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Composition of Polar Lipids of *Methanobrevibacter arboriphilicus* and Structure Determination of the Signature Phosphoglycolipid of *Methanobacteriaceae*

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Total lipid was extracted effectively by the acidified Blight and Dyer solvent system from *Methanobrevibacter arboriphilicus* A2 cells. The lipid content was 5.8% of dry cell weight. Cell disruption was required for the maximum yield of lipid from the cells. Eighteen polar lipids were detected and their composition was measured. Phosphoglycolipids from several species of *Methanobacteriaceae* which had the similar mobilities on thin-layer chromatograms were suggested as the common lipid of the family. The phosphoglycolipid (PGL1, 30%) from *M. arboriphilicus* was identified as gentiobiosyl caldarchaetidylinositol, which was identical to PGL1 of *Methanobacterium thermautotrophicum*. This confirmed that the lipid could be designated as the signature lipid of the family. The structure of the other major polar lipids were also identified as follows: gentiobiosyl caldarchaeol (GL1a, 9.9%), gentiobiosyl archaeol (GL1b, 12.6%), caldarchaetidylinositol (PL2a, 10.6%) and archaetidylinositol (PL2b, 3.1%).

Recently novel polar lipids were reported in several methaogenic bacteria such as *Methanospirillum hungatei*,[1,2] *Methanobrevibacter arboriphilicus*,[3,4] *Methanobacterium thermautotrophicum*,[5~7] (The names *Methanobrevibacter arboriphilius* and *Methanobacterium thermoautotrophicum* have been corrected to *Methanobrevibacter arboriphilicus* and *Methanobacterium thermautotrophicum*, respectively, based on Latin grammar and spelling rules[8]) and *Methanococcus voltae*.[9] Although we have identified the structure of one of the major polar lipids (PNL2b) of *M. arboriphilicus* A2 as archaetidylserine[3,4] (see the nomenclature of archaebacterial lipids proposed by us[5]), all the other lipids in this bacterium are unknown. Because hydrocarbon chains of lipids in methanogens are simple [only two kinds, phytanyl (C_{20}) and biphytanediyl (C_{40}) are present], it is interesting to elucidate complete structures of most of the major lipids in one species of methanogen.

We have reported that the presence and absence of some particular polar lipids are related to the taxonomic position of methanogens at the family or genus level.[10] *M. thermautotrophicum* and *M. arboriphilicus* belong to different genera of the same family. A highly polar phosphoglycolipid designated as PGL1 in ref. 10 is commonly found as the most predominant lipid in the genera *Methanobacterium* and *Methanobrevibacter* (*Methanobacteriaceae*) but not in other families. It could, therefore, be considered that the lipid was the signature lipid of the family. However, PGL1 extracted from various organisms of the family has not been identified. The thin-layer chromatographic profiles of the two organisms resembled each other but a few differences were observed.[10] It is, hence, important to elucidate the complete structures of major polar lipids including PGL1 of *M. arboriphilicus* to clarify the differences and similarities in the composition. This will serve to clarify the availability of the polar lipid composition for methanogen systematics.

To detect a whole set of polar lipids of *M. arboriphilicus*, conditions of lipid extraction

have to be reinvestigated because the modified Bligh-Dyer extraction has been reported to greatly improve the yield of lipids from *M. thermautotrophicum*.[11] As a result, not only a greater quantity of lipids was extracted but also more kinds of lipids were found.

This report describes an effective method of lipid extraction, the polar lipid composition, and the structures of five major polar lipids of *M. arboriphilicus*.

MATERIALS AND METHODS

Extraction of lipids from M. arboriphilicus cells. *M. arboriphilicus* A2 (DSM 2462) was grown as described.[12] Lipids were extracted essentially by the method of Bligh and Dyer[13] from intact cells, frozen-and-thawed cells, or disrupted cells suspended either in water (neutral extraction) or in 5% trichloroacetic acid (TCA, acid extraction).[11] Cells were disrupted by two successive passages of a French pressure cell (American Instrument Company, U.S.A.) operated at 1400 kg/cm^2.

Thin-layer chromatography (TLC). TLC was done on a Silica Gel 60 plate (Merck) with the following solvents (composition in volume ratios): solvent A, chloroform–methanol–7 M aqueous ammonia (65 : 35 : 8); solvent B, chloroform–methanol–acetic acid–water (85 : 30 : 15 : 5); solvent C, light petroleum–diethyl ether–acetic acid (50 : 50 : 1). Solvents A and B were used for two-dimensional TLC in the first and second directions, respectively. Lipids were detected as described in our previous paper.[3] Water-soluble products of phospholipase C treatment were chromatographed on a cellulose TLC plate (Avicell SF plate, Asahi Kasei Co., Inc.) which was developed with solvent D, ethanol–1.4 M aqueous ammonia (3 : 2). Spots on a cellulose plate were visualized with salicylsulfonic acid/$FeCl_3$ reagent for phosphate esters[14].

Purification of lipids. Total lipids (400 ~ 500 mg) were fractionated on a column (2.5 × 40 cm) of DEAE-cellulose (Brown, acetate form), using the following elution sequence: I, chloroform (1600 ml); II, chloroform–methanol (9 : 1, 2000 ml); III, chloroform–methanol (7 : 3, 1000 ml); IV, chloroform–methanol (1 : 1, 1000 ml); V, methanol (1000 ml); VI, chloroform–acetic acid (3 : 1, 2000 ml); VII, acetic acid (2000 ml); VIII, methanol (1000 ml); and IX, chloroform–methanol–aqueous ammonia–acetic acid (400 : 100 : 10 : 3, 2000 ml). GL1a and GL1b were eluted in fraction II and PL2a, PL2b and PGL1 in fraction IX. Each lipid of the fractions was further purified by TLC with solvent B. The *Rf* values of GL1a, GL1b, PL2a, PL2b, and PGL1 with this solvent were 0.78, 0.71, 0.50, 0.45, and 0.13, respectively.

Analytical methods and degradative procedures. GLC was done as described previously[3,5] except as described below. A trimethylsilyl derivative of inositol (TMS-inositol) was analyzed on a 2-m glass column of 2% OV-101 at 190°C. Inositol was measured by GLC after acetylation on a 3-m glass column of 2% OV-225 with an increasing temperature from 160°C to 220°C at a rate of 2°C/min. Acetylated mannitol was used as an internal standard.

The lipid composition was measured as described previously[11] essentially based on phosphorus and sugar measurements.[15,16] Glycerol was measured by the method of Kates.[17] Acid methanolysis, acetolysis, HI treatment, and BCl_3 cleavage of ether bonds were already reported.[3,18] The water-soluble product (glycerophosphoinositol) of BCl_3 cleavage of PL2a, PL2b, or PGL1 was hydrolyzed with 2 M HCl at 125°C for 48 hr to obtain glycerol and inositol. Permethylation analysis and identification of the anomeric configuration of sugar were described previously.[5]

Hydrolysis with phosphatidylinositol-specific phospholipase C. PL2a, PL2b, and PGL1 were hydrolysed with phosphatidylinositol-specific phospholipase C.[19] The reaction mixture (0.5 ml) contained 0.5 μmol of lipid, 0.8 mg of sodium deoxycholate, 49 μmol of tris-(hydroxymethyl)aminomethane (pH 7.5), and 0.06 units of the enzyme. The mixture was incubated for 6 hr at 37°C. The reaction was stopped by the addition of 5.3 ml $CHCl_3$–methanol–0.27 M HCl (3 : 3 : 2). The water-soluble product was extracted with neutral Bligh and Dyer solvent.

Physical measurements. Optical rotations of archaeol, caldarchaeol, and sugar were measured at 25°C at 589 nm with a high sensitivity polarimeter (PM-71, Union Scientific Eng. Co. Japan). The infrared spectra and the fast-atom bombardment (FAB)-mass spectra of lipids were recorded as described.[3]

Materials. Authentic archaeol, caldarchaeol, phytanyl chloride, and biphytanediyl dichloride were prepared from cells of *M. hungatei* GP1 (DSM1101) as described previously.[1,3] The phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis* was a gift from Dr. H. Ikezawa. *myo*-Inositol and *myo*-inositol-2-monophosphate were purchased from Tokyo Kasei Kogyo Co. and the Sigma Chemical Co., respectively. *myo*-Inositol cyclic 1,2-phosphate was prepared from *myo*-inositol-2-phosphate by dicyclohexylcarbodiimide treatment.[20] Standard sugar derivatives for GLC analysis were prepared from commercially available glucose, galactose, maltose, and dextran.

RESULTS

Extraction of total lipid from M. arboriphilicus cells

In our previous paper[3], lipids were extracted by the unmodified method of Bligh and Dyer,[13] which was satisfactory for obtaining archaetidylserine from *M. arboriphilicus* cells. In the case of *M. thermautotrophicum*, the modified extraction method with an acidified solvent was the most effective.[11] Since the total lipid obtained by the usual Bligh and Dyer method accounted for only 1% of the dry weight of *M. arboriphilicus* cells, which was one fifth of the lipid content of *M. thermautotrophicum*, a similar modification of the method was tried. As shown in Table I, the maximum amount of lipid was obtained when the disrupted cells of *M. arboriphilicus* were extracted with the acidified solvent. Only a limited amount of lipid-phosphorus and lipid-sugar were measured in the case of acid extraction from the intact cells or neutral extraction from the disrupted cells. Almost the same level of or slightly less lipid (5.3% lipid of dry cell weight) was extracted by the acidified solvent from frozen-and-thawed cells. This is of use as an alternative for the most effective extraction of lipids from the organism.

TABLE I. RECOVERY OF LIPIDS EXTRACTED FROM *M. arboriphilicus* CELLS BY VARIOUS MODIFICATIONS OF THE BLIGH & DYER METHOD

Cell	Aqueous phase[a]	Recovery		
		Lipid (mg/g cell)	P[b] (mmol/g cell)	Sugar[b] (mmol/g cell)
Intact	Water	ND	3.3	3.1
Intact	TCA	ND	6.1	7.3
Frozen-&-thawed	Water	12	6.5	11.3
Frozen-&-thawed	TCA	53	17.8	31.7
Disrupted	Water	30	10.1	21.9
Disrupted	TCA	58	20.2	40.1

[a] The lipid extraction method was essentially as described by Blight and Dyer[13] in which the aqueous phase was water or 5% TCA.

[b] Chloroform-soluble phosphorus and chloroform-soluble sugar.

Composition of alkyl chain

To see whether fractional extraction occurred in the less effective extractions, the compositions of alkyl chains in the various lipid extracts were compared. Biphytanediyl (C_{40}) chains consistently predominated in the alkyl groups of total lipid obtained by the acid extraction from disrupted cells or frozen-and-thawed cells (biphytanediyl, 81 mol%; phytanyl, 19 mol%). On the other hand, an apparent high proportion of phytanyl (C_{20}) chain was observed in the neutral extracts (biphytanediyl, 48 mol%; phytanyl, 52 mol%). This result shows that the tetraether type of polar lipids is predominant in the cells of *M. arboriphilicus* as in *M. thermautotrophicum*,[11] but it is less extractable by the neutral solvent than the diether type.

Polar lipid composition

The total lipid obtained by the acid extraction from disrupted cells was composed of 16% neutral lipid and 84% polar lipid. A two-dimensional TLC pattern of the total lipid is

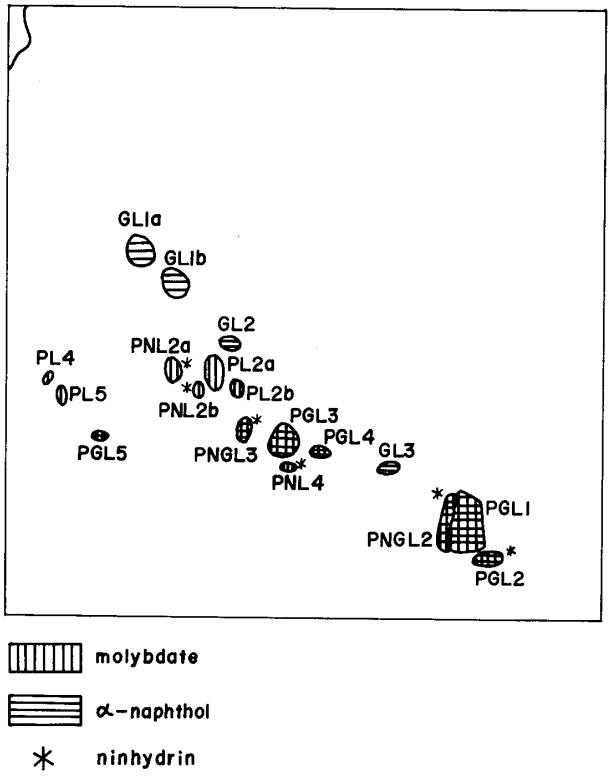

FIG. 1. TLC Profile of the Polar Lipids of *M. arboriphilicus*.

TABLE II. COMPOSITION OF POLAR LIPIDS EXTRACTED WITH AN ACIDIFIED SOLVENT FROM DISRUPTED *M. arboriphilicus* A2 CELLS

Lipid	P (%)	Sugar (%)	mol %[a]
PL2a	14.5		10.6
PL2b	4.2		3.1
PL4	1.0		0.7
PL5	2.8		2.1
PNL2a	4.4		3.2
PNL2b	4.2		3.1
PNL4	<0.5		<0.5
PNGL2	6.6	10.2	4.8
PNGL3	1.4	1.4	1.0
PGL1	41.1	40.8	30.0
PGL2	<0.5	<0.5	<0.5
PGL3	17.0	10.4	12.4
PGL4	1.7	<0.5	1.2
PGL5	1.3	<0.5	0.9
GL1a		13.6	9.9
GL1b		17.5	12.6
GL2		2.6	1.8
GL3		3.4	2.5

[a] mol% was calculated assuming that each lipid contained 1 phosphate moiety and/or 2 glucose moieties as established for the major lipids, PL2, PL2b, PNL2a,[7] PNL2b, [3] PGL1, GL1a, and GL1b.

shown in Fig. 1. At least 18 spots of polar lipids were detected by acid charring. The designations of the lipid spots in Fig. 1 correspond to those of *M. thermautotrophicum* ΔH.[11] Table II shows the composition of these polar lipids. The most predominant polar lipid was a tetraether type of phosphoglycolipid designated as PGL1 (30 mol%). A glycolipid which had a glucosylucose residue (GL1b, 13 mol%) was the most abundant in the diether type of polar lipids. The sum of the contents of GL1a, GL1b, PL2a, PL2b, and PGL1, whose structures are described below, was 66.2 mol%.

Stereochemical configurations of hydrophobic portions

Archaeol and caldarchaeol were prepared from the total lipid for the measurement of optical rotation. Acetolysis cleaves phosphodiester bonds but not glycosidic bonds. On the other hand, acid methanolysis breaks down glycosidic bonds but not phosphodiester bonds of archaebacterial ether aminophospholipids.[3] That is, the lipidous acetolysis products from the total lipid contained acetylated glycolipids and acetylated alkyl glycerol ethers, from which archaeol, caldarchaeol, and sugar were released by acid methanolysis. Alkyl glycerol ethers were extracted with light petroleum from the methanolysate, and archaeol and caldarchaeol were purified by preparative TLC. The alkyl glycerol diether prepared as above comigrated with the authentic archaeol from *M. hungatei* on TLC. The optical rotation of *M. arboriphilicus* archaeol was $[\alpha]_D$ +8.4° which was identical with that of 2,3-di-*O*-phytanyl-*sn*-glycerol ($[\alpha]_D$ +8.6°). The alkyl iodide prepared from the alkyl glycerol diether by HI treatment was analyzed by GLC. It showed a single peak and its retention time coincided with that of phytanyl iodide from *M. hungatei*.

The alkylene glycerol tetraether prepared from the total lipid showed the identical *Rf* value with the authentic caldarchaeol from *M. hungatei*. It showed the optical rotation $[\alpha]_D$ +8.8°, in agreement with that ($[\alpha]_D$ +8.7°) of *M. hungatei* in which the biphytanediyl groups are linked to the *sn*-2 and *sn*-3 positions of both glycerol residues. The alkyl chlorides prepared from the polar tetraether lipids (GL1a, PL2a, PGL1) by BCl_3 treatment were analyzed by GLC. These chromatograms coincided with that of biphytanediyl dichloride from *M. hungatei*. Thus, the hydrophobic portion (glycerol ethers) of each major lipid in the total lipid were identified as archaeol or caldarchaeol.

D-*Glucose as a sole sugar in the total lipid*

Free sugar was released by hydrolysis with 1 M HCl at 100°C for 3 hr from the methylglycoside resulted from the total lipid by succesive treatments of acetolysis and acid methanolysis as described above. It was then converted to alditol acetates and analyzed by GLC. Only the derivative of glucose was found on the chromatogram. The free sugar had an optical rotation of $[\alpha]_D$ +59.8°. These results show that sugar moieties of the main glycolipids and phosphoglycolipids in the total lipid

were D-glucose as a sole sugar.

PGL1

PGL1 was the most predominat polar lipid of this organism. It showed positive responses to acid molybdate, periodate-Schiff and α-naphthol reagents. The polar group of the lipid was identified as *myo*-inositol by GLC of trimethylsilyl and acetylated derivatives. Caldarchaeol, phosphoinositol, and glucose were the products of acid methanolysis. The infrared spectrum of PGL1 (Fig. 2) showed absorptions corresponding to groups of hydroxy –OH (3350 cm^{-1}) and –C–OH (1040 cm^{-1}), phosphate P=O (1220 cm^{-1}) and P–O–C (1020 cm^{-1}), ether C–O–C (1100 cm^{-1}), methyl $-CH_3$ and methylene $-CH_2-$ (1380, 1460, 2850 ~ 2950 cm^{-1}). There was no band indicative of an ester group (1740 cm^{-1}). The molar ratio of phosphorus, glycerol, glucose and inositol in PGL1 was 1.0 : 1.9 : 2.1 : 0.9. The partially methylated alditol acetate derived from permethylated PGL1 were identified as 1,5-diacetyl 2,3,4,6-tetramethyl glucitol and 1,5,6-triacetyl 2,3,4-trimethyl glucitol with GLC by comparison with standard samples prepared from maltose and dextran. The two peaks were present in an approximately equimolecular ratio. CrO_3 oxidation of acetylated PGL1 completely destroyed the glucose in the

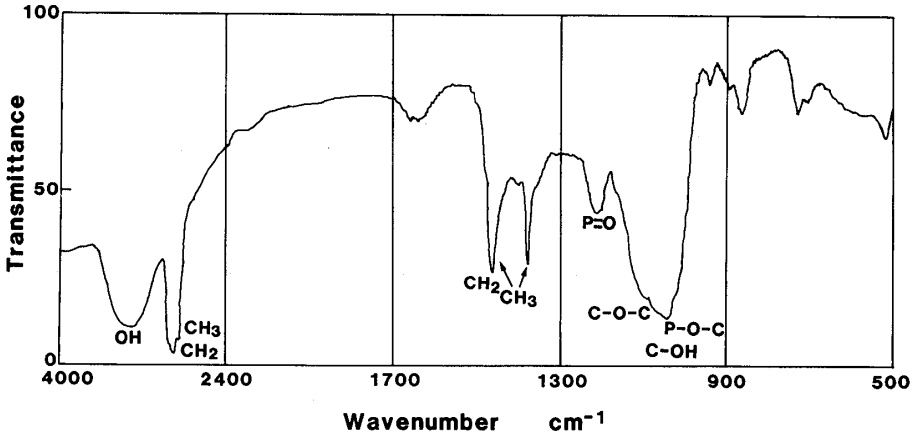

FIG. 2. Infrared Spectrum of Ammonium Salt of PGL1.

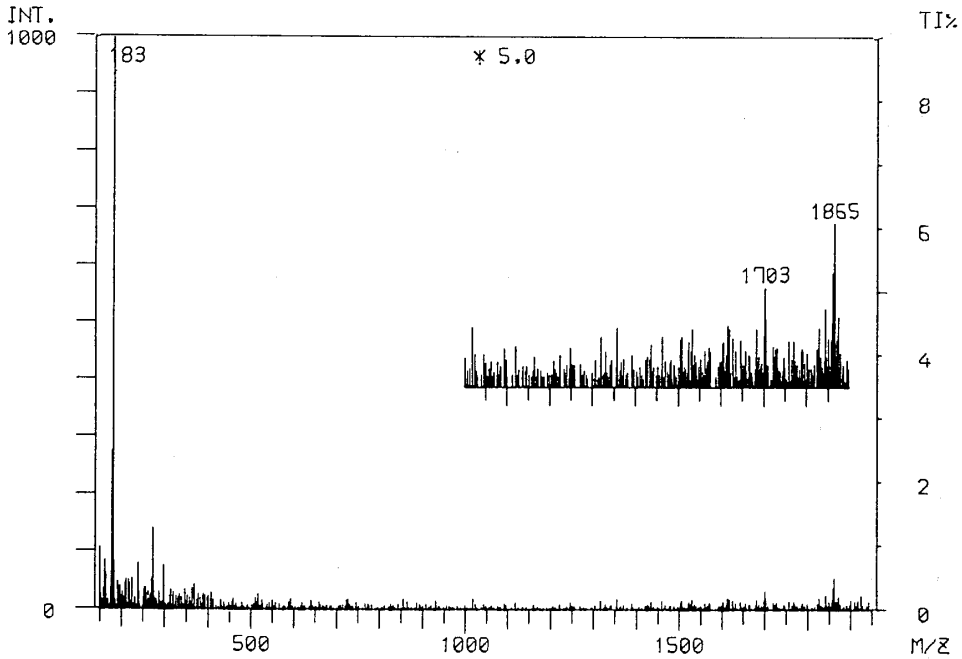

FIG. 3. Negative-ion FAB-mass Spectrum of PGL1.

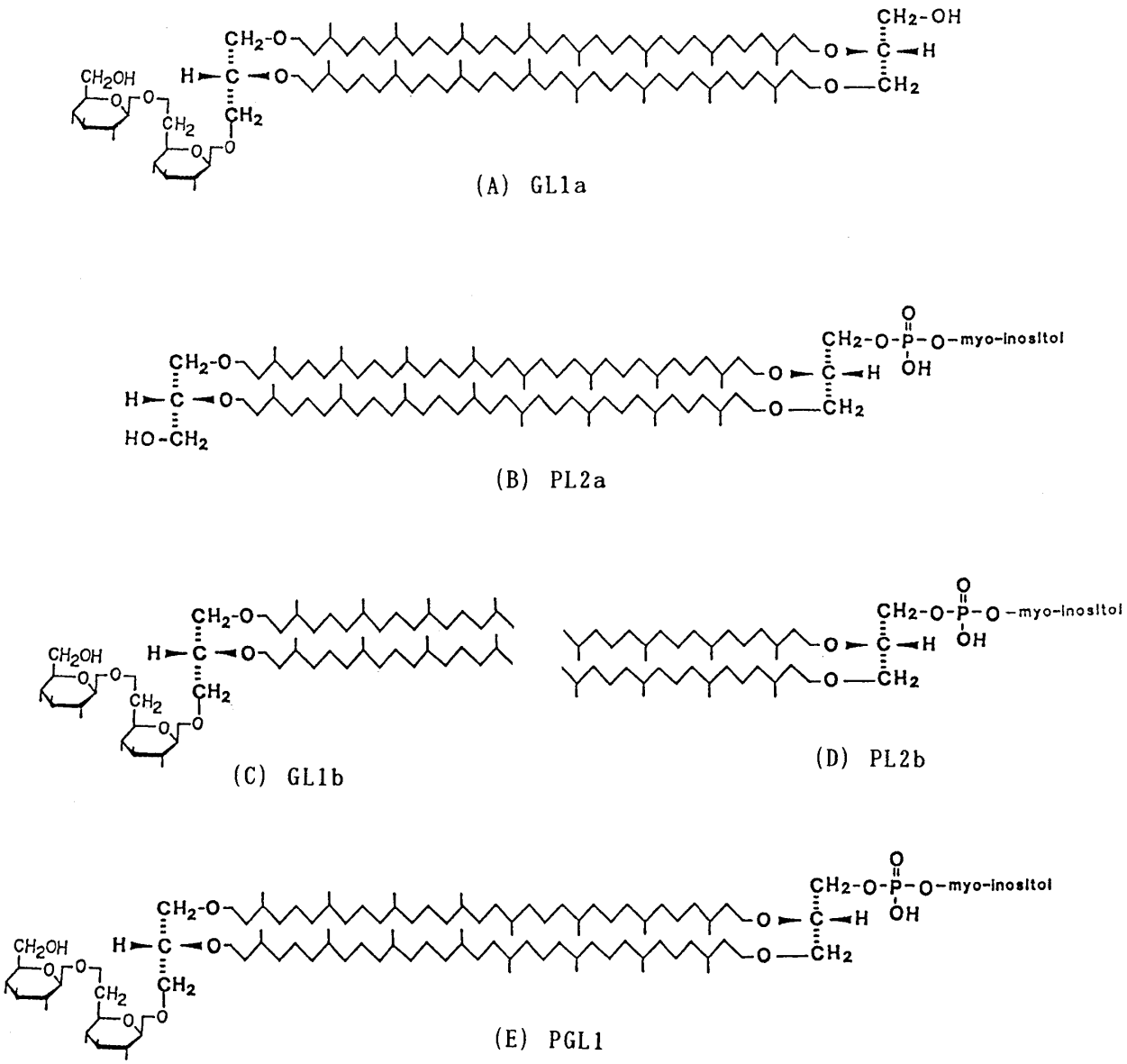

FIG. 4. Proposed Structures of Five Polar Lipids from *M. arboriphilicus*.

A, GL1a (gentiobiosyl caldarchaeol); B, PL2a (caldarchaetidylinositol); C, GL1b (gentiobiosyl archaeol); D, PL2b (archaetidylinositol); E, PGL1, (gentiobiosyl caldarchaetidylinositol).

lipid. Both glucoside residues of PGL1 were thus found to be linked by a (1→6) linkage and to have β-configurations (this is a gentiobiosyl residue).

PGL1 was almost completely hydrolyzed to *myo*-inositol cyclic 1,2-monophosphate and gentiobiosyl caldarchaeol with phosphatidylinositol-specific phospholipase C. These results indicated that PGL1 has a phosphoinositol moiety at one glycerol residue of caldarchaeol and a gentiobiose moiety at the other glycerol residue. The negative ion FAB-mass spectrum of PGL1 (Fig. 3) showed peaks of m/z 1865 ($[M-H]^-$) and m/z 1703 ($[M-\text{inositol}]^-$) which agreed with the molecular weight (1866) expected from the structure shown in Fig. 4E.

Finally the structure of PGL1 was established as caldarchaeol having monophospho-*myo*-inositol and β-glucopyranosyl-(1→6)-β-glucopyranosyl residues at each glycerol residue of caldarchaeol (gentiobiosyl caldarchaetidylinositol, Fig. 4E).

GL1a, GL1b

These lipids were major glycolipids and gave

positive responses to α-naphthol and periodate-Schiff reagents and were negative to acid molybdate and ninhydrin on a TLC plate. Chloroform-soluble products of acid methanolysis of GL1a and GL1b yielded caldarchaeol and archaeol, respectively. Glucose was identified as an aqueous methanol-soluble product of the reaction. The molar ratio of glycerol and glucose were 1.0 : 1.2 for GL1a and 1.0 : 1.9 for GL1b. The negative ion FAB-mass spectra of GL1a and GL1b showed peaks of *m/z* 1623 ($[M-H]^-$) and *m/z* 975 ($[M-H]^-$), respectively. These peaks agreed with the molecular weights (1624 and 976) expected from the structures shown in Fig. 4A and 4C, respectively.

GLC analysis of the partially methylated alditol acetates derived from permethylated GL1a or GL1b and CrO_3 oxidation analysis showed the same results as that of PGL1. Thus the structure of the glucoside of GL1a and GL1b was identified as gentiobiose.

These results showed that the structure of GL1a was identified as β-glucopyranosyl-(1→6)-β-glucopyranosyl caldarchaeol (gentiobiosyl caldarchaeol, Fig. 4A), and GL1b was as 2,3-di-*O*-phytanyl-1-*O*-[β-D-glucopyranosyl-(1′→6′)-β-D-glucopyranosyl]-*sn*-glycerol (gentiobiosyl archaeol) (Fig. 4C).

PL2a, PL2b

PL2a and PL2b showed positive response to acid molybdate and periodate-Schiff reagents on a TLC plate and were negative to α-naphthol reagent. The polar group of the lipid was identified as *myo*-inositol by GLC of trimethylsilyl and acetylated derivatives.

Incubation of the lipids with phosphatidylinositol-specific phospholipase C for 6 hr resulted in 92% (PL2a) and 98% (PL2b) hydrolysis. Under the same condition 95% of phosphatidylinositol (diacyl type) was hydrolyzed within 30 min. Only *myo*-inositol cyclic 1,2-phosphate was detected on a cellulose plate as the water-soluble product from either PL2a or PL2b. As the chloroform-soluble product, caldarchaeol from PL2a, and archaeol from PL2b were identified by TLC.

Acid methanolysis splitted PL2a and PL2b into caldarchaol and phosphoinositol, and archaeol and phosphoinositol, respectively. The molar ratio of phosphorus, glycerol, and inositol in PL2a and PL2b were 1.0 : 1.8 : 1.0 and 1.0 : 0.9 : 0.9, respectively. The negative ion FAB-mass spectra showed peaks of *m/z* 1541 ($[M-H]^-$) and *m/z* 1379 ($[M-\text{inositol}]^-$) for PL2a, and 893 ($[M-H]^-$) and *m/z* 731 ($[M-\text{inositol}]^-$) for PL2b. Finally it was concluded that PL2a was caldarchaetidylinositol (Fig. 4B) and PL2b was archaetidylinositol (Fig. 4D).

DISCUSSION

As in *M. thermautotrophicum*, TCA was required for the effective extraction of lipids from *M. arboriphilicus*. Moreover, in the latter case, cell disruption was another requirement. The maximum yield of lipids (5.8%) from *M. arboriphilicus* was at almost the same level of lipid content of *M. thermautotrophicum* (25.3 mmol lipids-P/g cell; 36.8 mmol lipids-sugar/g cell)[11] and was comparable with the total lipid content of most bacteria (4 to 6% of dry weight[21~23]). These two methanogens belong to the same family and both have pseudomurein in their cell walls. The ratio of diether and tetraether polar lipid in the organisms were identical. The difficulty in extracting lipids with neutral solvent and the requirement of acidification are probably due to the same reason discussed in our previous paper.[11] The requirement of cell disruption in *M. arboriphilicus* may reflect higher rigidity of the cells.

Although the TLC patterns of polar lipids and lipid composition of *M. arboriphilicus* and *M. thermautotrophicum* are similar, a few differences were recognized. That is, ethanolamine-containing phospholipids (PNL1a, PNL1b and PNGL1) were present in *M. thermautotrophicum* but not in *M. arboriphilicus*. Phosphoglycolipids and glycolipids were more complex in *M. arboriphilicus* A2 than in *M. thermautotrophicum*. Recently, structures of glycolipids (GL1a and GL1b), inositol-containing phospholipids (PL1a and PL1b), and

inositol-containing phosphoglycolipid (PGL1) from *M. thermautotrophicum* were identified.[7] These five lipids were completely identical with the corresponding ones from *M. arboriphilicus* even in the stereochemical details of polar groups. Although distribution of inositol-lipids in methanogenic bacteria has not been extensively examined, PGL1 has been suggested as the common lipid of *Methanobacteriaceae*[10] and these results confirmed this based on the chemical structure of the lipid. Moreover, it showed that the other two inositol-lipids occurred commonly in the two genera. In *M. hungatei* which lipid constituents are well known, no inositol-lipid has been found so far. The two inositol-phospholipids were found in sulfur-dependent thermophilic archaebacteria, that is, archaetidylinositol (PL-2b) in *Thermococcus celar*[24] and caldarchaetidylinositol (PL2a) in *Sulfolobus solfataricus*[25] have been reported. Phospho-*myo*-inositol are common to sulfur-dependent thermophilic archaebacteria[26,27]. It is interesting that the methanogens share inositol lipids with sulfur-dependent archaebacteria but not with extreme halophilic archaebacteria, while methanogens are phylogenetically more related with the latter than with the former.[28]

The heptad hypothesis was proposed by us for *M. thermautotrophicum* lipids.[7] It postulates that diether and tetraether types of neutral, phospho-, and phosphoglycolipids (phosphoglycolipid is only the tetraether type) with identical glycosyl and/or phosphate-containing polar residues can be grouped as a set of 7 lipids, which may reflect a possible biosythetic relationship.[7] The five polar lipids of *M. arboriphilicus* in combination with archaeol and caldarchaeol are another example of the heptad. This can reinforce the factual basis of the heptad concept.

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