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NOTE

2,6,10,15,19-Pentamethylicosenes in *Methanolobus bombayensis*, a marine methanogenic archaeon, and in *Methanosarcina mazei*

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Abstract—2,6,10,15,19-Pentamethylicosenes (PMEs) containing three to five double bonds have been found in the methanogenic archaea *Methanosarcina mazei* (DSM 3338), a strain isolated from sewage sludge, and in *Methanolobus bombayensis* (OCM 438), a non-extremophilic archaeon isolated from a marine sediment. This finding gives additional support for the use of compounds with the PME carbon skeleton as markers for methanogenic activity in marine environments. © 1997 Elsevier Science Ltd

Key words—2,6,10,15,19-pentamethylicosane, methanogenic archaea, *Methanolobus bombayensis*, *Methanosarcina mazei*, archaeal biomarkers

INTRODUCTION

Steroids and hopanoids are specific biomarkers for eukaryotes and prokaryotes, respectively (e.g. Ratledge and Wilkinson, 1989). Biomarkers for the third domain of life, archaea, include ether-linked lipids with isoprenoid side chains such as phytanyl (**I**) and biphytanyl (**II**) (e.g. de Rosa and Gambacorta, 1988). These compounds have been encountered frequently in hypersaline sediments, where they have been attributed to halophilic archaea (e.g. Teixidor *et al.*, 1993), and in lacustrine sediments, where they have been attributed to methanogenic archaea (e.g. Pauly and van Vleet, 1986).

Another compound considered to be a biomarker for methanogenic archaea is the irregular tail-to-tail isoprenoid 2,6,10,15,19-pentamethylicosane (PME*, **III**; Brassell *et al.*, 1981). This assumption was based on the identification of PME and 2,6,10,15,19-pentamethylicosanes with one to four double bonds in several methanogens, including the thermophilic methanogenic archaeon *Methanobacterium thermoautotrophicum* (Holzer *et al.*, 1979; Tornabene *et al.*, 1979). However, Risatti *et al.* (1984) showed, using authentic standards, that this thermophilic archaeon (strain ΔH) indeed biosynthesizes C_{25} isoprenoid alkenes with one to two double bonds but possesses the 2,6,10,14,18-pentamethylicosane carbon skeleton (**IV**). The reason for these contradictory findings is unclear and may be due to the use of different strains of *Methanobacterium thermoautotrophicum* or different culture conditions. However, the mesophilic methanogenic archaeon *Methanosarcina barkeri*, which is considered to be predominantly a freshwater species (Maestrojuan *et al.*, 1992) but which can also live under more saline conditions (Sowers and Gunsalus, 1988), was unambiguously shown to biosynthesize PME (Holzer *et al.*, 1979; Risatti *et al.*, 1984). Holzer *et al.* (1979) also reported the tentative identification of pentamethylicosenes in low amounts in *Methanobacterium ruminantium*, a methanogenic archaeon isolated from bovine rumen, and *Methanococcus vannielii*, a mesophilic methanogenic archaeon.

PME (**III**) is frequently encountered in the marine water column (e.g. Wakeham, 1990) and in marine sediments (e.g. Brassell *et al.*, 1981; Kohnen *et al.*, 1992). In these environments its origin remains unclear since organisms which biosynthesize PME, and commonly live under non-extreme marine conditions, have not yet been conclusively identified. The stable carbon isotopic composition of PME is sometimes similar to that of photoautotrophic biomarkers (Kohnen *et al.*, 1992; Freeman *et al.*, 1994), which suggests an origin other than from

*Earlier literature refer to this compound as 2,6,10,15,19-pentamethyleicosane. However, IUPAC rules are now different but for reason of compatibility the acronym PME is still used.

Table 1. Cultured methanogenic archaea

Species	Culture no.	Origin
Methanosarcina mazei	DSM 3338	Sewage sludge
Methanolobus bombayensis	OCM 438	Sea sediment, Indian Ocean
Methanosarcina acetivorans	DSM 2834	Marine mud, U.S.A.
Methanococcoidus methylutens	DSM 2657	Marine sediment, U.S.A.
Methanosarcina sp. MPT4	DSM 6636	Salt marsh sediment, France
Methanosarcina siciliae	DSM 3028	Lake sediment, Sicily

methanogenic archaea. However, in other instances the ^{13}C content of PME may differ substantially from that of photoautotrophic biomarkers (Schouten *et al.*, in press).

Here we report the presence of unsaturated compounds with the PME carbon skeleton not only in a strain of *Methanosarcina mazei*, but also in the marine methanogenic archaeon *Methanolobus bombayensis*, confirming previous ideas on a methanogenic archaeal origin for compounds possessing the PME carbon skeleton in marine environments.

EXPERIMENTAL

Culture conditions

Cell masses of *Methanosarcina mazei* G1 were grown at 37°C with stirring (50 rpm) at pH 6.9 in a 100 l enamel-protected fermentor (HTE Bioengineering, Wald, Switzerland) pressurized with 200 kPa N_2/CO_2 (80:20, v/v). For cultivation, modified M3-medium (Balch *et al.*, 1979) supplemented with methanol (0.5%, final concentration) was used. Cells were harvested by continuous centrifugation (3000 rpm).

The following strains (see Table 1) were cultivated according to the 1993 catalog of the Deutsche Sammlung von Mikroorganismen und Zellkulturen: *Methanosarcina acetivorans* and *Methanosarcina* sp. strain MTP4 (30°C), *Methanosarcina siciliae* and *Methanococcoides methylutens* (30°C). *Methanolobus bombayensis* (30°C) was grown in mineral medium according to the Oregon Collection of Methanogens guidelines. All strains were grown on a combination of (tri)methylamine (50 mM) and methanol (50 mM) as growth substrates at 37°C, unless otherwise indicated. Cells were harvested by centrifugation at 16 000 *g* for 10 min and the pellet was washed twice with a 25 mM potassium phosphate buffer (pH 7.1) containing 30 g/l NaCl and 3.5 g/l $MgCl_2.6H_2O$. After washing, the pellet was resuspended in 1 ml sterile doubly-distilled water. Subsequently the cells were lyophilized.

Analysis of lipids

The freeze-dried biomass was ultrasonically extracted with methanol (3×), methanol/dichloromethane (DCM) (1:1, v/v; 3×) and DCM (3×). The extracts were combined and separated into an apolar and a residual fraction using column chromatography (Al_2O_3 as stationary phase) with hexane/DCM (9:1, v/v) and DCM/methanol (1:1, v/v) as eluents. Fractions were hydrogenated by dissolving in ethyl acetate, adding PtO_2 and bubbling H_2 through for 1 h. The mixture was then stirred for an additional 24 h. The samples were analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS).

Gas chromatography

GC was performed using a Hewlett-Packard 5890 equipped with an on-column injector. A fused-silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (film thickness 0.12 μm) was used with helium as carrier gas. A flame ionization detector (FID) was used for detection. The samples were dissolved in ethyl acetate and injected at 70°C. Subsequently the oven was programmed to 130°C at 20°C/min and then at 4°C/min to 320°C, at which it was held for 15 min.

Gas chromatography–mass spectrometry

GC–MS was performed using a Hewlett-Packard 5890 gas chromatograph interfaced with a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of m/z 40–800 and a cycle time of 1.7 s (resolution 1000). The gas chromatograph was equipped with a fused-silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (film thickness 0.2 μm). The carrier gas was helium. The samples were injected on column at 60°C and subsequently the oven was programmed to 130°C at 20°C/min and then at 4°C/min to 300°C at which it was held for 10 min.

RESULTS AND DISCUSSION

Six different methanogenic archaea were cultured and analysed for apolar lipids (see Table 1). Only two cultures, *Methanosarcina mazei* and *Methanolobus bombayensis*, were found to contain significant amounts of apolar hydrocarbons.

Table 2. Pseudo Kovats indices of unsaturated PMEs

Archaea	Number of double bonds	Pseudo Kovats index (CP Sil 5)
Methanosarcina mazei	4	2336
	5	2347
Methanolobus bombayensis	3	2290, 2314
	4	2326, 2333
	5	2347, 2366

Methanosarcina mazei, a strain isolated from sewage sludge, was found to contain only a limited number of compounds which, on the basis of their mass spectra and retention indices (Table 2), are thought to be 2,6,10,15,19-pentamethylicosenes containing four or five double bonds (Figs 1 and 2B). The carbon skeleton of these compounds was confirmed by hydrogenation of the apolar fraction,

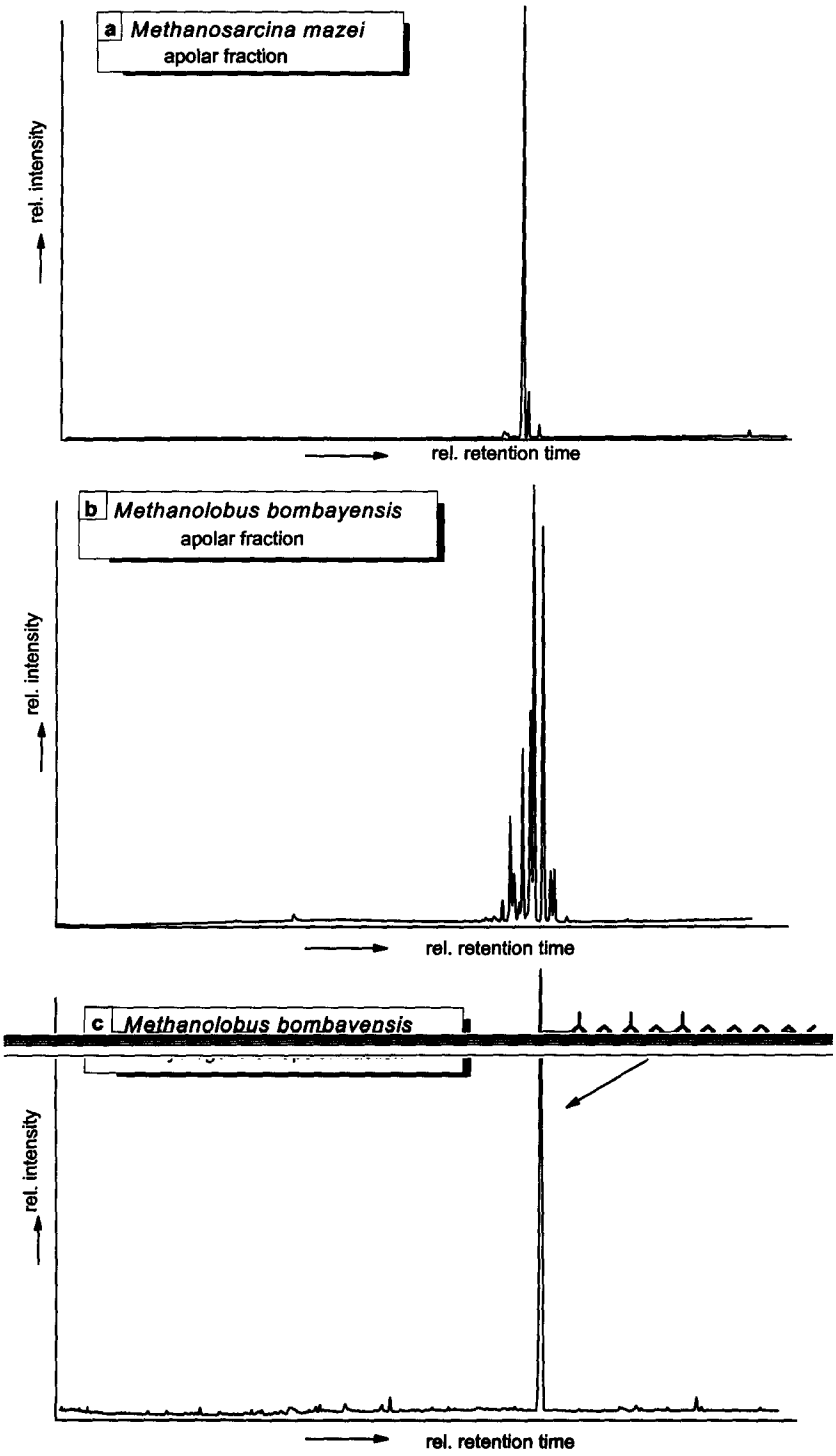

Fig. 1. Gas chromatograms of (a) apolar fraction of lipid extract from *Methanosarcina mazei*, (b) apolar fraction of lipid extract from *Methanolobus bombayensis* and (c) hydrogenated apolar fraction of lipid extract from *Methanolobus bombayensis*.

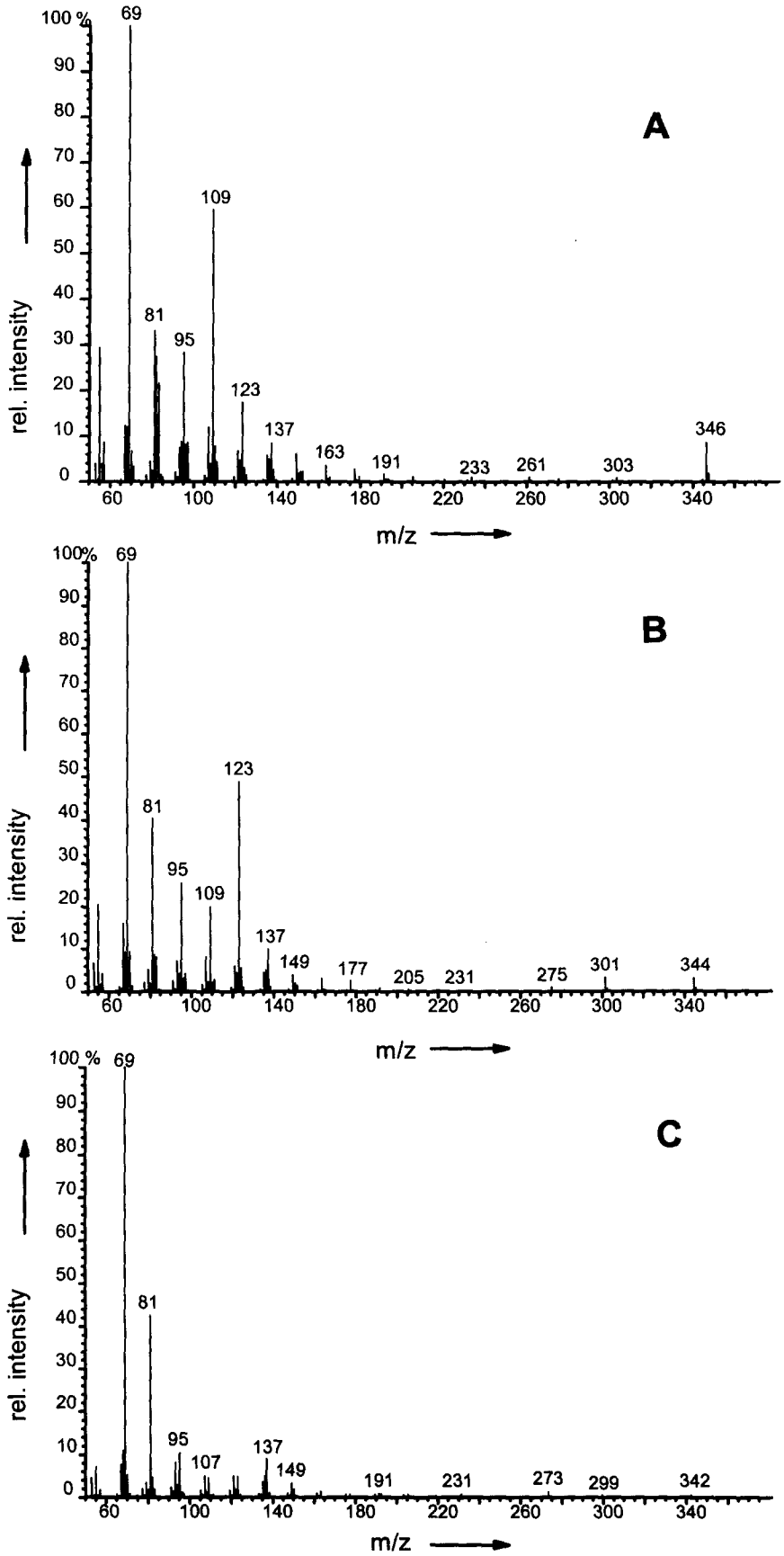

Fig. 2. Mass spectrum of (A) 2,6,10,15,19-pentamethylicosene with three double bonds present in *Methanolobus bombayensis*, (B) 2,6,10,15,19-pentamethylicosene with four double bonds present in *Methanosarcina mazei* and (C) 2,6,10,15,19-pentamethylicosene with five double bonds present in *Methanolobus bombayensis*.

which yielded PME as the only compound as identified by comparison with the mass spectrum and retention index of synthetic PME (Rowland *et al.*, 1982; Risatti *et al.*, 1984). When the apolar fraction of the extract of *Methanolobus bombayensis* was analysed, a cluster of compounds was found which were identified as 2,6,10,15,19-pentamethylicosenes containing three to five double bonds (Fig. 1b, 2b and 2c). Indeed, hydrogenation of the fraction yielded PME as the only compound (Fig. 1c). *Methanosarcina* sp. MPT4 and *Methanosarcina siciliae* contain only trace amounts of pentamethylicosenes with three and four double bonds, respectively, but their low amounts prevented any firm identification through hydrogenation of the fractions. The apolar fractions of the lipid extracts of *Methanosarcina acetivorans* and *Methanococcoidus methylutens* did not contain any significant amounts of apolar hydrocarbons.

The identification of alkenes with the PME carbon skeleton in *Methanosarcina mazei*, a species which can thrive under mesophilic conditions (Maestrojuan *et al.*, 1992), and, more importantly, in *Methanolobus bombayensis*, an archaeon isolated from a marine sediment of the Indian Ocean (Kadam *et al.*, 1994), gives additional support for the hypothesis that compounds with this carbon skeleton can serve as a marker for methanogenic activity in marine environments (Brassell *et al.*, 1981). Previous unambigous identifications of compounds possessing the PME carbon skeleton were either in an archaeon living at temperatures of 60–80°C (*Methanobacterium thermoautotrophicum*) or in archaea living predominantly in freshwater or mesophilic conditions (*Methanosarcina barkeri*, *Methanobacterium ruminantum*, *Methanococcus vannielii*). Although it cannot be excluded that other organisms are biosynthesizing this compound, methanogenic archaea are the only organisms known to date which biosynthesize this particular carbon skeleton. Furthermore, it has been shown for a few marine sediments that the stereochemistry of the sedimentary PME is in full accordance with a biological origin from methanogenic archaea (Rowland *et al.*, 1982; Risatti *et al.*, 1984). The fact that this compound has been found in oxygenated upper parts of the water column (Wakeham, 1990) does not invalidate this hypothesis since methanogenic archaea have been isolated from such environments (Cynar and Yayanos, 1991; Sieburth, 1993); also methanogenic activity is known to occur in oxygen-rich waters (Karl and Tilbrook, 1994), possibly in the guts of zooplankton or in anoxic microenvironments inside particulate organic matter.

To date, only PME has been reported in sediments or in the water column (e.g. Brassell *et al.*, 1981; Wakeham, 1990; Kohnen *et al.*, 1992) whilst there are no reports of unsaturated PMEs or of sulphur-bound PME (incorporation of sulphur requires the presence of functionalities in the original lipid; e.g. Kohnen *et al.*, 1992). Our results and those of Tornabene *et al.* (1979) show that methanogens in culture produce abundant unsaturated PMEs, although Risatti *et al.* (1984) found only the saturated compound in their culture of *Methanosarcina barkeri*. It is unclear why in their case and possibly also in the natural environment only PME is biosynthesized. This may be due to the particular environments where they thrive or due to the nutrients and carbon sources supplied to the methanogenic archaea. Growth of methanogen cultures under different conditions may help to explain this observation.

CONCLUSION

2,6,10,15,19-Pentamethylicosenes containing three to five double bonds have been found in high amounts in the methanogenic archaeon *Methanosarcina mazei*, a strain isolated from sewage sludge, and in *Methanolobus bombayensis*, a strain isolated from marine sediments of the Indian Ocean. This result gives additional support to the contention that compounds possessing a PME carbon skeleton are biomarkers for past methanogenic activity in marine environments.

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APPENDIX