Methanolobus profundus sp. nov., a methylotrophic methanogen isolated from deep subsurface sediments in a natural gas field

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A mesophilic, methylotrophic methanogen, strain MobM^T, was isolated from a natural gas field in Japan. Strain MobM^T grew on methanol and methylamines, but not on H₂/CO₂, formate, acetate or dimethyl sulfide. The cells were motile, irregular cocci (diameter, 0.9–1.2 µm) and occurred singly, in pairs, as tetrads or (occasionally) as aggregates. Strain MobM^T grew at 9–37 °C (optimally at 30 °C) and at pH 6.1–7.8 (optimally at pH 6.5). Sodium and magnesium were required for growth, at 0.1–1.0 M Na⁺ (optimally at 0.35 M) and 10–400 mM Mg²⁺ (optimally at 15–25 mM). The G + C content of the genomic DNA was 42.4 mol%. 16S rRNA gene sequencing revealed that the isolate is a member of the genus *Methanolobus*, but distinct from its closest neighbours, *Methanolobus tindarius* DSM 2278^T (sequence similarity, 98.0 %) and *Methanolobus vulcani* DSM 3029^T (98.1 %). On the basis of phenotypic and phylogenetic features of MobM^T, it is clear that this strain represents a novel species of the genus *Methanolobus*, for which the name *Methanolobus profundus* sp. nov. is proposed. The type strain is MobM^T (=DSM 21213^T=NBRC 104158^T).

The genus *Methanolobus* comprises coccoid, methylotrophic methanogens that grow optimally in media containing approximately 0.5 M NaCl (Boone *et al.*, 2001; Table 1). To date, five species have been described; all have been found in various saline environments. *Methanolobus bombayensis*, *Methanolobus oregonensis* and *Methanolobus taylorii* can use dimethyl sulfide as a substrate for methanogenesis (Kadam *et al.*, 1994; Liu *et al.*, 1990; Oremland & Boone, 1994), unlike the other two species, *Methanolobus tindarius* and *Methanolobus vulcani* (Konig & Stetter, 1982; Kadam & Boone, 1995). In this study, a slightly halophilic, methylotrophic methanogen, designated strain MobM^T, was isolated from subsurface sediments below 350 m in the Minami-Kanto Gas

Field (Mobara, Chiba prefecture, Japan). This natural gas field is a dissolved-in-water type, and analyses of the stable carbon (¹³C/¹²C) and deuterium/hydrogen (D/H) isotopic composition of the methane and the ratio of methane to ethane and propane suggest that the methane is biogenic in origin (Igari & Sakata, 1989). The reservoir rocks are turbidite sandstones deposited around 1 Ma (million years ago) in a bathyal environment, being filled with ancient seawater (Sudo, 1967; Kunisue *et al.*, 2002). The chemical composition of the formation water was conspicuously different from that of common seawater, i.e. it contained large amounts of iodine, bicarbonate and ammonia along with negligible amounts of sulfate. Culture-independent analysis of archaeal 16S rRNA gene sequences revealed that the methanogenic community residing in the formation water is diverse and includes close relatives of members of the genera *Methanolobus*, *Methanohalophilus*, *Methanosaeta*, *Methanocalculus*, *Methanobacterium* and *Methanococcus* (Mochimaru *et al.*, 2007). Using a culture-

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MobM^T is AB370245.

A supplementary figure showing the effects of variations in temperature and salinity on the specific growth rate of strain MobM^T is available with the online version of this paper.

Table 1. Differential characteristics for strain MobM^T and species in the genus *Methanobolus*

Taxa: 1, MobM^T (this study); 2, *M. vulcani* PL-12/M^T (Kadam & Boone, 1995); 3, *M. tindarius* Tindari 3^T (Konig & Stetter, 1982); 4, *M. taylorii* GS-16^T (Oremland & Boone, 1994); 5, *M. oregonensis* WAL1^T (Liu *et al.*, 1990); 6, *M. bombayensis* B-1^T (Kadam *et al.*, 1994). All strains were positive for utilization of methanol and methylamines. +, Positive result; –, negative result; ND, not determined.

Characteristic	1	2	3	4	5	6
Habitat	Saline, deep subsurface sand	Sea sediments	Marine black sediment	Estuarine sediments	Saline, alkaline aquifer	Sea sediments
Cell diameter (µm)	0.9–1.2	1.0–1.25	0.8–1.25	0.5–1.0	1.0–1.5	1.0–1.5
Flagella	+	–	+	–	–	–
Optimum growth conditions (range allowing growth):						
Temperature (°C)	30 (9–37)	37 (13–45)	25 (10–45)	37 (11–40)	35–37 (20–42)	37 (22–40)
pH	6.5 (6.1–7.8)	7.2 (6.0–7.5)	6.5 (5.5–8.0)	8.0 (6.8–9.0)	8.6 (7.6–9.4)	7.2 (6.5–8.0)
Na ⁺ concentration (M)	0.35 (0.1–1.0)	0.5 (0.1–1.2)	0.47 (0.06–1.27)	0.5 (0.2–1.2)	0.48 (0.1–1.6)	0.5 (0.3–2.0)
Mg ²⁺ concentration (mM)	15–25 (10–400)	13 (0.5–85)*	ND	40 (13–40)*	40 (13–220)*	33 (30–80)*
Utilization of dimethyl sulfide	–	–	–	+	+	+
Growth factor(s)	–	Biotin	–	Biotin	Biotin, thiamine†	–
Stimulation by yeast extract and/or peptone	–‡	+	–	+	+	+
DNA G + C content (mol%)	42.4	39	45.9	40.8	40.9	39.2
16S rRNA gene sequence similarity (%)* with respect to strain MobM ^T	–	98.1	98.0	97.3	96.9	97.1

*Data are from Kadam & Boone (1995).

†Data are from Oremland & Boone (1994).

‡Growth was inhibited slightly.

based method, we observed that methanol was converted to methane and that a *Methanobolus*-like methanogen dominated the primary enrichment culture (Mochimaru *et al.*, 2007), indicating the presence of viable methylotrophic methanogens in the subsurface gas reservoir. A methanogen inhabiting the subsurface zone in the natural gas field was then isolated.

A sample for isolation purposes was obtained from a sand separator in the commercial gas/water-producing well at Mobara in June 2003. The sample, comprising sediment and formation water, came through the side-facing slit of the well at a depth of 347–795 m. The temperature of the formation water was 22 °C. The pH was 8.0 and the redox potential was –232 mV.

To enrich for methanogens, saline mineral medium, as described by Sekiguchi *et al.* (2000), was used, but with the following slight modifications: the concentration of MgCl₂·6H₂O was changed to 15 mM, and 350 mM NaCl was added. Enrichment was carried out at 25 °C in 50 ml serum vials containing approximately 10 ml sediment sample and 20 ml medium (pH 7.0 at 25 °C) with 20 mM methanol as the sole catabolic substrate, under an atmosphere of N₂/CO₂ (80:20, v/v).

Growth in the enrichments was confirmed by the formation of methane, and cultures showing methane production were transferred to fresh medium periodically.

The concentration of methane in the headspace of the bottle was measured at intervals of several weeks, using a gas chromatograph (GC-8A; Shimadzu) equipped with a thermal conductivity detector and a 60/80-mesh Unibeads column.

Isolation of strain MobM^T was accomplished from repeated inoculations using plates containing 0.8 % agar with 20 mM methanol and vancomycin (50 µg ml^{–1}) under an atmosphere of N₂/CO₂ (80:20, v/v). Visible colonies approximately 1 mm in diameter were produced after incubation at 30 °C for 10 days. Colonies on the surface of solid medium were circular with entire margins, shiny, convex and yellowish-white.

Cells in the late-exponential phase were observed by means of phase-contrast and epifluorescence microscopy, using an epifluorescence microscope (AX80; Olympus). Under phase-contrast microscopy, it was apparent that cells of the isolate were irregular cocci 0.9–1.2 µm in diameter. Cells occurred singly, in pairs, as tetrads or (occasionally) as aggregates (Fig. 1a). Under phase-contrast microscopy, a few cells showed motility. Transmission electron microscopy was performed with a Hitachi H7000 microscope (Nakamura *et al.*, 2006). Negative staining indicated that cells of strain MobM^T each have multiple flagella (Fig. 1b). In thin-section electron micrographs, neither an outer membrane nor a thick cell wall was observed (Fig. 1c).

Fig. 1. Phase-contrast photomicrograph (a), electron micrograph of negatively stained cells (b) and electron micrograph of a thin section (c) of cells of strain MobM^T in the late-exponential growth phase. Bars, 10 µm (a); 1 µm (b, c).

The Gram reaction and tests of susceptibility to lysis by SDS were performed as described previously (Boone & Whitman, 1988). The Gram reaction of strain MobM^T was negative and cells lysed in a 0.01 % (w/v) SDS solution and in a hypotonic solution (distilled water).

The growth effects of variations in temperature, pH and concentrations of Na⁺ and Mg²⁺ were tested in medium containing 20 mM methanol under an atmosphere comprising N₂/CO₂ (80:20, v/v). Duplicate cultures (1 % inoculum) were incubated under each set of conditions and the OD₆₀₀ was measured with an Ultrospec 500 pro (GE Healthcare) to ascertain growth rates. To evaluate the effect of temperature on growth, duplicate cultures were incubated at temperatures in the range 4–40 °C. Strain MobM^T grew at 9–37 °C and pH 7.0, with an optimum growth temperature of 30 °C (Supplementary Fig. S1a,

available in IJSEM Online). No growth occurred at 4 or 40 °C within 4 months. The isolate grew at pH 6.1–7.8, with an optimum at pH 6.5 (initial pH of culture medium). No growth was observed at pH 5.5 or 8.1 within 2 months. Strain MobM^T grew in the presence of 0.1–1.0 M Na⁺, the optimum concentration being 0.35 M (Supplementary Fig. S1b). It also grew in the presence of 10–400 mM Mg²⁺, the optimum concentration being 15–25 mM. No growth was observed in medium supplemented with 0 or 1.5 M Na⁺ or with 5 or 500 mM Mg²⁺ within 2 months incubation. The doubling time for the culture on methanol under the optimum conditions was 5 h.

Strain MobM^T used methanol (20 mM), monomethylamine (20 mM), dimethylamine (20 mM) and trimethylamine (20 mM) as energy and carbon sources. Growth and methane formation were not observed on the following substrates: H₂/CO₂ (80:20, v/v; 100 kPa), acetate (20 mM), formate (20 mM), ethanol (20 mM) and dimethyl sulfide (5 mM). The addition of yeast extract (2 g l⁻¹) or peptone (2 g l⁻¹) inhibited growth slightly. Acetate (5 mM) did not stimulate growth in the presence of methanol. The isolate did not require any vitamins and did not require tungsten or selenium.

The sensitivities of strain MobM^T to antibiotics such as ampicillin, penicillin G, vancomycin, kanamycin and tetracycline (each at a final concentration of 100 µg ml⁻¹) were tested at 30 °C (pH 7.0). Strain MobM^T was resistant to all five antibiotics, but growth in the presence of tetracycline was extremely slow.

The G + C content of the genomic DNA was determined by means of HPLC (LC-10A; Shimadzu) with a UV detector (Kamagata & Mikami, 1991): the value obtained was 42.4 mol%.

The 16S rRNA gene of strain MobM^T was amplified using a PCR with a forward primer (*Escherichia coli* positions 8–27; Kamagata *et al.*, 1997) and a slightly modified version of universal primer 1490R (Weisburg *et al.*, 1991) and sequenced as described previously (Mochimaru *et al.*, 2007). The phylogenetic tree obtained by using almost-complete 16S rRNA gene sequences (1405 bp) indicated that strain MobM^T was closely related to *M. tindarius* DSM 2278^T and *M. vulcani* DSM 3029^T, having sequence similarity of 98.0 and 98.1 %, respectively (Table 1; Fig. 2).

Strain MobM^T and its closest phylogenetic relatives, *M. tindarius* and *M. vulcani*, are all methylotrophic methanogens unable to use dimethyl sulfide as a catabolic substrate (Table 1). Although these three methanogens have similar cell sizes and pH and NaCl requirements, there are significant differences among them. The genomic G + C content of MobM^T clearly differs from those of *M. tindarius* and *M. vulcani*. The maximum growth temperature is the most remarkable feature distinguishing strain MobM^T from the two related species: MobM^T cannot tolerate temperatures above 40 °C (Supplementary Fig.

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain MobM^T with respect to members of the genus *Methanobus* and related taxa. The tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with the CLUSTAL_X software package (Thompson *et al.*, 1997). The tree topologies were evaluated by bootstrap analysis (based on 1000 replicates) with the neighbour-joining/maximum-parsimony/maximum-likelihood methods, using the PAUP* package (Swofford, 2003). *Methanococcus aeolicus* was used as an outgroup (not shown). Bar, 2 substitutions per 100 nt.

S1a, available in IJSEM Online), whereas *M. tindarius* and *M. vulcani* are able to grow at temperatures up to 45 °C. The other distinctive feature of MobM^T was that the optimum NaCl concentration (0.35 M) was slightly lower (Supplementary Fig. S1b) than those (0.48–0.5 M) of all other known species within the genus *Methanobus*. This may reflect the fact that the *in situ* NaCl concentration of the gas-associated ancient seawater (0.2 M) in which the isolate resides is slightly lower than that in seawater (0.5 M) (Mochimaru *et al.*, 2007).

On the basis of phenotypic and phylogenetic data, therefore, strain MobM^T represents a novel species of the genus *Methanobus*, for which the name *Methanobus profundus* sp. nov. is proposed.

Description of *Methanobus profundus* sp. nov.

Methanobus profundus sp. nov. (pro.fun'di. L. gen. n. *profundi* of an abyss).

Cells are irregular, motile cocci (diameter, 0.9–1.2 µm) and occur singly, in pairs, in tetrads and in aggregates. Strictly anaerobic. Gram-negative. Lysed by 0.01 % (w/v) SDS and hypotonic solution (distilled water). Colonies on the surface of solid medium are circular with entire margins, shiny, convex and yellowish-white. Methane is produced from methanol, monomethylamine, dimethylamine and trimethylamine, but not from H₂/CO₂, formate, acetate, ethanol or dimethyl sulfide. Growth occurs between 9 and 37 °C, with an optimum at 30 °C. The pH range for growth is 6.1–7.8 and the optimum is pH 6.5. Sodium and magnesium are required for growth: the ranges are 0.1–1.0 M Na⁺ (optimum, 0.35 M) and 10–400 mM Mg²⁺ (optimum, 15–25 mM). Vitamins, tungsten and selenium are not required. Resistant to ampicillin, penicillin G, vancomycin, kanamycin and tetracycline at concentrations of at least 100 µg ml⁻¹. The DNA G+C content of strain MobM^T is 42.4 mol%.

The type strain, MobM^T (=DSM 21213^T=NBRC 104158^T), was isolated in Mobara, Japan, from deep subsurface sediments from a natural gas field filled with ancient seawater.

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