[Original]

Lipid Component Parts Analysis of the Hyperthermophilic Sulfate-Reducing Archaeon *Archaeoglobus fulgidus*

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Abstract : *Archaeoglobus (A.) fulgidus* is a hyperthermophilic, anaerobic, sulfate-reducing archaeon. Although the polar lipid composition of various archaea has been reported, no information has been available for *A. fulgidus* polar lipids. The present paper reports the results of lipid component parts analysis applied to the archaeon. Lipid component parts analysis is a simplified analytical method developed by the authors to obtain a rough outline of information about the polar lipid of a species of a microorganism. Unfractionated total lipid is subjected to several chemical degradation procedures to release lipid component parts (core lipids, glycolipid sugars and phospholipid polar head groups), which are identified by appropriate chromatography. Archaeol and caldarchaeol were found as core lipids along with an unknown core lipid. The major glycolipid sugars were galactose and mannose. A trace amount of glucose was also detected. The phosphodiester-linked polar head groups of phospholipids were inositol and ethanolamine. The presence of these lipid components is consistent with the occurrence of polar lipid-synthesizing enzymes detected by a BLAST search of the whole genome sequence of the organism. An amino group containing phospholipid was found for the first time in an archaeon other than methanogenic archaea.

Key words : *Archaea, Archaeoglobus fulgidus*, ether lipid, phospholipid, glycolipid.
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Introduction

Archaea are defined primarily by small subunit ribosomal RNA sequences [1], and is found to be unique in many biochemical properties compared with Bacteria and Eucarya [2]. Therefore, Archaea, Bacteria and Eucarya are the highest taxa of all living organisms, and the taxonomic rank is called "domain" [1] (the term "domain" has no relation to the term "domain" used in protein structure). In other words, Archaea is the third group of living organisms on the Earth. Among the three domains, Archaea and Bacteria are prokaryotes from a morphological point of view.

One of the most remarkable biochemical differences between organisms of the domains Archaea and Bacteria is the structure of polar glycerolipids. Polar lipids from Archaea consist exclusively of di- and tetra-ethers of glycerol and isoprenoid alcohols, which are bound at the *sn*-2 and *sn*-3 positions of the glycerol moiety [3] (Fig. 1). This configuration is opposite to that of bacterial polar lipids. That is, archaeal and bacterial polar lipids have enantiomeric glycerol backbones. These characteristics are used to discriminate between Archaea and Bacteria. Furthermore, tetraether type polar lipids are found in many, but not all, Archaea [4]. These characteristics have been based on the analyses of lipids from archaeal organisms belonging to 19 families out of the 22 archaeal families [5]. Polar lipids show a unique composition depending on the family or the genus to which the archaeal organism belongs. Polar lipid composition is, therefore, used as one of the useful chemotaxonomic markers [6]. However, no information about lipids from the sulfate-reducing hyperthermophilic archaeon, *Archaeoglobus (A) fulgidus*, is available. The polar lipid composition of this organism is, therefore, needed for comparison with that of other archaeal organisms. Because of the poor growth of *A. fulgidus*, it is not easy to collect enough biomass of the organism to analyze complete structures of its individual polar lipids. As an alternative, a simplified analytical method, called "lipid component parts analysis" [7], was applied to this organism.

The polar lipids (phospholipids, glycolipids and phosphoglycolipids) of Archaea are composed of glycerol or glycerophosphate backbone with ether-linked isoprenoid hydrocarbon chains (core lipid) and polar groups (phosphodiester-linked alcohols or sugar groups). These are lipid component parts (Fig. 1). One species of Archaea contains several to 20 or more kinds of polar lipids. Determination of the complete structure of these lipids requires a large quantity of cell mass and laborious and time-consuming work. To know the outline of the lipid constituents for taxonomic purposes, we have developed a simplified lipid component parts analysis. That is, without separating individual lipids, lipid component parts are liberated from the total lipid by appropriate chemical degradation methods and identified by adequate chromatography [7]. The results of the analysis are qualitatively recorded only for "presence" or "absence" of the individual component parts. The present paper reports the results of the lipid component analysis of *A. fulgidus*.

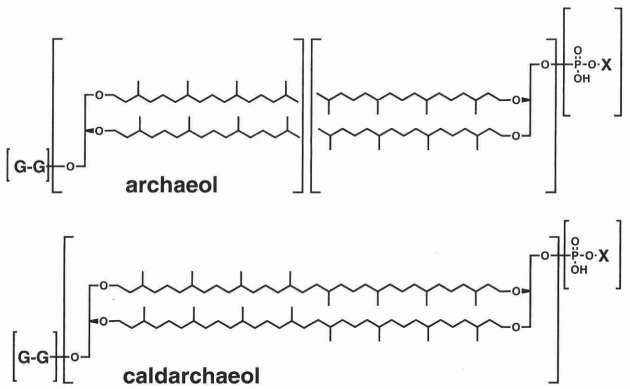

Fig. 1. Typical polar lipid structures of Archaea and their component parts. Archaeol and caldarchaeol are core lipids. G-G and X represent a diglycosyl residue and a phosphodiester-linked polar head group (for example, ethanolamine and *myo*-inositol), respectively.

Materials and Methods

Growth of A. fulgidus cells

A. fulgidus DSM4304 (VC-16) [8] cells were grown statically and anaerobically in 600 ml of DSM399 Archaeoglobus medium [9] in a 1-liter bottle with N_2-CO_2 (4 : 1) as a gas phase at 83℃ for 2 or 3 days. The cells were collected by centrifugation, were resuspended in a small amount of the spent growth medium, and were stored under refrigeration (-20℃).

Lipid component parts analysis

Total lipid was extracted from the thawed cell suspension by the method of Bligh and Dyer [10]. The lipid component parts were analyzed according to the methods described by Koga *et al.* [7]. Briefly, total lipid was analyzed by two-dimensional TLC on a silica gel 60 plate (Merck 5721) using the solvent systems, chloroform-methanol-conc.-aqueous ammonia (65:35:8) for the first direction, and chloroform-methanol-acetic acid-water (85:30:15:5) for the second direction. Spots were visualized with ninhydrin for amino lipids, acid molybdate for phospholipids [11], α-naphthol reagent for glycolipids [12], and charring for all lipids. The core lipids liberated from total lipid by acetolysis followed by methanolysis [13] were chromatographed on a silica gel plate with the solvent system, light petroleum-diethylether-acetic acid (50:50:1). The spots were detected by charring after spraying sulfuric acid-water (95:5). Inositol phospholipids and glycolipids were methanolyzed with 5% HCl-methanol at 100℃ for 3 h. Because the product from inositol phospholipids was phosphoinositol, the phosphate group was hydrolyzed off under a strong acid hydrolysis condition (6 M HCl at 100℃ for 2 h). The resultant free inositol was acetylated with acetic anhydride in pyridine at 80℃ for 1 h and analyzed by the use of a gas-liquid chromatograph Shimadzu GC-17A equipped with a column DB-1 at 210℃. Another portion of the first methanolyzed sample

served as a sample for sugar analysis. The products of the methanolysis of the glycolipids were 1-methylglycosides. They were hydrolyzed under mild acid hydrolysis (1 M HCl at 100℃ for 3 h). The liberated monosaccharides were reduced with $NaBH_4$ by room temperature for 2 h. After removal of excess $NaBH_4$ by decomposition with acetic acid and evaporating in methanol, the reduced sugar alcohol was acetylated and analyzed similarly to the case of inositol. In order to prepare glycerophosphodiesters from the total lipid, ether bonds were cleaved with BCl_3 treatment [14]. The resultant glycerophosphodiesters were used for the identification of phosphodiester-linked polar head groups by two-dimensional electrophoresis and TLC on a thin-layer cellulose plate [7]. Spots were identified by reactions to specific staining reagents ($FeCl_3$ and sulfosalycilic acid for phosphate-containing compounds [15], and ninhydrin for amino group-containing compounds).

Results and Discussion

Two-dimensional TLC (Fig. 2) revealed at least 9 polar lipid spots, of which two were glycolipids, one was phosphoglycolipids, and six were phospholipids identified by reactions with acid molybdate and α-naphthol spray reagents. Tentative identification of the spots on the TLC is summarized in Table 1. One ninhydrin-positive spot (PNL1) was comigrated with standard phosphatidylethanolamine. Two ninhydrin-negative, α-naphthol-negative

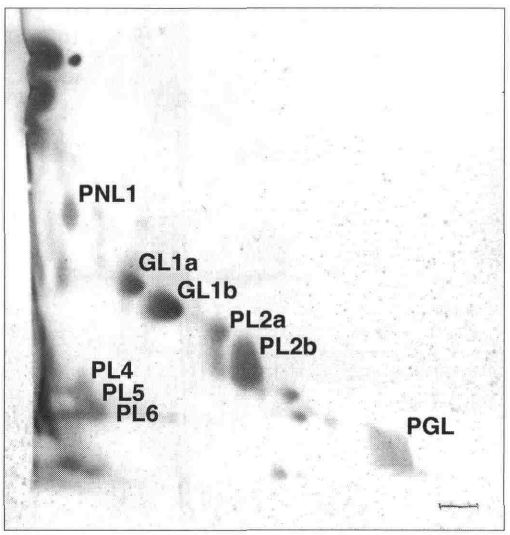

Fig. 2. Two-dimensional TLC profile of *A. fulgidus* total lipid on a silica gel plate (Merck) developed with a solvent chloroform-methanol-conc. aqueous ammonia (65:35:8) for the vertical direction and chloroform-methanol-acetic acid-water (85:30:15:5) for the horizontal direction. The starting point of chromatography is the bottom-right corner. Spots are designated as shown. Tentative identifications are shown in Table 1.

phospholipids (PL2a and PL2b) showed similar mobilities to standard phosphatidylinositol. These two spots had slightly different mobilities. Such paired spots are phospholipids with the same polar head group and with a diether or tetraether core lipid. That is, these are probably archaetidylinositol and caldarchaetidylinositol. One phosphoglycolipid(PGL) spot and two phospholipid spots (PL4 and PL5) showed mobilities close to diglucosyl caldarchaetidylinositol, archaetidic acid and caldarchaetidic acid, respectively, of *Methanothermobacter (M.) thermautotrophicus* [16] (see ref. [17] for the nomenclature of archaeal lipids). The two-dimensional TLC profile of the *A. fulgidus* total lipid resembled that of *M. thermautotrophicus* shown in Fig. 2 of the reference [18]. The same spot number in Fig. 2 of the present paper is designated to the spot of *A. fulgidus* that is assumed to be identical with the corresponding lipid of *M. thermautotrophicus*. There are a few additional lesser spots on the TLC, which were not identified and are not discussed here.

The acetolyzed total lipid of *A. fulgidus* showed three major spots on TLC (data not shown). Two of them were archaeol and caldarchaeol (Fig. 1), and one was unknown. Among them, the caldarchaeol and the unknown were predominant and the archaeol was less in quantity. The unknown core lipid migrated more slowly than the caldarchaeol on the TLC, but no further information is available.

Inositol liberated from the phospholipids and monosaccharides (its reduced products) from

Table 1. Possible identification of spots on two-dimensional TLC of total lipid from *A. fulgidus*

Spot	Reaction to			Mobility close to*	Tentative identity
	Nynhydrin	Acid molybdate	α-Naphthol		
PNL1	+	+	−	PE	Ethanolamine PL
GL1a	−	−	+	DGC	DGC
GL1b	−	−	+	DGA	DGA
PL2a	−	+	−	PI	CI
PL2b	−	+	−	PI	AI
PGL	−	+	+	DGCI	DGCI
PL4	−	+	−	PA	CA
PL5	−	+	−	PA	AA
PL6	−	+	−	PA	

See Fig. 2 for spot disignations. +: positive reaction, −: negative reaction, *: Standard lipids; PE, PI and PA were commercially obtained. Standard lipids; DGC, DGA and DGCI were obtained from *M. thermautotrophicus*[16]. PE: phosphatidylethanolamine (ester type), DGC: diglycosylcaldarchaeol (ether type), DGA: diglycosylarchaeol (ether type), PI: phosphatidylinositol (ester type), DGCI: diglycosylcaldarchaetidylinositol, PA: phsophatidic acid (ester type), PL: phospholipid, CI: caldarchaetidylinositol, AI: archaetidylinositol, CA: caldarchaetidic acid, AA: archaetidic acid

glycolipids were analyzed by gas-liquid chromatography (GLC) (Fig. 3). As sugars, mannose and galactose were detected along with a trace amount of glucose. The presence of inositol was clearly shown on the chromatogram. This supports the inference of the identity of polar lipids by two-dimensional TLC.

The phosphodiester-linked polar head groups were analyzed by BCl_3 treatment and two-dimensional electrophoresis and TLC on a thin-layer cellulose plate. A large spot of glycerophosphoinositol and a faint spot of glycerophosphoethanolamine were detected. This result confirmed the presence of inositol phospholipid(s) and ethanolamine phospholipid(s) shown by silica gel TLC of the total lipid and GLC of the hydrolyzate.

The present work showed the presence in *A. fulgidus* cells of archaeol and caldarchaeol as core lipids, inositol and ethanolamine as phosphodiester-linked polar groups, and galactose and mannose as glycolipid sugars. Although a complete structure determination has not been carried out for the individually separated lipids, a combination of these lipid component parts and the profile of two-dimensional TLC of the total lipid may enable us to infer the presence of diether- or tetraether-type ethanolamine phospholipid(s), archaetidylinositol (AI), caldarchaetidylinositol (CI), diglycosyl caldarchaetidylinositol (DGCI), diglycosylarchaeol (DGA), diglycosylcaldarchaeol (DGC), archaetidic acid (AA) and caldarchaetidic acid (CA).

Amino group-containing ether-type phospholipids had been found only in methanogenic archaea. This is the first report to suggest the occurrence of such lipid(s) in other than

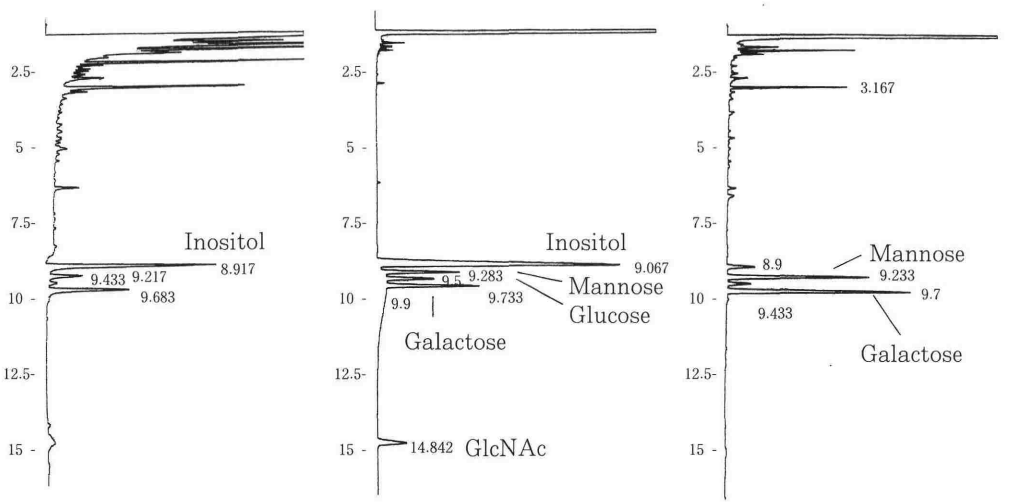

Fig. 3. Gas-liquid chromatography of glycolipid sugars and inositol. Methanolyzed total lipid was divided into two portions; one was subjected to strong acid hydrolysis for the analysis of inositol. The other one was hydrolyzed under a milder condition to recover monosaccharides, which were then reduced and acetylated for GLC. Left panel: analysis of inositol, center panel: authentic standard samples treated as above, GlcNAc: N-acetylglucosamine, right panel: acetylated glycitol analysis (sugar analysis).

methanogens. It is reasonable because of the close phylogenetic relationship of *A. fulgidus to* methanoarchaea [19]. Both are Euryarchaeota.

The occurrence of mannose as a glycolipid sugar was also limited to organisms of Euryarchaeota (*Halobacteriaceae, Thermoplasmataceae, Methanopyraceae and Methanosaetaceae*) [5]. *Archaeoglobaceae* is the fifth family in archaea that has mannosyl lipids.

The whole genome sequence of *A. fulgidus* has been published [20], in which the following genes for phospholipid synthesizing enzymes were tentatively identified: archaetidylserine synthase (GI2648482), archaetidylserine decarboxylase (GI2648481) (archaetidylethanolamine synthesizing enzyme from archaetidylserine) and archaetidylinositol synthase (GI2649447, GI2648807) [21]. The results of our lipid component parts analysis are consistent with the presence of these genes.

Lipid component parts analysis cannot determine the structure of unknown lipid or lipid component parts, because they are identified by the identical mobility on a chromatogram and reactions to detection reagents. The lipid component parts identified above are known compounds. An unidentified core lipid was detected on a TLC. Although its mobility resembled the H-shaped caldarchaeol [22], the exact structure determination of it remains for future work.

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超好熱硫酸還元古細菌 *Archaeoglobus fulgidus* の脂質部品分析

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要　旨：この40年間の脂質分析の蓄積によってほとんどすべての科の古細菌の極性脂質の種類が知られるようになってきたが，硫酸還元古細菌 *Archaeoglobus (A.) fulgidus* の脂質についてはまったく情報がない．そこで，われわれが開発した脂質部品分析という簡便分析法を用いて，*A. fulgidus* の極性脂質の構成部品を解析した．今回の分析の結果，コア脂質としてはテトラエーテル型のカルドアーキオールがほとんどで，少量のジエーテル型アーキオールも存在していた．リン脂質のリン酸ジエステル結合している極性基は，イノシトールとエタノールアミンが検出された．糖脂質の糖は，マンノースとガラクトースが主要成分であり，グルコースも少量存在していた．2次元 TLC により，これらの脂質部品をもつと思われる約9種類の主要なリン脂質，糖脂質，リン糖脂質の存在が確認された．*A. fulgidus* の全ゲノムの BLAST searchにより，これらのリン脂質の合成に関与する遺伝子が確認され，脂質分析の結果と一致した．

キーワード：古細菌，*Archaeoglobus fulgidus*，エーテル型脂質，リン脂質，糖脂質．

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