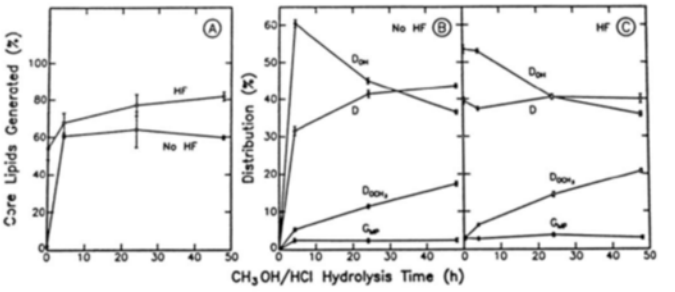

FIG. 8. **Generation and distribution of *M. barkeri* core lipids as a function of hydrolysis time in methanolic HCl.** Lipids were labeled during growth in medium containing [2-^{14}C]mevalonate. Core lipids were generated (*panel A*) by hydrolysis in 0.18% methanolic HCl at 50 °C for the times indicated either without (*panel B*) or with (*panel C*) prehydrolysis with HF (0 °C, 16 h). Samples were spotted on Silica Gel 60 plates and separated using petroleum ether/diethyl ether/acetic acid (50:50:1). *Spots* were located by autoradiography (5000 dpm/lane) and lipid cores removed for counting. G_{MP}, 3-mono-*O*-phytanyl-*sn*-glycerol.

than IV (Fig. 7). The second site methylated incompletely even after 29 h of refluxing (*lane 4*). Methylation of IV to product VI occurred readily. Analysis of the products by ^{13}C NMR (Fig. 6) revealed that V corresponded to methylation on the *sn*-1 carbon of glycerol and that VI was the dimethyl derivative (Fig. 1). These results are as predicted from the structures proposed for III and IV.

Hydrolytic Conditions—HF and/or weak methanolic HCl was evaluated as a means to generate unmodified core lipids from *M. barkeri* (Fig. 8). Following acid hydrolysis, the lipids were partitioned into ether for TLC. Spots located by autoradiography were counted, including the origin (unhydrolyzed polar lipid). HF alone released head groups from approximately 55% of the total polar lipid; methanolic HCl within 4 h was similarly effective. HF followed by methanolic HCl hydrolysis yielded up to 80% of the lipid cores. During methanolic HCl hydrolysis, the hydroxydiether declined as its monomethylated product D_{OCH_3} was formed. From these data the distribution of core lipids was estimated as 43% diether and 57% hydroxydiether.

DISCUSSION

A sampling of 15 methanogenic bacteria was screened by NMR and TLC methods for hydroxyl groups on the phytanyl

chains of polar ether lipids. Hydroxylation was confirmed for lipids extracted from *M. concilii* and extended to include *M. barkeri, M. mazei,* and tentatively several other methanogens. The formation of major amounts of hydroxydiether lipids is presently restricted to these two genera, which share a degree of phylogenetic (20) and metabolic (21) relatedness. Based on TLC evidence alone, a core lipid corresponding to a hydroxydiether may occur in *M. voltae,* with still lesser amounts in certain other methanogens.

There is a high degree of selectivity in the carbon position hydroxylated. The *Methanosaeta* and *Methanosarcina* species (that were tested) are distinguished by the position of the hydroxy chain, specifically, *sn*-3 in *Methanosaeta* and *sn*-2 in *Methanosarcina.* In both genera, hydroxylation was detected only on carbon-3 of the phytanyl chains. Hydroxylation of other methine carbons (C-7, C-11, or C-15) could be detected easily by the ^{13}C NMR methods used, but were absent. Further, biosynthesis of the 3-hydroxydiether was independent of widely differing growth conditions since the identical structure was found in *M. barkeri* grown on CO_2/H_2 in a defined medium and in *M. mazei* grown on methanol in complex medium. Evaluation of this promising taxonomic feature will require the detailed structural analysis of hydroxy lipids from other methanogens.

Hydroxylation of the diether is likely to confer differences in cytoplasmic membrane properties. The hydrophilic 3-hydroxyl group may either extend the polar head group region of the polar lipids to cause a local increase of surface area and effective shortening of the lipid tail or remain within the membrane to generate a hydrophilic pocket.

In organic solvents, the hydroxyl group of the phytanyl chain must be hydrated since ^{13}C NMR signals are broadened, and transverse relaxation times are short.[3] Most probably there is an interaction with paramagnetic ions, which are very difficult to remove.

Both this and our previous report (8) demonstrate clearly that the 3-hydroxydiether lipid structures are very acid labile and that the pattern of products of core lipids obtained depends on the conditions of hydrolysis. We propose the use of 48% HF (0 °C, 16 h) or 0.18% methanolic HCl (50 °C, 16 h) to minimize the formation of artifacts. Under these conditions only diether and hydroxydiether were detected in appreciable amounts in *M. barkeri, M. mazei,* and *M. concilii.* Both diether lipids could be detected by NMR in total polar lipid extracts never subjected to harsh conditions, supporting their designation as true metabolic products. The possible presence of traces of other core structures is difficult to exclude. In particular, the *sn*-3 monophytanylglycerol is found at about 3% of the lipid in HF extracts (Fig. 8) and is not detected as a product following HF hydrolysis of the hydroxydiether (Fig. 5), suggesting that it may be a minor metabolite.

No evidence was obtained for dibiphytanyldiglycerol tetraethers, tetritol diethers, or $C_{20,25}$-diethers that were reported in *M. barkeri* by others (5, 6). Although the possibility of strain variations is not excluded, the fact that the hydroxylated core degrades to a variety of products during strong methanolic HCl and methanolic H_2SO_4 procedures and that indeed *sn*-3 monophytanylglycerol (a product produced from the hydroxydiether) has been reported as a major lipid core (5) make it most probable that the previous studies were complicated by the formation of degradation products. We were able to detect only the diether and hydroxydiether core lipids in both species of *Methanosarcina* grown on different carbon sources.

The two hydroxyl groups of the 3-hydroxydiether core lipid methylate in CH_3I at different rates. The glycerol *sn*-1 hydroxyl methylates readily whereas methylation of the phytanyl C-3 hydroxyl is relatively slow and incomplete (Ref. 8 and Fig. 7), presumably because of steric hindrance. By contrast, methylation that occurs in methanolic HCl occurs exclusively at the phytanyl C-3 hydroxyl.

To avoid the description of artifacts in future studies on the core lipids of methanogens, the possibility of unstable hydroxydiether lipids must be documented carefully.

[3] I. Ekiel and G. D. Sprott, unpublished observations.

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