Extremophiles (2000) 4:275–277

ORIGINAL PAPER

Masateru Nishihara · Satsue Nagahama · Mami Ohga
Yosuke Koga

Straight-chain fatty alcohols in the hyperthermophilic archaeon *Pyrococcus furiosus*

Received: March 8, 2000 / Accepted: May 8, 2000

Abstract Two straight-chain fatty alcohols (*n*-hexadecanol and *n*-octadecanol) were found in the neutral lipid fraction extracted from *Pyrococcus furiosus* cells. They were identified by thin-layer and gas-liquid chromatography, mass and infrared spectra, and chemical modification. The fatty alcohols accounted for 54% of the neutral lipid of the cell.

Key words Archaea · *Pyrococcus furiosus* · Lipid · Fatty alcohol · Neutral lipid · Hexadecanol · Octadecanol

Introduction

Archaeal lipids have been analyzed for a number of species including extreme halophiles (Kates 1993), methanogens (Koga et al. 1993), thermoacidophiles (De Rosa and Gambacorta 1986), and hyperthermophiles (De Rosa et al. 1987; Lanzotti et al. 1989; Sprott et al. 1997). All the archaeal polar lipids analyzed so far are composed of core lipids with ether linkages between isoprenoid alcohol and glycerol. Several studies on lipids of the hyperthermophilic archaea of the *Thermococcaceae* have been reported. The major polar lipid of *Thermococcus celer*, *Pyrococcus woesei*, and *Thermococcus zilligii* AN1 is the diether type of phosphatidylinositol (archaetidylinositol) (De Rosa et al. 1987; Lanzotti et al. 1989). 3-Phosphoglucosyl archaeol has been identified as the additional major polar lipid in the latter organism (Lanzotti et al. 1989). In these organisms the core lipid is mainly composed of archaeol, but the closely related organisms *Thermococcus chitonophagus* and *Thermococcus hydrothermalis* contain caldarchaeol as the core lipid as well as archaeol (Huber et al. 1995; Lattuati et al. 1998). Although several possible polar lipid structures have been proposed on the basis of FAB-MS studies of total lipids of *Pyrococcus furiosus* (Sprott et al. 1997), the individual lipids have not been isolated nor have complete structures have been determined. Because *P. furiosus* is the hyperthermophilic archaeon that has been most studied biochemically, we tried to study lipid structures of its cells. During the course of our structural studies of *P. furiosus* lipids, we found an unusual spot on a thin-layer chromatogram of the methanolyzed lipid fraction between caldarchaeol and archaeol. Although it was initially presumed to be a new core lipid, it was subsequently found to consist of two straight-chain fatty alcohols. Although most hydrocarbon chains in Archaea are isoprenoid derivatives and fatty acids have been reported in *P. furiosus* (Carballeira et al. 1997), this is the first example of a straight-chain fatty alcohol in Archaea. We report its identification here. The nomenclature for archaeal ether-linked lipids proposed by Nishihara et al. (1987) is used in this article.

Materials and methods

Extraction and chromatography of lipids

Pyrococcus furiosus JCM8422 cells were grown as described (Uemori et al. 1993). Total lipid was extracted from the cells by the method of Bligh and Dyer (Bligh and Dyer 1959). Thin-layer chromatography (TLC) was developed on a Silica Gel 60 thin-layer plate (Merck l.05721) with solvent A (chloroform/methanol/acetic acid/water, 85:35:15:5) for polar lipids, and solvent B (light petroleum/diethyl ether/acetic acid, 50:50:1) for neutral lipids. Spots were visualized by spraying acid molybdate reagent for phospholipid, or 30% H_2SO_4 followed by charring for all lipid. The neutral lipid composition was determined densitometrically on a TLC chromatogram of the total lipid after development with solvent B followed by charring with H_2SO_4 by use of a scanning densitometer (Shimadzu CS-9300PC). Hydrocarbons were analyzed by gas-liquid chromatography (GLC).

Communicated by J. Wiegel

M. Nishihara · S. Nagahama · M. Ohga · Y. Koga (✉)
Department of Chemistry, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan
Tel. +81-93-691-7215; Fax +81-93-693-9921
e-mail: kogay@med.uoeh-u.ac.jp

GLC analysis was carried out with a 30-m DB-1 capillary column (film thickness, 0.25 μm; J & W Scientific, Folsom, CA, USA) at a temperature increasing from 120° to 300°C at a rate of 20°C/min. The total lipid was roughly fractionated by DEAE-cellulose chromatography as described by Nishihara et al. (1989).

Analytical procedures and physical measurements

HI cleavage of ether lipid followed by $LiAlH_4$ reduction was as described by Kates (Kates et al. 1965). The hydroxyl group of the unknown lipids was acetylated with pyridine/acetic anhydride (1:1) at a room temperature for 1, 5, or 60 min. Acetolysis was carried out with acetic acid/acetic anhydride (3:2) at 160°C for 16 h as described by Renkonen (1965). Gas chromatography-mass spectrometry (GC-MS) analysis was performed with a mass spectrometer (JMX-DX303; Japan Electron Optics, Akishima, Japan) equipped with a Hewlett Packard gas chromatograph (HP5890). Infrared (IR) spectra of the lipid were recorded as thin films using an IR spectrometer (Shimadzu IR470).

Materials

Authentic *n*-hexadecanol, *n*-octadecanol, and hexadecanoic acid methyl ester were purchased from Nacalai Tesque (Kyoto, Japan). di-*O*-Hexadecylglycerol, di-*O*-oleoylglycerol, and mono-*O*-oleoylglycerol were the products of Serdary Research (London, Ontario, Canada). Archaeol and caldarchaeol were prepared from lipids of *Methanobacterium thermoautotrophicum* as described previously (Nishihara et al. 1987).

Results and discussion

To analyze the core lipid composition of *Pyrococcus furiosus*, the total lipid was subjected to methanolysis in HCl-methanol and the resultant lipidic fraction was developed by TLC on a silica gel plate with solvent B. Archaeol (*R*f = 0.54) and caldarchaeol (*R*f = 0.24) were detected as major core lipids. Additionally, an unknown spot (*R*f = 0.33; designated lipid X) was found. Because it might be a new core lipid or an unknown neutral lipid, we tried to identify the structure. To identify the intact (original) lipid from which lipid X was derived, total polar lipid was separated with one-dimensional TLC with solvent A and the whole chromatogram was divided into four fractions. Each fraction was isolated and subjected to acetolysis followed by methanolysis to remove polar groups. Lipid X was not detected in any of the four polar lipid fractions. When total lipid was separated by DEAE-cellulose column chromatography with solvents chloroform (fraction 1), methanol (fraction 2), acetic acid (fraction 3), and methanol containing 0.25 M ammonium acetate (fraction 4), only fraction 1 (chloroform eluate) contained lipid X, and all other fractions did not. This result indicated that lipid X would be the neutral lipid itself. To confirm this possibility, intact total lipid of *P. furiosus* cells was developed on a TLC plate with solvent B to separate intact neutral lipid. The total lipid in fact did contain lipid X (Fig. 1, lane 1). Lipid X was proved not to be a derived lipid by methanolysis, but it was an intact lipid present in the total lipid.

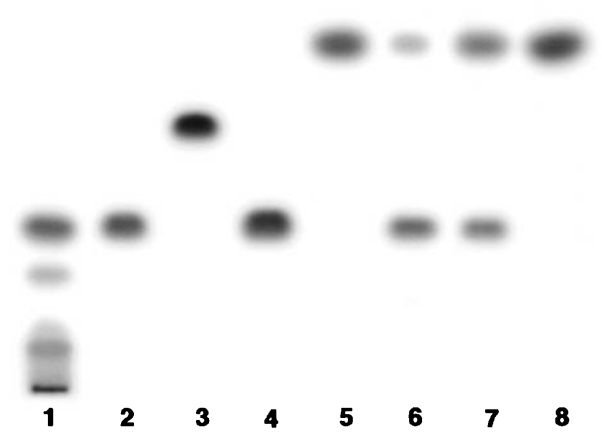

Fig. 1. Thin-layer chromatogram of total neutral lipid of *Pyrococcus furiosus* and purified lipid X. Thin-layer chromatography was developed with solvent B and spots were visualized by spraying 30% H_2SO_4 followed by charring. *Lane 1*, total neutral lipid of *P. furiosus*; *lane 2*, purified lipid X; *lane 3*, archaeol from *Methanobacterium thermoautotrophicum*; *lane 4*, hexadacanol; *lane 5*, hexadecyl acetate; *lanes 6–8*, lipid X acetylated with pyridine/acetic anhydride (1:1) at room temperature for 1, 5, and 60 min, respectively

Lipid X was purified from methanolyzed total lipid or from intact total lipid by TLC with solvent B (Fig. 1, lane 2). Acetylated lipid X (Fig. 1, lane 8) coincided with *n*-hexadecanyl acetate (Fig. 1, lane 5) on TLC. Acetylated lipid X migrated on TLC differently from hexadecanoic acid methyl ester, di-*O*-hexadecylglycerol, archaeol, caldarchaeol, diacylglycerol, or monoacylglycerol. During acetylation of lipid X, no intermediate spot was detected between full (60 min) and no (0 min) acetylation (Fig. 1, lanes 2, 6–8). This time course of acetylation suggested that lipid X had only one hydroxyl group.

Hydrocarbon was prepared from lipid X by HI treatment followed by $LiAlH_4$ reduction. Two peaks of hydrocarbons were detected by GLC, which eluted from the column at retention times identical to those of *n*-hexadecane (peak 1) and *n*-octadecane (peak 2) but different from those of phytane and biphytane (C-40 isoprenoid) derived from archaeol and caldarchaeol, respectively. Hydrocarbons prepared from lipid X were analyzed by GC-MS. Peak 1 and peak 2 showed molecular ion peaks of *m/z* 226 and 254, respectively, which are consistent with the molecular weights of *n*-hexadecane (226) and *n*-octadecane (254), respectively. Moreover, fragment ion patterns of peak 1 and

peak 2 coincided with those of authentic *n*-hexadecane and *n*-octadecane, respectively. Intact lipid X was also separated into two peaks by GLC. These two peaks were tentatively designated as peak 1′ (retention time, 4.97 min; 76%) and peak 2′ (retention time 5.95 min, 24%). When lipid X was mixed with either *n*-hexadecanol or *n*-octadecanol, peak 1′ or peak 2′ became larger, respectively, but no third peak appeared. These results suggested that lipid X was a mixture of C-16 and C-18 fatty alcohols. Finally, an IR spectrum of intact lipid X showed signals of only CH_3, CH_2, and OH groups.

The content of the fatty alcohols was determined by densitometry of a TLC chromatogram of the total lipid after development with solvent B followed by charring with H_2SO_4. The fatty alcohols accounted for 54% of neutral lipid of the cell. Other neutral lipids were archaeol (6%), caldarchaeol (13%), and unknown (28%). This value shows that the fatty alcohols are two of the major components of the total lipid.

Because this is the first report of straight-chain fatty alcohols from an archaeon, it was then necessary to determine if some fatty alcohols could have arisen from the medium. Therefore, a control experiment was done in which we analyzed the medium for fatty alcohols before and after *P. furiosus* was grown. The medium (80 ml each) before inoculation and after growth (supernatant) was lyophilized and lipid was extracted by the Bligh and Dyer method. In both samples, no spot coincided with lipid X was detected on TLC developed with solvent B, while a trace amount of caldarchaeol was detected only in the medium after growth of the organism.

Carotenoids, squalenes, and acyclic isoprenoid hydrocarbons have been identified as neutral lipids in *Halobacterium cutirubrum* (Kramer et al. 1972; Kushwaha et al. 1972, 1975) and in methanogenic and thermoacidophilic archaea (Tornabene et al. 1979). Although this is the first report of the presence of straight-chain fatty alcohols in Archaea, it is not surprising because straight-chain hydrocarbons have been reported in *M. thermoautotrophicum* (Tornabene et al. 1978). A similar spot that migrated on TLC with a solvent of hexane/ethyl acetate (78:22) a little faster than caldarchaeol but significantly slower than archaeol has been reported in the neutral lipid fraction of *T. chitonophagus* (Huber et al. 1995). The chemical nature has not been described in the literature, but it may be a related or the same compound because *Thermococcus* is a close relative of *Pyrococcus*. Straight-, branched-, or hydroxylated-chain fatty acids including fatty acids with the same carbon numbers as the fatty alcohols reported here are found in an esterified form in the total lipid of *P. furiosus* (Carballeira et al. 1997). We also confirmed the presence of such fatty acids. The present fatty alcohols might be derived from the carboxylic acids with the same hydrocarbon chains.

References

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Carballeira NM, Reyes M, Sostre A, Huang HS, Verhagen MFJM, Adams MWW (1997) Unusual fatty acid compositions of the hyperthermophilic archaeon *Pyrococcus furiosus* and the bacterium *Thermotoga maritima*. J Bacteriol 179:2766–2768

De Rosa M, Gambacorta A (1986) Lipid biogenesis in archaebacteria. Syst Appl Microbiol (Archaebacteria '85) 7:278–285

De Rosa M, Gambacorta A, Trincone A, Basso A, Zillig W, Holz I (1987) Lipids of *Thermococcus celer*, a sulfur-reducing archaebacterium: structure and biosynthesis. Syst Appl Microbiol 9:1–5

Huber R, Stohr J, Hohenhaus S, Rachel R, Burggraf S, Jannasch HW, Stetter KO (1995) *Thermococcus chitonophagus* sp. nov., a novel, chitin-degrading, hyperthermophillic archaeum from a deep-sea hydrothermal vent environment. Arch Microbiol 164:255–264

Kates M (1993) Membrane lipids of archaea. In: Kates M, Kushner DJ, Matheson AT (eds) The biochemistry of Archaea (Archaebacteria). Elsevier, Amsterdam, pp. 261–295

Kates M, Yengoyan LS, Sastry PS (1965) A diether analog of phosphatidyl glycerophosphate in *Halobacterium cutirubrum*. Biochim Biophys Acta 98:252–268

Koga Y, Nishihara M, Morii H, Akagawa-Matsushita M (1993) The ether polar lipids of methanogenic bacteria. Structures, comparative aspects, and biosynthesis. Microbiol Rev 57:164–182

Kramer JKG, Kushwaha SC, Kates M (1972) Structure detmination of the squalene, dihydrosqualene and tetrahydrosqualene in *Halobacterium cutirubrum*. Biochim Biophys Acta 270:103–110

Kushwaha SC, Pugh EL, Kramer JKG, Kates M (1972) Isolation and identification of dehydrosqualene and C40-carotenoid pigments in *Halobacterium cutirubrum*. Biochim Biophys Acta 260:492–506

Kushwaha SC, Kramer JKG, Kates M (1975) Isolation and characterization of C_{50}-carotenoid pigments and other polar isoprenoids from *Halobacterium cutirubrum*. Biochim Biophys Acta 398:303–314

Lanzotti V, Trincone A, Nicolaus B, Zillig W, De Rosa M, Gambacorta A (1989) Complex lipids of *Pyrococcus* and AN1, thermophilic members of Archaebacteria belonging to *Thermococcales*. Biochim Biophys Acta 1004:44–48

Lattuati A, Guezennec J, Metzger P, Largeau C (1998) Lipids of *Thermococcus hydrothermalis*, an archaea isolated from a deep-sea hydrothermal vent. Lipids 33:319–326

Nishihara M, Morii H, Koga Y (1987) Structure determination of a quartet of novel tetraether lipids from *Methanobacterium thermoautotrophicum*. J Biochem (Tokyo) 101:1007–1015

Nishihara M, Morii H, Koga Y (1989) Heptads of polar ether lipids of an archaebacterium, *Methanobacterium thermoautotrophicum*: structure and biosynthetic relationship. Biochemistry 28:95–102

Renkonen O (1965) Individual molecular species of different phospholipid classes. Part II. A method of analysis. J Am Oil Chem Soc 42:298–304

Sprott GD, Agnew BJ, Patel GB (1997) Structural features of ether lipids in the archaeobacterial thermophiles *Pyrococcus furiosus*, *Methanopyrus kandleri*, *Methanothermus fervidus*, and *Sulfolobus acidocaldarius*. Can J Microbiol 43:467–476

Tornabene TG, Wolfe RS, Balch WE, Holzer G, Fox GE, Oro J (1978) Phytanyl-glycerol ethers and squalenes in the archaebacterium *Methanobacterium thermoautotrophicum*. J Mol Evol 11:259–266

Tornabene TG, Langworthy TA, Holzer G, Oro J (1979) Squalenes, phytanes and other isoprenoids as major neutral lipids of methanogenic and thermoacidophilic archaebacteria. J Mol Evol 13:73–83

Uemori T, Ishino Y, Toh H, Asada K, Kato I (1993) Organization and nucleotide sequence of the DNA polymerase gene from the archaeon *Pyrococcus furiosus*. Nucleic Acids Res 21:259–265