Core Lipids of Hyperthermophilic Archaeon, *Pyrococcus horikoshii* OT3

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Abstract: Core lipids of the new hyperthermophilic archaeon, *Pyrococcus horikoshii* OT3, purified from hot sediment at the Iheya Ridge in the Mid-Okinawa Trough were investigated. More than 80% of core lipids in the isolate were composed of caldarchaeol (dibiphytanyldiglycerol tetraether). Archaeol (diphytanylglycerol diether) and the trialkyl type of caldarchaeol were present as minor components. A core lipid similar to that of the H form caldarchaeol found in *Methanothermus fervidus* was detected at a total content of 15%. The negative FAB-MS spectrum of caldarchaeol from strain OT3 showed the major molecular ionic peak (M-H)- to be at m/z 1300. The isoprenoid chain of the caldarchaeol was composed primarily of a C40 hydrocarbon without cyclopentane rings. Isoprenoid chains prepared from total lipids of strain OT3 consisted of 9% C20, 82% acyclic-C40, 7% monocyclic-C40 and 2% bicyclic-C40. Tricyclic-C40 and tetracyclic-C40, observed in the order Sulfolobales of thermophilic archaea, were not found in the isolate. The core lipids from strain OT3 thus more resemble those of thermophilic methanogen in Euryarchaeota rather than those of the order, Sulfolobales in Crenarchaeota.

Key words: Archaea, Hyperthermophile, *Pyrococcus horikoshii*, lipid, core lipid, caldarchaeol

1 Introduction

In recent years, several distinct types of extremely thermophilic microorganisms have been isolated from geothermal active terrestrial and marine habitats. In the Japanese sea area, a new strain of hyperthermophilic archaea, *Pyrococcus horikoshii* OT3, was isolated from the Okinawa Trough hydrothermal vents, at a depth of 1395 m, and they can grow at 105°C3. These strains are currently of interest because they are capable growing at highly elevated temperatures and almost all of them are Archaea3. The prospect of obtaining a highly stable biocatalyst that can influence bioengineering design is an intriguing possibility. The thermophilic archaea have been classified into several order up to now. In the order Sulfolobales, the lipids represent the important biochemical characteristics for their classification. For example, the presence of calditocaldarchaeol (calditol-glycerol-dibiphytanyltetraether) in the lipids suggests an identification of the organisms belonging to the order Sulfolobales. The polar lipids in them were also similar to each other3. In contrast, the composition of the core lipids in the strains from the order Thermococcales were...
reported as different from each other, that is, some of them were diether lipids and the others were tetraether lipids\(^4\),\(^5\),\(^6\). It has been shown that there is a tendency in which the major core lipid of archaea existing in the high temperature environment were the tetraether type, caldarchaeol (diglycerol-dibiphytanyl-tetraether), while those in the mesophilic temperature range were of the diether type, archaeol (glycerol-diphytanyl-diether). We have been interested in the difference in the core lipids among the strains of the order Thermococcales grown at high temperature. To clarify the problems involved, it is necessary to investigate the lipids of another strain in the order Thermococcales. In this paper, we discussed the core lipids composition of P. horikoshii OT3 belonging to the order Thermococcales\(^7\).

2 Experimental

2-1 Materials

Chloroform and methanol were distilled before use. All other chemicals and solvents were of analytical grade.

2-2 Growth Conditions

Strain OT3 (JCM 9974) was cultured in Pyrococcus medium\(^7\), which contains 13.5 g of NaCl, 4 g of Na\(_2\)SO\(_4\), 0.7 g of KCl, 0.2 g of NaHCO\(_3\), 0.1 g of KBr, 30 mg of H\(_3\)BO\(_3\), 10.8 g of MgCl\(_2\)·6H\(_2\)O, 1.5 g of CaCl\(_2\), 25 mg of SrCl\(_2\), 1.0 ml of 0.02\% (w/vol) resazurin stock solution, 1 g of Bactoyeast extract (Difco, USA), and 5 g of Bactotryptone (Difco, USA) in 1 L. The pH was adjusted to 6.8, then autoclaved for 15 min. After autoclaving, the medium was added 0.05\% (w/vol) elemental sulfur that had been sterilized at 100°C in a drying oven for 1 h on 3 successive days, and was saturated with argon gas. The anaerobic condition was finally completed by adding the drops of sterilized Na\(_2\)S·9H\(_2\)O solution (0.2 g/L) until the color of the medium changed from pink to colorless. The cultures were anaerobically incubated at 98°C.

2-3 Lipid Extraction

The total lipids were extracted from harvested cells with weak alkaline solvents as follows: cells (5 g, wet weight) were harvested from the culture medium by centrifugation, washed twice with 20 mL of distilled water and suspended in 10 mL of methanol. After the addition of 5 mL of chloroform, the mixture was sonicated for 20 min, and then centrifuged. The supernatant was then transferred to a recovery flask. The precipitated cells were suspended in 30 mL of chloroform/methanol/1M NH\(_4\)OH (1:2:0.5, by vol) and sonicated for 20 min. Each suspension was centrifuged and the supernatant was combined with that previously obtained. The extraction was repeated three times. The solvent in the collected supernatant was removed using a rotary evaporator at 40°C. Residual lipids were dissolved by adding and thoroughly mixing 5 mL of chloroform/methanol (2:1, by vol) and 0.5 mL of water. Methanol was then added to the mixture until the solution became clear. The solution was used for the lipid analysis as crude total lipids.

2-4 Preparation of Total Lipids

The crude total lipids solution contained sulfur derived from the medium. To remove the sulfur in the solution, a gel filtration was performed using a column (ID 1.3 cm×10 cm) of Toyopearl HW-40 C (Tosoh, Japan). After the solvent in the crude total lipids solution was removed, the residue was dissolved in 1 mL of chloroform/methanol/water (5:5:1, by vol), and applied onto the column. The same solvent mixture was used to elute the lipids. The total lipids were eluted with the first 39 mL, and then subsequent elutions contained the sulfur and salts.

The total lipids (10 mg) were fractionated on a column (ID 1.5 cm×10 cm) of DEAE-Sephadex A-25 (Pharmacia, acetate form) into neutral lipids and acidic lipids. After the total lipids were applied onto the column, the neutral lipids were eluted with 100 mL of chloroform/methanol/water (3:4:1, by vol), and the acidic lipids were eluted with 100 mL of chloroform/methanol/0.8 M sodium acetate solution (3:4:1, by vol).

2-5 Hydrocarbon Chains Analysis

The total lipids (10 mg) were heated at 100°C in 1.5 mL of 5% methanolic-HCl in a stopped reaction tube for 2 h to release the polar head groups from the intact lipids. The core lipids were obtained from the organic phase after partition between chloroform and water. The core lipids from the total lipids were treated with 57% HI (1 mL) at 100°C for 2 h. The product, alkyl iodides, taken up in hexane, was successively washed with 15\% (w/w) aq. Na\(_2\)S\(_2\)O\(_3\) and water. After removal of the solvent, the alkyl iodides were reduced with CH\(_3\)COOH (1 mL) and zinc powder (30 mg) for 2 h at 100°C. Hydrocarbons were obtained from the...
upper phase after partition between hexane and water. After being purified by column chromatography on silica gel in hexane, the passed fraction was concentrated. In the fraction, the composition of the hydrocarbon chains was analyzed by GLC which was performed using a Hitachi 163 gas chromatograph (Hitachi, Tokyo, Japan) equipped with 2% DEXsil 300 GC glass column (ID 3 mm × 1 m) by increasing the temperature from 100°C to 300°C at the rate of 15°C/min.

2-6 Acetylation of Core Lipids

Core lipids (2 mg) were acetylated with 0.4 mL of acetic anhydride/pyridine (2:1, v/v) solution in a stoppered reaction tube at 100°C for 2 h. The reaction mixtures were dried under a nitrogen stream and the acetylated compounds were purified by silica gel column chromatography with hexane/chloroform (1:1, by vol).

2-7 Thin-layer Chromatography

The TLC of the core lipids was carried out on precoated silica gel 60 plates (HPTLC, Art. 5641, Merck, Germany) which were activated at 120°C for 2 h before use. The TLC of the core lipids was developed using solvent A (hexane/ethyl acetate = 7:3, by vol) or solvent B (chloroform/ethanol = 9:1, by vol). After spraying the TLC plates with 50% H2SO4, it was charred at 140°C for 15 min to detect the core lipids. The core lipid contents were estimated using a Dual-Wavelength Chromato Scanner CS-930 (Shimadzu corporation, Kyoto, Japan). Two dimensional thin-layer chromatography (2D-TLC) of the intact lipids were carried out on same HPTLC plates which were activated after treatment with methanol solution containing 3% H3BO3. The separation of the polar lipids was performed by 2D-TLC on HPTLC plates (10 cm × 10 cm). Solvent system C consisting of chloroform/methanol/0.2% CaCl2 (20 : 20 : 3, by vol) was used to develop the plates in the first direction and solvent D of chloroform : methanol : 2% (NH4)2CO3 (55 : 45 : 7, by vol) was used in the second direction. The phospholipids were visualized after spraying the chromatogram with Dittmer reagent8), then charred at 140°C for 15 min to detect all of the lipids.

2-8 Fast Atom Bombardment Mass Spectrometry

Negative and positive fast atom bombardment mass spectrometries (FAB-MS) of the core lipids were performed using a JMS AX-505H (Japan Electron Optics Laboratory, Tokyo, Japan), with a matrix of glycerol or 3-nitrobenzylalcohol.

3 Results and Discussion

3-1 Polar Lipid Contents and Composition

Since the cultivated medium contained elemental sulfur, the harvested cells were recovered together with sulfur powder. Due to the difficulty in separating the cells from sulfur, the cell weights could not exactly measured. Also, the sulfur containing crude total lipids solution is soluble in chloroform/methanol (2 : 1, by vol). For the determination of the lipid contents, however, an exact separation of the sulfur from the lipids extract is required. Thus, the gel filtration was performed, and the total lipids were purified. The result showed that the total lipids of strain OT3 accounted for 2% of the cellular dry weight which contained sulfur. In fact, it is considered that if the sulfur can be completely removed from dried cells, the amount of the lipid content will be increased to the same extent.

Fractionation of total lipids on a DEAE-Sephadex A-25 column gave 20% neutral lipids and 80% acidic lipids. On the 2D-TLC plate of total lipids, seven spots (PLα~PLg) of polar lipids were stained blue with Dittmer reagent, indicating the presence of several phospholipids (Fig. 1). No aminolipid was detected by the ninhydrin reagent. Glucose, inositol and phosphate were detected in aqueous phase of the methanolysate of the total lipid. At this stage, only PLd was identified as archaetidylinositol. Structural characterizations of other phospholipids are still not complete.

3-2 Core Lipid Contents

Strain OT3 contained the typical archaeal ether lipids instead of ester lipids. The polar lipids of strain OT3 were based on two types of core ether lipids. To determine the distribution of each core lipid, the total polar lipid fraction was subjected to mild acid methanolysis and the core lipids were separated by TLC using solvent A (Fig. 2). The composition of the core lipids calculated by TLC densitometry showed that 2.8% of sn-2,3-diphytanylglycerol diether (archaeol), 81.0% of dibiphytanlydgclyeryl tetraether (caldarchaeol), 0.3% of trialkyl-caldarchaeol, and 15.9% of unknown core lipid X. Calditocaldarchaeol (dibiphytanlycalcdiol-glycerol tetraether), which was typically detected in the strains in
Fig. 1 Two-dimensional TLC of Polar Lipids of Strain OT3. TLC plate: silica gel 60 HPTLC plate (Merck Art. 5641) treated with 3% H3BO3. Solvent systems: (1) chloroform : methanol: 2% CaCl2 (20 : 20 : 3, by vol), (2) chloroform : methanol: 2% (NH4)2CO3 (55 : 45 : 7, by vol).

Fig. 2 TLC of Core Lipids from Strain OT3. Lane (1), core lipids fraction of OT3; lane (2), core lipids fraction of Sulfolobus acidocaldarius. TLC plate: silica gel 60 HPTLC plate (Merck Art. 5641). Solvent systems: hexane/ethyl acetate (7:3, by vol).

*Sulfolobales*3), was not detected in the lipids of strain OT3 by TLC developed with solvent B (data not shown). Caldarchaeol of *S. acidocaldarius* was separated into several bands with solvent A, dependent on the number of cyclopentane rings in the hydrocarbon chains. Caldarchaeol of strain OT3 did not separate into more than two bands with solvent A (Fig. 2). The results of our experiment revealed that the main core lipid was the tetraether type, caldarchaeol, instead of the diether type as seen in *P. woeseii*5 and *T. celer*6. It has been reported that *Thermococcus alcalophilus*, *Pyrococcus abyssi*6 and several *Pyrococcus*-like isolates from the Southwestern Pacific Ocean7 growing at nearly 90°C also possessed caldarchaeol as a major core lipid. In *P. woeseii*, it was reported that more than 90% of the polar lipids was the diether type, the 2,3-di-O-phytanyl-sn-glycero-1-phosphoinositol derivatives. On the contrary, strain OT3 contained the diether type lipid analogue of phosphatidylinositol as a minor component (PLd). It might be considered that this difference was due to using different extraction solvents, the former used a solvent mixture of chloroform/methanol and the latter used a mixture of chloroform/methanol/1 M NH4OH.

3.3 Isoprenoid Chains of Core Lipids

The isoprenoid chains prepared from the total lipids of strain OT3 were directly analyzed by GLC (Fig. 3). They consisted of 9% C20, 82% acyclic-C40, 7% monocyclic-C40 and 2% bicyclic-C40. It has been known that the isoprenoid chains of the tetraether lipids in *Sulfolobus solfataricus* contain from zero to four cyclopentane rings in which more than 60% are bicyclic11). The number of cyclopentane rings increased with an increase in the growth temperature. The same result was obtained for *S. acidocaldarius* in our study. The degree of cyclization of the isoprenoid chain played an important role in the stability of the membrane in the high temperature environment12). It seemed likely that strain OT3 had more cyclopentane rings than *S. acidocaldarius*, because the optimum growth temperature of the former (98°C, pH 7.0) is considerably higher than that of the latter (75°C, pH 2.0). In contrast with our anticipation, it was shown that more than 80% of caldarchaeol of strain OT3 was composed of C40 hydrocarbons without cyclopentane rings as determined from the hydrocarbon GLC pattern of the total lipids. These facts were also supported by the results of the core lipids TLC patterns and the FAB-MS spectra of the caldarchaeol (Fig. 4). Most of the thermophilic methanogen also contained acyclic-C40 hydrocarbon chains mainly in the caldarchaeol derivatives13).

3.4 The Structures of the Tetraether Core Lipids

The results of the negative FAB-MS spectrum of caldarchaeol of strain OT3 showed that the major
molecular ionic peak (M-H)$^-$ was at m/z 1300 (Fig. 4) and the structure of the major part of strain OT3 caldarchaeol did not contain a cyclopentane ring in its hydrocarbon chain. The FAB-MS spectrum of core lipid X showed that the molecular ionic peak, (M-H)$^-$ of X appeared at m/z 1298 (data not shown). The acetylated core lipid X also gave a peak at m/z 1384 (M+H)$^+$ on the positive FAB-MS that was 84 mass units (2 CH$_3$COO-2 H) larger than the original X, indicating the presence of two free hydroxyl groups in the original X. We could not detect the hydrocarbon of core lipid X using our GLC system from 100°C ~ 300°C (Max. 350°C). From these results, it seems that the core lipid X of strain OT 3 is similar to that of the H form caldarchaeol (H-caldarchaeol) in Methanothermus fervidus$^{14}$ which grows at 83°C optimally. We also estimated the structure of this core lipid as follows: two isoprenoid chains were linked to each other by a covalent bond around the center of both isoprenoid chains. It could be considered that the fluidity of the
membrane constituted with the H-caldarchaeol should be decreased and the stability of the core lipid should be increased. The existence of H-caldarchaeol may be one of the factors that attribute to the heat-resistance properties observed in these organisms. We are planning to estimate the properties of phase transition and heat stability of H-caldarchaeol. The profile of the core lipids from strain OT3 is much closer to that of the thermophilic methanogen in Euryarchaeota rather than that from the order Sulfolobales in Crenarchaeota.

Acknowledgment

This work was supported in part by the Shiseido Research Fund.

The author (Yaeko Masuchi) is grateful to Toyo Suisan Kaisha for generous endowment of the Toyo Suisan Chair of Marine Biotechnology to the Research Center for Advanced Science and Technology, University of Tokyo.

(Received Jan. 24, 2000 ; Accepted Mar. 9, 2000)

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日本油学会誌本号掲載 論文要旨

【総説】 変性リボタンパク質と動脈硬化
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現代生活においては動脈硬化のコントロールが大変重要な課題になってきた。食事中のリボタンパク質が動脈硬化の発症進展と密接な関係があることが分かってきており、特に酸化変性によるリボタンパク質の質的な変化が、動脈硬化を引き起こす一つの可能性として注目されている。酸化LDLはマクロファージの泡沫化、血管内皮と単球の粘着性亢進、平滑筋細胞の分化・増殖など種々の細胞活性化作用をもち、これらが動脈硬化病巣形成の引き金になり得ると考えられている。最近、ヒト血中に微量の酸化LDLが存在し、この酸化LDLが循環器疾患患者などで変動する病態マーカーとなる可能性が示唆された。生体内の酸化LDLの性質や挙動が少しずつ明らかにされつつある最新の知見にも触れることとする。

（連絡者：板部洋之） Vol.49, No.7, 687 (2000)

【報文】 超好熱性 Archaeon, Pyrococcus horikoshii OT3 の脂質骨格
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沖縄トラフの深海熱水噴出口から分離された新しい超好熱性古細菌，Pyrococcus horikoshii OT3 の脂質骨格について調べた。分離株の脂質骨格の80%以上がテトラエーテルタイプのcaldarchoeal（diphytanyldiglycerol tetraether）で構成されていた。ジエーテルタイプのarchaeol（diphytanyl diether）やcaldarchoealのトリアルキルタイプの脂質骨格も微量成分として存在していた。さらに，Methanothermus fervidus 中に見出された H 型の caldarchoeal と同等構造の脂質骨格が、15%含まれていた。OT3 株のcaldarchoealを除イオンモードで測定した FAB-MS スペクトルは, m/z 1300 に分子イオンピークを示し，caldarchoealの主要なイソプレノイド鎖は，シクロペンタン環を含まない C27 炭化水素であることわかった。OT3 株の全脂質から調製したイソプレノイド鎖は，9%の C27, 82%のacyclic-C27, 7%のmonocyclic-C27, 2%のbicyclic-C27 から構成されていた。好熱性古細菌の Sulfolobales 目の菌株にみられる tricyclic-C27 と tetracyclic-C27 の炭化水素鎖は，OT3 株からは検出されなかった。これらの結果は，OT3 株の脂質の性状が，Crenarchaeota の Sulfolobales 目よりもむしろ Eur亚archaeota の好熱性メタン生成菌に近いことを示している。

（連絡者：伊藤佑子） Vol.49, No.7, 695 (2000)