A Simple Chromatographic Procedure for the Detection of Cyclized Archaeobacterial Glycerol-Bisdiphytanyl-Glycerol Tetraether Core Lipids

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(Received 26 May 1988)

Archaeobacterial glycerol-bisdiphytanyl-glycerol tetraether core lipids containing from one to eight cyclopentane rings could be resolved from each other and from the parent uncyclized C₄₀,C₄₀ lipid by TLC. The core lipids of examples from the genera *Methanobacterium*, *Methanobrevibacter*, *Methanogenium* and *Methanoplanus* did not contain cyclized forms of glycerol-bisdiphytanyl-glycerol tetraethers, whereas the core lipids of *Methanosarcina barkeri* contained glycerol-bisdiphytanyl-glycerol tetraethers with from one to three cyclopentane rings in each C₄₀ isoprenoid chain.

INTRODUCTION

All membrane lipids of archaeobacteria characterized to date are based on saturated isoprenoid ethers formed by condensation of glycerol, or more complex polyols, with isoprenoid alcohols containing 20, 25 or 40 carbon atoms (De Rosa *et al.*, 1986*a*). Most halophilic archaeobacteria have membrane lipids based exclusively on 2,3 di-*O*-phytanyl-*sn*-glycerol (C₂₀,C₂₀), although a few isolates, notably the alkaliphiles, also have significant amounts of C₂₅ (sesterterpanyl) chains as C₂₀,C₂₅ glycerol diethers (De Rosa *et al.*, 1982, 1983*a*). The situation is more complex for methanogenic and thermophilic archaeobacteria, since, in most cases, the membranes have a significant component of glycerol-bisdiphytanyl-glycerol tetraethers. These are formed by dimerization of diphytanyl diethers, where head-to-head linkage between the terminal methyls occurs.

Methanogen core lipid structures are particularly diverse, in that in some isolates, glycerol may be replaced by a tetritol, both in the core lipid structures (De Rosa *et al.*, 1986*b*) and in complex lipids (Ferrante *et al.*, 1987). Furthermore, one isolate has a macrocyclic diether with a 36-member ring, which originates from the condensation of a glycerol, in the 2,3-*sn*-configuration, with the 3,7,11,15,18,22,26,30-octamethyldotriacontane-1-32-diol (Comita & Gagosian, 1983).

Thermophilic archaeobacteria of the genera *Desulfurococcus*, *Thermoproteus*, *Thermofilum* and *Pyrodictum* have ether lipids based on C₂₀ and C₄₀ chains (Stetter *et al.*, 1983; Zillig *et al.*, 1981, 1982, 1983) whereas *Thermococcus celer* contains only C₂₀,C₂₀ lipids (De Rosa *et al.*, 1987). In *Sulfolobus* spp. the lipids are based on C₄₀,C₄₀ tetraethers, but there are two classes; one class consists of glycerol dialkyl glycerol tetraethers (GDGT), while the second differs from the first in that, nonitol, a nine-carbon polyol, replaces one of the glycerols (GDNT). These C₄₀,C₄₀ lipids have the additional feature of containing up to four cyclopentane rings in each C₄₀ chain. The degree of cyclization depends on the growth temperature of the organism (De Rosa *et al.*, 1986*a*).

Abbreviations: GDGT, glycerol dialkyl glycerol tetraethers; GDNT, glycerol dialkyl nonitol tetraethers.

Cyclization of C_{40} , C_{40} core lipids has only been found in one methanogen to date (De Rosa *et al.*, 1986b), but detailed structural analyses of C_{40} , C_{40} lipids have only rarely been done (De Rosa *et al.*, 1986b; Kramer *et al.*, 1987; Kushwaha *et al.*, 1981; Comita & Gagosian, 1983). The resolution and identification of cyclized forms currently involves a lengthy combination of column chromatography and HPLC, or GLC of the hydrocarbon chains after hydrolysis and derivatization (De Rosa *et al.*, 1983b; Tornabene & Langworthy, 1979; Makula & Singer, 1978). We report here a simple TLC procedure for the resolution of different cyclized forms of GDGT and the use of this procedure to survey the core lipids of a number of different methanogens.

METHODS

Micro-organisms and culture conditions. Details of methanogens are shown in Table 1. Growth conditions were as previously described (Grant *et al.*, 1985). *Sulfolobus solfataricus* MT-4 was grown in the standard medium at 87 °C as previously described (De Rosa *et al.*, 1975). Cells were harvested at the stationary phase of growth and freeze-dried.

Extraction and isolation of lipids. Polar lipids were extracted by the procedure of Bligh & Dyer (1959) as modified by Minnikin *et al.* (1979). Core lipids were extracted into chloroform or hexane, after hydrolysis of complex lipids by acid methanolysis (Ross *et al.*, 1985). Purified samples of 2,3-di-*O*-phytanyl-*sn*-glycerol (C_{20} , C_{20} diether), 3-*O*-phytanyl-*sn*-glycerol (C_{20} monoether), tetritol C_{20} , C_{20} diether, uncyclized C_{40} , C_{40} tetraether and the glycerol-bisdiphytanyl-glycerol tetraether (C_{40} , C_{40} tetraether) fraction from *Methanosarcina barkeri* (De Rosa *et al.*, 1986b) were available for comparison by TLC.

TLC. This was done on 0.25 mm layers of silica gel F 254 (Merck), activated by heating at 100 °C for 2 h. The solvent was *n*-hexane/ethyl acetate (7:3, v/v), single development. Compounds were detected by spraying with 0.1% (w/v) $Ce(SO_4)_2$ in 1M- H_2SO_4 followed by heating at 150 °C for 5 min.

RESULTS AND DISCUSSION

In a previous study (Grant *et al.*, 1985) the core lipids of a considerable number of different methanogens were analysed by TLC. Glycerol ethers with 20, 25 and 40 carbon atoms were detected, along with a number of new structures. In a later study, two of these new structures from *Ms. barkeri* were characterized as a C_{20} glycerol monoether and C_{20} , C_{20} tetritol diether (De Rosa *et al.*, 1986b). Furthermore, the C_{40} , C_{40} tetraether core lipids of this organism were shown to be of the GDGT class and to contain from one to three cyclopentane rings (De Rosa *et al.*, 1986b). To date, *Ms. barkeri* is the only known methanogen known to possess cyclized core lipids; indeed this organism contains no uncyclized C_{40} , C_{40} lipids. The TLC system used by

Table 1. Core lipids occurring in a range of methanogens

Culture collection no.*	Organism	Core lipids	
		C_{40} , C_{40}	C_{20} , C_{20} †
DSM 862	<i>Methanobacterium bryantii</i>	Acyclic	+
DSM 1535	<i>Methanobacterium formicicum</i>	Acyclic	+
DSM 1053	<i>Methanobacterium thermoautotrophicum</i>	Acyclic	+
DSM 861	<i>Methanobrevibacter smithii</i>	Acyclic	+
DSM 1093	<i>Methanobrevibacter ruminantium</i>	Acyclic	+
DSM 744	<i>Methanobrevibacter arboriphilicus</i>	Acyclic	+
DSM 1224	<i>Methanococcus vannielii</i>	—	+
DSM 1537	<i>Methanococcus voltae</i>	—	+
DSM 2095	<i>Methanococcus thermolithotrophicus</i>	—	+
DSM 1497	<i>Methanogenium cariaci</i>	Acyclic	+
DSM 1498	<i>Methanogenium marisnigri</i>	Acyclic	+
DSM 2373	<i>Methanogenium thermophilicum</i>	Acyclic	+
DSM 2279	<i>Methanoplanus limicola</i>	Acyclic	+
DSM 800	<i>Methanosarcina barkeri</i>	Cyclic	+‡

—, No C_{40} , C_{40} components present.

* DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, FRG.

† Data from Grant *et al.* (1985).

‡ Also contains C_{20} glycerol monoether, C_{25} , C_{20} glycerol diether and C_{20} , C_{20} tetritol diether (De Rosa *et al.*, 1986b).

Fig. 1. TLC of core ether lipids using a simple development system (*n*-hexane/ethyl acetate, 7:3, v/v). Lanes: A, 2,3-di-*O*-phytanyl-*sn*-glycerol; B, uncyclized C₄₀,C₄₀ derivative of GDGT; C, core lipids of *S. solfataricus* (structures of compounds 1–9 are indicated in Fig. 2); D, C₄₀,C₄₀ lipids of *Ms. barkeri*; E, 3-*O*-phytanyl-*sn*-glycerol (lower band) and tetritol C₂₀,C₂₀ diether (upper band).

Grant *et al.* (1985) does not resolve cyclized forms of C₄₀,C₄₀ core lipids from uncyclized forms and it is, therefore, not clear whether cyclized C₄₀,C₄₀ core lipids are present in the hydrolysed lipid extracts of those methanogens that have not been subject to detailed chemical characterization.

Chromatography of core lipid extracts on 0.25 mm layers of silica gel F 254 in *n*-hexane/ethyl acetate 7:3 (v/v) resolved both uncyclized GDGT and the eight different cyclized variations of this C₄₀,C₄₀ tetraether lipid found in the core lipids of *S. solfataricus* (Fig. 1). The structures of these compounds are shown in Fig. 2. Purified samples (De Rosa *et al.*, 1983*b*) were used to confirm the separation order shown in Fig. 1. A purified C₄₀,C₄₀ core lipid fraction from *Ms. barkeri* was readily resolved into the three fractions corresponding to the cyclized forms of GDGT containing four, five or six cyclopentane rings, characterized in the previous study (De Rosa *et al.*, 1986*b*). Samples of C₂₀,C₂₀ diether, tetritol C₂₀,C₂₀ diether and C₂₀ monoether have different *R_F* values and were clearly distinct from C₄₀,C₄₀ tetraethers (Fig. 1). GDNT ethers remained at the origin using this solvent system.

Table 1 shows the results of a survey of the core lipids of several different methanogens using this TLC procedure. Of the strains tested, only *Ms. barkeri* possessed cyclized GDGT core

Fig. 2. Structures of C_{40},C_{40} core lipids (GDGT type), the backbone of complex lipids of thermophilic archaeobacteria. The numbers 1–9 correspond to the bands in Fig. 1, lane C.

lipids. Included in the survey were *Methanobacterium thermoautotrophicum* and *Methanobrevibacter arboriphilicus*, whose complex lipids have been extensively characterized as C_{20},C_{20} diethers and uncyclized C_{40},C_{40} (GDGT) derivatives (Kramer *et al.*, 1987; Morii *et al.*, 1986). This preliminary survey suggests that cyclization of C_{40},C_{40} tetraether lipids is likely to be uncommon amongst methanogenic archaeobacteria that contain C_{40},C_{40} tetraether lipids of the GDGT class. This supports and extends the results of the early surveys of Tornabene & Langworthy (1979) and Makula & Singer (1978).

The pattern of complex lipids and core lipids of archaeobacteria has some value in the taxonomy of the group (Ross *et al.*, 1985; Langworthy & Pond, 1986; Grant *et al.*, 1985). The use of this simple TLC procedure for the detection of cyclized GDGT core lipids extends the range of techniques that can readily be applied in non-specialist laboratories.

We thank Dr M. R. Vaccaro and Mr G. Pinch for their technical assistance.

REFERENCES

- BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.
- COMITA, P. B. & GAGOSIAN, R. B. (1983). Structural elucidation of a unique macrocyclic membrane lipid from a new, extremely thermophilic deep-sea hydrothermal vent archaeobacterium, *Methanococcus jannaschii*. *Journal of Biological Chemistry* **259**, 15234–15241.
- DE ROSA, M., GAMBACORTA, A. & BU'LOCK, J. D. (1975). Extremely thermophilic acidophilic bacteria convergent with *Sulfolobus solfataricus*. *Journal of General Microbiology* **86**, 156–164.
- DE ROSA, M., GAMBACORTA, A., NICOLAUS, B., ROSS, H. N. M., GRANT, W. D. & BU'LOCK, J. D. (1982). An asymmetric diether lipid from alkaliphilic halophiles. *Journal of General Microbiology* **128**, 343–348.
- DE ROSA, M., GAMBACORTA, A., NICOLAUS, B. & GRANT, W. D. (1983a). A C_{25},C_{25} diether core lipid from archaeobacterial halophiles. *Journal of General Microbiology* **129**, 2333–2337.

- DE ROSA, M., GAMBACORTA, A., NICOLAUS, B., CHAPPE, B. & ALBRECHT, P. (1983*b*). Isoprenoid ethers: backbone of complex lipids of the archaeobacterium *Sulfolobus solfataricus*. *Biochimica et biophysica acta* **753**, 249–256.
- DE ROSA, M., GAMBACORTA, A. & GLIOZZI, A. (1986*a*). Structure, biosynthesis and physicochemical properties of archaeobacterial lipids. *Microbiological Reviews* **50**, 70–80.
- DE ROSA, M., GAMBACORTA, A., LANZOTTI, V., TRINCONI, A., HARRIS, J. E. & GRANT, W. D. (1986*b*). A range of ether core lipids from the methanogenic archaeobacterium *Methanosarcina barkeri*. *Biochimica et biophysica acta* **875**, 487–492.
- DE ROSA, M., GAMBACORTA, A., TRINCONI, A., BASSO, A., ZILLIG, W. & HOLZ, I. (1987). Lipids of *Thermococcus celer*, a sulfur-reducing archaeobacterium: structure and biosynthesis. *Systematic and Applied Microbiology* **9**, 1–6.
- FERRANTE, G., EKIEL, I. & SPROTT, G. D. (1987). Structure of diether lipids of *Methanospirillum hungatei* containing novel head group *N,N*-dimethyl amino and *N,N,N*-trimethyl amino pentanetetrol. *Biochimica et biophysica acta* **921**, 281–291.
- GRANT, W. D., PINCH, G., HARRIS, J. E., DE ROSA, M. & GAMBACORTA, A. (1985). Polar lipids in methanogen taxonomy. *Journal of General Microbiology* **131**, 3277–3286.
- KRAMER, J. K. G., SAUER, F. D. & BLACKWELL, B. A. (1987). Structure of two new amino lipids from *Methanobacterium thermoautotrophicum*. *Biochemical Journal* **245**, 139–143.
- KUSHWAHA, S. G., KATES, M., SPROTT, G. D. & SMITH, I. C. P. (1981). Novel polar lipids from the methanogen *Methanospirillum hungatei* GP1. *Biochimica et biophysica acta* **664**, 156–173.
- LANGWORTHY, T. A. & POND, J. L. (1986). Archaeobacterial ether lipids and chemotaxonomy. *Systematic and Applied Microbiology* **7**, 253–257.
- MAKULA, R. A. & SINGER, M. E. (1978). Ether-containing lipids of methanogenic bacteria. *Biochemical and Biophysical Research Communications* **82**, 716–722.
- MINNIKIN, D. E., COLLINS, M. D. & GOODFELLOW, M. (1979). Fatty acid and polar lipid composition in the classification of *Cellulomonas*, *Oerskovia* and related taxa. *Journal of Applied Bacteriology* **47**, 87–95.
- MORII, H., NISHIHARA, M., OHGA, M. & KOGA, Y. (1986). A diphytanyl ether analogue of phosphatidylserine from a methanogenic bacterium *Methanobrevibacter arborophilus*. *Journal of Lipid Research* **27**, 724–730.
- ROSS, H. N. M., GRANT, W. D. & HARRIS, J. E. (1985). Lipids in archaeobacterial taxonomy. In *Chemical Methods in Systematics*, pp. 289–300. Edited by M. Goodfellow & D. Minnikin. London & New York: Academic Press.
- STETTER, K. O., KÖNIG, H. & STACKEBRANDT, E. (1983). *Pyrodictium*, a new genus of submarine disc-shaped sulfur reducing bacteria growing optimally at 105 °C. *Systematic and Applied Microbiology* **4**, 535–541.
- TORNABENE, T. G. & LANGWORTHY, T. A. (1979). Diphytanyl and dibiphytanyl ether lipids of methanogenic archaeobacteria. *Science* **203**, 51–53.
- ZILLIG, W., STETTER, K. O., SCHAFER, W., JANEKOVIC, D., WUNDERL, S., HOLZ, I. & PALM, P. (1981). Thermoproteales: a novel type of extremely thermophilic archaeobacteria isolated from Iceland solfataras. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I Originale C)* **3**, 218–227.
- ZILLIG, W., STETTER, K. O., PRANGISHVILLI, P., SHAFER, W., WUNDERL, S., JANEKOVIC, P., HOLTZ, J. & PALM, P. (1982). *Desulfurococcaceae*, the second family of the extremely thermophilic anaerobic, sulfur-respiring *Thermoproteales*. In *Archaeobacteria*, pp. 304–317. Edited by O. Kandler. Stuttgart: Gustav Fisher Verlag.
- ZILLIG, W., GIERL, A., SHRIEBER, G., WUNDERL, S., JANEKOVIC, D., STETTER, K. O. & KLENK, H. P. (1983). The archaeobacterium *Thermofilum pendens* represents a novel genus of thermophilic anaerobic sulfur-respiring *Thermoproteales*. *Systematic and Applied Microbiology* **4**, 79–87.