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

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A mesophilic, methylotrophic methanogen, strain MobMT, was isolated from a natural gas field in Japan. Strain MobMT 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 \(\mu\)m) and occurred singly, in pairs, as tetracocci or (occasionally) as aggregates. Strain MobMT 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 2278T (sequence similarity, 98.0 %) and Methanolobus vulcani DSM 3029T (98.1 %). On the basis of phenotypic and phylogenetic features of MobMT, it is clear that this strain represents a novel species of the genus Methanolobus, for which the name Methanolobus profundi sp. nov. is proposed. The type strain is MobMT (=DSM 21213T=NBRC 104158T).

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 MobMT, 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 (\(^{13}C/^{12}C\)) 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, Methanoseta, Methanocalculus, Methanobacterium and Methanococcus (Mochimaru et al., 2007). Using a culture-
Table 1. Differential characteristics for strain MobMT\textsuperscript{T} and species in the genus Methanolobus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td><strong>Taxa:</strong></td>
<td>1, MobMT\textsuperscript{T} (this study); 2, M. vulcani PL-12/MMT (Kadam &amp; Boone, 1995); 3, M. tindarius Tindari 3\textsuperscript{T} (Konig &amp; Stetter, 1982); 4, M. taylorii GS-16\textsuperscript{T} (Oremland &amp; Boone, 1994); 5, M. oregonensis WAL1\textsuperscript{T} (Liu et al., 1990); 6, M. bombayensis B-1\textsuperscript{T} (Kadam et al., 1994). All strains were positive for utilization of methanol and methylamines. +, Positive result; -, negative result; ND, not determined.</td>
<td></td>
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</tr>
<tr>
<td><strong>Habitat</strong></td>
<td>Saline, deep subsurface sand</td>
<td>Sea sediments</td>
<td>Marine black sediment</td>
<td>Estuarine sediments</td>
<td>Saline, alkaline aquifer</td>
<td>Sea sediments</td>
</tr>
<tr>
<td><strong>Cell diameter (µm)</strong></td>
<td>0.9–1.2</td>
<td>1.0–1.25</td>
<td>0.8–1.25</td>
<td>0.5–1.0</td>
<td>1.0–1.5</td>
<td>1.0–1.5</td>
</tr>
<tr>
<td><strong>Flagella</strong></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Optimum growth conditions</strong> (range allowing growth):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td>30 (9–37)</td>
<td>37 (13–45)</td>
<td>25 (10–45)</td>
<td>37 (11–40)</td>
<td>35–37 (20–42)</td>
<td>37 (22–40)</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>6.5 (6.1–7.8)</td>
<td>7.2 (6.0–7.5)</td>
<td>6.5 (5.5–8.0)</td>
<td>8.0 (6.8–9.0)</td>
<td>8.6 (7.6–9.4)</td>
<td>7.2 (6.5–8.0)</td>
</tr>
<tr>
<td><strong>Na\textsuperscript{+}</strong> concentration (M)**</td>
<td>0.35 (0.1–1.0)</td>
<td>0.5 (0.1–1.2)</td>
<td>0.47 (0.06–1.27)</td>
<td>0.5 (0.2–1.2)</td>
<td>0.48 (0.1–1.6)</td>
<td>0.5 (0.3–2.0)</td>
</tr>
<tr>
<td><strong>Mg\textsuperscript{2+}</strong> concentration (mM)**</td>
<td>15–25 (10–400)</td>
<td>13 (0.5–85)*</td>
<td>ND</td>
<td>40 (13–40)*</td>
<td>40 (13–220)*</td>
<td>33 (30–80)*</td>
</tr>
<tr>
<td><strong>Utilization of dimethyl sulfide</strong></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Growth factor(s)</strong></td>
<td>–</td>
<td>Biotin</td>
<td>–</td>
<td>Biotin</td>
<td>Biotin, thiamine†</td>
<td>–</td>
</tr>
<tr>
<td><strong>Stimulation by yeast extract and/or peptone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DNA G + C content (mol%)</strong></td>
<td>42.4</td>
<td>39</td>
<td>45.9</td>
<td>40.8</td>
<td>40.9</td>
<td>39.2</td>
</tr>
<tr>
<td><strong>16S rRNA gene sequence similarity (%)</strong> with respect to strain MobMT\textsuperscript{T}</td>
<td>–</td>
<td>98.1</td>
<td>98.0</td>
<td>97.3</td>
<td>96.9</td>
<td>97.1</td>
</tr>
</tbody>
</table>

*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 Methanolobus-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 MgCl\textsubscript{2}, 6H\textsubscript{2}O 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 N\textsubscript{2}/CO\textsubscript{2} (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 MobMT\textsuperscript{T} was accomplished from repeated inoculations using plates containing 0.8% agar with 20 mM methanol and vancomycin (50 μg ml\textsuperscript{-1}) under an atmosphere of N\textsubscript{2}/CO\textsubscript{2} (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 tetracocci 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 MobMT\textsuperscript{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).
The Gram reaction and tests of susceptibility to lysis by SDS were performed as described previously (Boone & Whitman, 1988). The Gram reaction of strain MobMT 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 Mg\(^{2+}\) were tested in medium containing 20 mM methanol under an atmosphere comprising H\(_2\)/CO\(_2\) (80:20, v/v) 100 kPa, acetate (20 mM), formate (20 mM), ethanol (20 mM) and dimethyl disulfide (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 MobMT to antibiotics such as ampicillin, penicillin G, vancomycin, kanamycin and tetracycline (each at a final concentration of 100 \(\mu\)g ml\(^{-1}\)) were tested at 30 °C (pH 7.0). Strain MobMT 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 MobMT 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 MobMT 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).

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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 MobM² 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 *Methanolobus*. 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 MobM² represents a novel species of the genus *Methanolobus*, for which the name *Methanolobus profundi* sp. nov. is proposed.

**Description of Methanolobus profundi* sp. nov.**

*Methanolobus profundi* 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 tetracocci 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 range for growth is 6.1–7.8 and the optimum is pH 6.5.

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**References**


