JOURNAL OF BACTERIOLOGY, June 1991, p. 3907–3910
0021-9193/91/123907-04$02.00/0

Vol. 173, No. 12

Proportions of Diether, Macrocyclic Diether, and Tetraether Lipids in *Methanococcus jannaschii* Grown at Different Temperatures†

G. DENNIS SPROTT,* MICHELE MELOCHE,‡ AND JAMES C. RICHARDS

Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario, Canada K1A 0R6

Received 6 December 1990/Accepted 3 April 1991

Growth of *Methanococcus jannaschii* over a wide temperature range (47 to 75°C) is correlated with an ability to alter dramatically the proportions of three ether lipid cores. These lipids shifted from predominantly diether (2,3-di-*O*-phytanyl-*sn*-glycerol) at the lower growth temperatures to macrocyclic diether and tetraether at near optimal growth temperatures. Lipid head groups varied as well, especially with respect to an increase in phosphate at the higher temperatures.

Coping with life in harsh environments must, by necessity, involve alterations in cytoplasmic membrane structure. Alterations in the membrane lipids of those eubacteria which are associated with thermophily involve the degree of branching, degree of saturation, and degree of chain length in the fatty acylglycerol chains (1, 18). In general, these changes seem not to occur in archaeobacterial lipids where the alkylglycerol chains are fully saturated, branched, isoprenoid units of fixed chain length (4, 15, 18). Even the change from the eubacterial ester bond to the archaeobacterial ether bond does not, in itself, ensure thermophily, because many archaeobacteria are limited in growth to the mesophilic range. However, changes must occur within the membrane lipids of thermophilic archaeobacteria to allow the maintenance of essential membrane properties during large temperature fluctuations. One such change noted in *Caldariella acidophila* in response to rising growth temperatures was an increase in pentacyclic rings in the C_{40} chains of tetraether lipids (3).

In methanogens, it is unknown how membrane lipid synthesis responds to variations in growth temperature. With the recent discovery of specific hydroxylations in diether lipid cores (7, 21) and the presence of tetraether lipids in some species (22), it appears that most, if not all, methanogens have the capability of synthesizing more than one core structure. This allows the possibility for alterations in the proportions of the core lipids as needed. Here, we document the structures of three core lipids in *Methanococcus jannaschii*, a submarine hydrothermal vent isolate (11), and show dramatic shifts in their proportions in cells grown at various temperatures.

M. jannaschii was obtained from the German Collection of Microorganisms, Gottingen, Germany, as DSM 2661. Growth was conducted in either 100-ml (in 1-liter bottles) or 10-liter (in a 19-liter fermentor) aliquots of defined medium under an atmosphere of CO_2-H_2 (1:4, vol/vol) as described previously (10). Lipids were labeled by including 10 μCi of [2-^{14}C]mevalonate salt (50.1 mCi/mmol) in 100 ml of medium (21) just prior to inoculation. Inoculum consisted of cells grown to mid-exponential phase at the appropriate growth temperature. Cultures were pressurized twice daily to 10 lb/in^2 with a CO_2-H_2 gas mixture and incubated at various temperatures with shaking at 120 rpm. Cells were harvested in early stationary growth phase and stored at −20°C.

[^{14}C]mevalonate-labeled cells were washed and fractionated into cold CCl_3CO_2H-soluble (cell pool), alcohol-plus-ether-soluble (lipid), hot CCl_3CO_2H-soluble (nucleic acid), and residue (protein-rich) fractions, as described elsewhere for *Methanococcus voltae* (5). Aliquots counted in aqueous counting scintillant (Amersham) were corrected for quenching by ^{14}C spiking.

Total lipid extracts were prepared from thawed cells by a neutral Bligh and Dyer procedure (10). This neutral procedure resulted in lipid extraction efficiencies varying from 94 to 97%, determined with cells containing lipids labeled by incorporation of [^{14}C]mevalonate during growth. The percentage of neutral lipids present at each growth temperature was estimated by using thin-layer chromatography (TLC) to separate ^{14}C-labeled neutral lipids from ^{14}C-labeled polar lipids. Silica gel 60 plates were developed with petroleum ether-diethyl ether-acetic acid (50:50:1, by volume). Spots were located with I_2 vapor, and the ^{14}C-labeled adsorbent corresponding to the neutral lipid fraction (R_f near 0.9) was removed for liquid scintillation counting. Quenching was taken into account by spiking with ^{14}C and recounting. Neutral lipids were estimated as a percentage of the disintegrations per minute spotted. Core lipids were quantitated in a similar fashion, following head group removal (see below), by using [2-^{14}C]mevalonate-labeled total lipid extracts.

To measure the proportion of lipid cores after a shift in the growth temperature, cells were first grown in 1-liter bottles (with shaking at 155 rpm) for several transfers at 50 and 70°C. Each cell type was used to inoculate media containing [^{14}C]mevalonate and incubated at the appropriate growth temperature until the mid-exponential phase was achieved. These labeled cells were diluted anaerobically into an equal volume of fresh medium preheated to 70°C and containing [^{14}C]mevalonate. Growth was monitored at 660 nm. Samples of 50 ml were removed as a function of time and processed for quantitation of the core lipids.

Lipid cores were purified for structural determination from polar lipids precipitated with acetone from the total lipids extracted from cells grown at 65°C. Lipid head groups were removed as described previously (8, 9, 14) with 48% hydrofluoric acid (0°C, 16 h, taken to dryness over 6 to 7 h with N_2 at 48°C) followed by a 2.5% methanol-HCl solution (70°C, 2 h). The core lipids, so generated, were partitioned

* Corresponding author.

† NRCC publication no. 31950.

‡ Present address: Department of Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5.

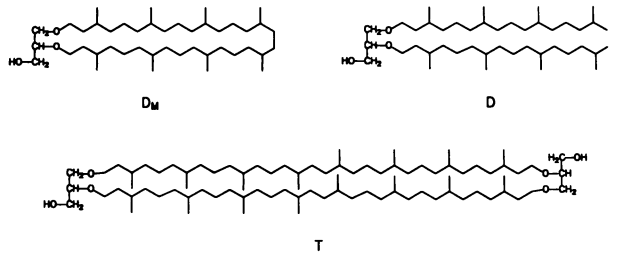

FIG. 1. Structures of the ether lipid cores in *M. jannaschii*. D_M, macrocyclic diether; D, diether; T, tetraether.

into petroleum ether, dried under an N_2 stream, and resuspended into a known volume of chloroform. Core lipids were separated by TLC (described above), detected with I_2, and recovered from the adsorbent in chloroform-methanol (2:1, vol/vol).

Carbohydrates (16) and phosphate (8) were quantitated colorimetrically in total lipid extracts. Polar lipids were separated on Silica Gel G plates (Brinkmann Instruments, Ltd.) developed with chloroform-methanol-acetic acid-water (85:22.5:10:4, by volume). For two-dimensional TLC, the second solvent was chloroform-methanol-acetic acid-water (80:12:15:4, by volume).

Purified core lipids were characterized by optical rotation performed in chloroform with a Perkin-Elmer 243 polarimeter. Ammonium chemical ionization mass spectrometry was carried out on unmodified lipid cores with JEOL (JMS-AX505H) or Kratos Concept IS instruments. Nuclear magnetic resonance data was acquired as distortionless enhancement by polarization transfer (DEPT) spectra recorded in $CDCl_3$ with a Bruker AM500 instrument. Standard tetraether and diether lipids were prepared from *Methanospirillum hungatei* (17), and macrocyclic diether lipids were prepared from *M. jannaschii* (10). The source of materials used in this study was reported previously (10).

Comita et al. (2) reported that the hydrophobic lipid cores of *M. jannaschii* consist primarily of a macrocyclic diether and small amounts of the diether first found in extreme halophiles (13). These structures (Fig. 1) were confirmed here and previously (10), by ^{13}C DEPT spectra and optical rotation measurements, to be 2,3-di-*O*-phytanyl-*sn*-glycerol (diether) and 2,3-di-*O*-cyclic-biphytanyl-*sn*-glycerol (macrocyclic diether). Molecular weights were found to differ by 2 protons (650 and 652), providing final confirmation.

TLC patterns clearly showed that a third core lipid was synthesized by *M. jannaschii* with an R_f value identical to the tetraether standard prepared from *M. hungatei* (Fig. 2). A ^{13}C DEPT spectrum of this purified lipid was identical to those of the macrocyclic diether (10) and of tetraether from *M. hungatei* (6), strongly indicating the compound to be a tetraether core lipid. The 2,2′,3,3′-dibiphytanyl-*sn*-diglycerol tetraether structure shown in Fig. 1 was confirmed by its molecular weight measurement of 1,300 and molecular rotation of +102 degrees (+110 reported in reference 17). Another thermophilic methanogen isolated from hydrothermal vent sediment and grown at 75°C was found to contain these same three lipid cores (12). A minor unknown component with an R_f of 0.42 accounted for <5% of the lipid cores at all growth conditions tested and was not studied further.

[^{14}C]mevalonate was incorporated with a high uptake efficiency of 27% from the medium (pH 6.0) during growth to the early stationary phase at 65°C. Of the label taken up, 78%

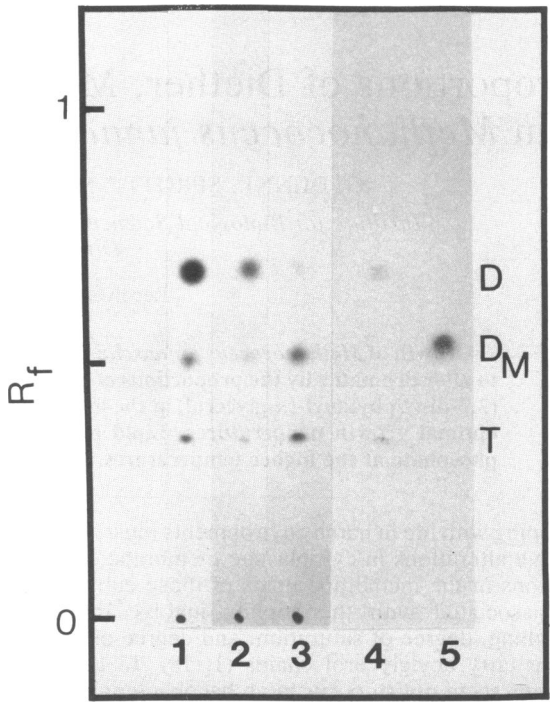

FIG. 2. TLC of ether lipid cores. An autoradiogram of [^{14}C]mevalonate-labeled lipid cores from *M. jannaschii* grown at 45°C (lane 1), 50°C (lane 2), and 75°C (lane 3) is shown. Unlabeled standards, visualized by acid charring (14), are diether (D) plus tetraether (T) lipids from *M. hungatei* (lane 4) and purified macrocyclic diether (D_M) (10) from *M. jannaschii* (lane 5).

appeared as lipid counts (651 μCi/g of lipid) and 19.8% appeared as protein, with the small amount of counts remaining being distributed in the cell pool and nucleic acid fractions. The appearance of significant counts in the protein fraction may reflect some instability of mevalonate under these growth conditions. After hydrolysis to remove head groups, 98% of the lipid counts partitioned into ether, indicating a high specificity for the phytanyl chains. This was similar for cells grown from 47 to 78°C.

Shifts in the proportions of ether lipid cores after growth at different temperatures can be seen in autoradiograms of TLC plates (Fig. 2). Radioactivity remaining at the origin accounted for 10 to 15% of that spotted, probably representing unhydrolyzed polar lipid, lipid degradation products, or nonlipid, and was deleted from calculations of core lipid distribution. The relative amount of each lipid as quantitated over a 30°C range is shown in Fig. 3. Error bars represent three separate growth studies at each temperature; variation within any repeated analysis of a single extract was small, usually <5%. Clearly, the predominantly diether lipid membrane of *M. jannaschii* grown near the lower temperature limit shifted at the higher growth temperatures to contain predominantly macrocyclic diether and tetraether lipids. A cyclization of the chains would tend to decrease their freedom of motion and presumably contribute at elevated growth temperatures to an acceptable membrane fluidity. The elevated content of C_{40}-tetraether lipid should stabilize the membranes at higher temperatures by forming covalently bonded bilayers (monolayers) that span the cytoplasmic membrane. Evidence has been presented for such a membrane spanning monolayer organization of the C_{40}-tetraether

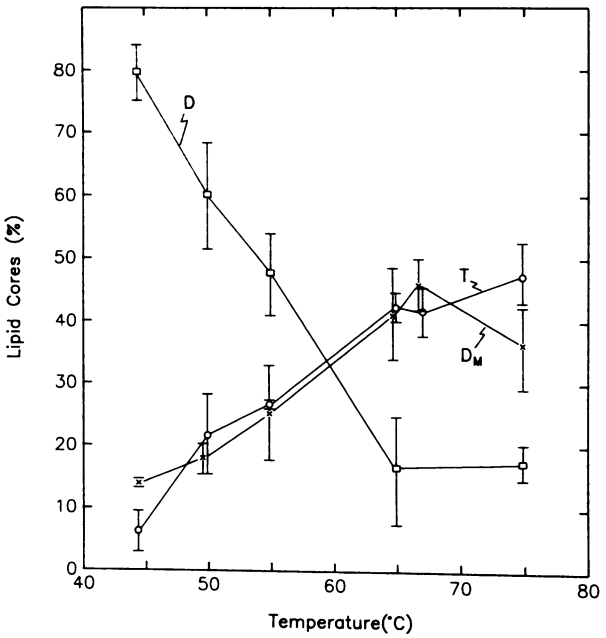

FIG. 3. Proportions of ether lipid cores in *M. jannaschii* grown at various temperatures. D, diether; T, tetraether; D_M, macrocyclic diether.

lipids in the thermoacidophilic *Sulfolobus sulfataricus* (4), but similar evidence is lacking thus far for the methanogens.

Inoculum grown at 50°C to the mid-exponential phase exhibited a 4- to 6-h lag period prior to the initiation of growth at 70°C. This lag was absent when the inoculum consisted of cells grown at 70°C. The proportion of each core lipid was determined at various times after the shift from 50 to 70°C (Fig. 4). Prior to the initiation of growth, diether lipids declined and both macrocyclic diether and tetraether lipids increased to reach approximately equal proportions (Fig. 4A). During the exponential growth phase, this trend continued until the same proportions of core lipids were found as had been found in the control cells (Fig. 4B). These data provide strong correlative evidence linking the predominance of both macrocyclic diether and tetraether lipids to growth at high temperature.

The temperature of growth affected greatly neither the total lipid content of the cells (Table 1) nor the ratio of polar to neutral lipids. At 44°C, the neutral fraction was 8% of the total lipids and increased for the extracts shown in Fig. 3 to 14% at 75°C. The correlation coefficient for this data was only 0.57 by linear regression analysis.

TABLE 1. Polar head groups in lipids extracted from *M. jannaschii* grown at 48 and 75°C[a]

Analysis	Lipid components (% [dry wt] of total lipid extract)[b]	
	48°C	75°C
Elemental		
C	61.36	69.19
H	10.02	11.33
N	1.96	1.48
Phosphate	6.09 ± 0.21	12.58 ± 0.26
Carbohydrate	9.98 ± 0.092	10.67 ± 1.00

[a] Generation times were 15.6 h (48°C) and 2.0 h (75°C).
[b] Extracts represented 2.5% (48°C) and 2.8% (75°C) of cell dry weight. Standard errors are shown for four analyses.

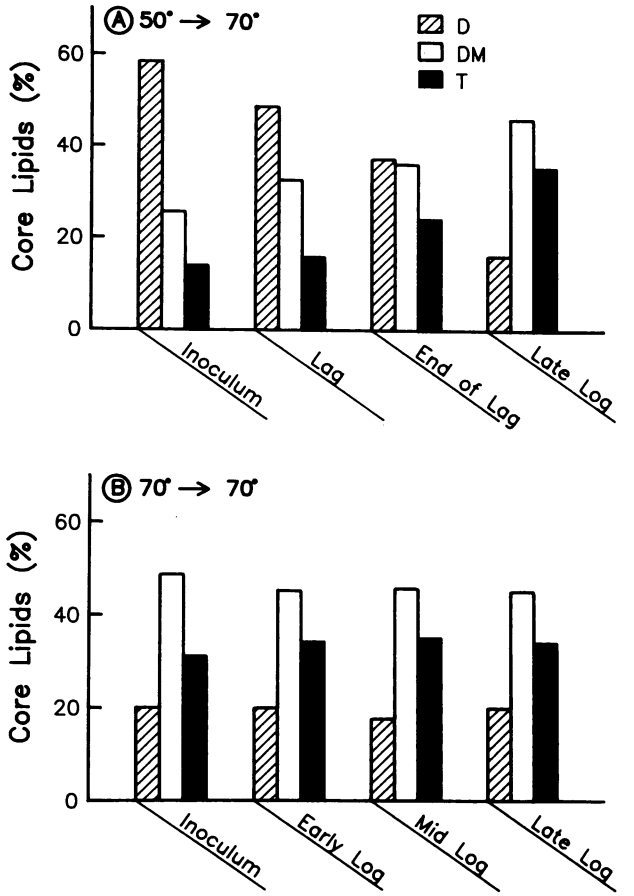

FIG. 4. Changes in the proportions of ether lipid cores following a shift in the growth temperature from 50 to 70°C. Inocula were grown at either 50°C (A) or 70°C (B) in media containing [^{14}C]mevalonate. Growth was continued at 70°C in similar media also containing [^{14}C]mevalonate. D, diether; DM, macrocyclic diether; T, tetraether.

Only one of the diether and four of the macrocyclic diether polar lipids of *M. jannaschii* have been characterized fully (10). Autoradiograms of two-dimensional TLC plates revealed a complex pattern with greater than 30 polar lipid spots. Polar lipids from cells grown at 78°C were more numerous than from cells grown at 47°C (not shown). This correlates with an increase in phosphate (Table 1) as expected from the increased abundance of the tetraether core, considering that tetraether lipids of other methanogens are largely phospholipids (17, 19). A eubacterial thermophilic *Thermus* sp., growing at its upper growth temperature, differed in having an elevated glycolipid level, with little change in phospholipid content (20). In the case of *M. jannaschii*, large structural changes in the lipid cores may obviate the need for a similar head group modification.

Fred Cooper provided expert analysis of lipid cores by mass spectrometry, and we thank Kratos Analytical, Ramsey, N.J., for the mass spectrum of the tetraether. Elemental analyses were conducted by A. C. Webb, and the cell biomass was grown, in part, by K. M. Shaw.

REFERENCES

1. **Amelunxen, R. E., and A. L. Murdock.** 1978. Microbial life at high temperatures: mechanisms and molecular aspects, p. 217–278. *In* D. J. Kushner (ed.), Microbial life in extreme environments. Academic Press, Inc., New York.
2. **Comita, P. B., R. B. Gagosian, H. Pang, and C. E. Costello.** 1984. Structural elucidation of a unique macrocyclic membrane lipid from a new, extremely thermophilic, deep-sea hydrothermal vent archaebacterium, *Methanococcus jannaschii*. J. Biol. Chem. **259**:15234–15241.
3. **DeRosa, M., E. Esposito, A. Gambacorta, B. Nicholaus, and J. D. Bu'Lock.** 1980. Effects of temperature on ether lipid composition of *Caldariella acidophila*. Phytochemistry **19**:827–831.
4. **DeRosa, M., A. Gambacorta, and A. Gliozzi.** 1986. Structure, biosynthesis, and physicochemical properties of archaebacterial lipids. Microbiol. Rev. **50**:70–80.
5. **Ekiel, I., K. F. Jarrell, and G. D. Sprott.** 1985. Amino acid biosynthesis and sodium-dependent transport in *Methanococcus voltae*, as revealed by ^{13}C NMR. Eur. J. Biochem. **149**:437–444.
6. **Ekiel, I., I. C. P. Smith, and G. D. Sprott.** 1983. Biosynthetic pathways in *Methanospirillum hungatei* as determined by ^{13}C nuclear magnetic resonance. J. Bacteriol. **156**:316–326.
7. **Ferrante, G., I. Ekiel, G. B. Patel, and G. D. Sprott.** 1988. A novel core lipid isolated from the aceticlastic methanogen, *Methanothrix concilii* GP6. Biochim. Biophys. Acta **963**:173–182.
8. **Ferrante, G., I. Ekiel, and G. D. Sprott.** 1986. Structural characterization of the lipids of *Methanococcus voltae*, including a novel N-acetylglucosamine 1-phosphate diether. J. Biol. Chem. **261**:17062–17066.
9. **Ferrante, G., I. Ekiel, and G. D. Sprott.** 1987. Structures of diether lipids of *Methanospirillum hungatei* containing novel head groups N,N-dimethylamino- and N,N,N-trimethylaminopentanetetrol. Biochim. Biophys. Acta **921**:281–291.
10. **Ferrante, G., J. C. Richards, and G. D. Sprott.** 1990. Structures of polar lipids from the thermophilic, deep-sea archaeobacterium *Methanococcus jannaschii*. Biochem. Cell Biol. **68**:274–283.
11. **Jones, W. J., J. A. Leigh, F. Mayer, C. R. Woese, and R. S. Wolfe.** 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. **136**:254–261.
12. **Jones, W. J., C. E. Stugard, and H. W. Jannasch.** 1989. Comparison of thermophilic methanogens from submarine hydrothermal vents. Arch. Microbiol. **151**:314–318.
13. **Kates, M.** 1978. The phytanyl ether-linked polar lipids and isoprenoid neutral lipids of extremely halophilic bacteria. Prog. Chem. Fats Other Lipids **15**:302–342.
14. **Kates, M.** 1986. Techniques of lipidology: isolation, analysis and identification of lipids, 2nd revised ed. North-Holland Publishing Co., Amsterdam, The Netherlands.
15. **Kates, M.** 1988. Structure, physical properties, and function of archaebacterial lipids, p. 357–384. *In* M. L. Karnovsky, A. M. Leaf, and L. C. Bolis (ed.)., Biological membranes: aberrations in membrane structure and function. Alan R. Liss, Inc., New York.
16. **Kushwaha, S. C., and M. Kates.** 1981. Modification of phenol-sulfuric acid method for the estimation of sugars in lipids. Lipids **16**:372–373.
17. **Kushwaha, S. C., M. Kates, G. D. Sprott, and I. C. P. Smith.** 1981. Novel polar lipids from the methanogen *Methanospirillum hungatei* GP1. Biochim. Biophys. Acta **664**:156–173.
18. **Langworthy, T. A., and J. L. Pond.** 1986. Membranes and lipids of thermophiles, p. 107–135. *In* T. D. Brock (ed.), Thermophiles: general, molecular, and applied microbiology. Wiley Interscience, New York.
19. **Nishihara, M., H. Morii, and Y. Koga.** 1989. Heptads of polar ether lipids of an archaebacterium, *Methanobacterium thermoautotrophicum*: structure and biosynthetic relationship. Biochemistry **28**:95–102.
20. **Prado, A., M. S. DaCosta, and V. M. C. Madeira.** 1988. Effect of growth temperature on the lipid composition of two strains of *Thermus* sp. J. Gen. Microbiol. **134**:1653–1660.
21. **Sprott, G. D., I. Ekiel, and C. Dicaire.** 1990. Novel, acid-labile, hydroxydiether lipid cores in methanogenic bacteria. J. Biol. Chem. **265**:13735–13740.
22. **Tornabene, T. G., and T. A. Langworthy.** 1979. Diphytanyl and dibiphytanyl glycerol ether lipids of methanogenic archaebacteria. Science **203**:51–53.