**Methanomicrobium antiquum** sp. nov., a hydrogenotrophic methanogen isolated from deep sedimentary aquifers in a natural gas field

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A mesophilic, hydrogenotrophic methanogen, designated strain MobH1, was isolated from sediments derived from deep sedimentary, natural-gas-bearing aquifers in Japan. Strain MobH1 utilized H2/CO2 or formate, but not ethanol, 1-propanol, 2-propanol, 2-butanol or cyclopentanol, for growth and methane production. In addition, acetate and tungsten were required for growth. Yeast extract stimulated the growth, but was not required. The cells were weakly motile with multiple flagella, presented as a curved-rod-shaped (0.8×2.0 µm) and occurred singly or in pairs. Strain MobH1 grew at 15–40 °C (optimum 35 °C) and at pH 5.9–7.9 (optimum pH 7.0–7.5). The sodium chloride range for growth was 0–5.8% (optimum 2%). The G+C content of the genomic DNA was 37.6 mol%. In the phylogenetic tree based on the 16S rRNA gene sequences, strain MobH1 clustered together with Methanomicrobium mobile (95.4% in sequence similarity) and formed a distinct clade from Methanolacinia petrolearia SEBR 4847T (95.6%) and Methanolacinia paynteri G-2000T (95.4%). The two species of the genus Methanolacinia utilized 2-propanol, whereas strain MobH1 and Methanomicrobium mobile, the sole species of the genus Methanomicrobium, do not. Based on phenotypic and phylogenetic features, we propose a novel species for the isolate with the name, Methanomicrobium antiquum sp. nov. The type strain is MobH1 (=DSM 21220T=NBRC 104160T).

The family Methanomicrobiaceae currently comprises the following six genera: Methanoculleus, Methanolfis, Methanogenium, Methanolacinia, Methanomicrobiuim and Methanoplanus. All species within the family Methanomicrobiaceae are hydrogenotrophic methanogens that utilize H2 and CO2 as a substrate for methanogenesis. Because the species of the family Methanomicrobiaceae share many common phenotypic features, a phylogenetic analysis is a prerequisite for the taxonomic assignment of micro-organisms to genera and species (Boone et al., 2001). In fact, based on the phylogeny of 16S rRNA gene sequences, Methanoplanus petrolearius was recently reclassified as Methanolacinia petrolearia (Göker et al., 2014). In addition, Methanomicrobium paynteri (Rivard et al., 1983) was excluded from the genus Methanomicrobium and reclassified as Methanolacinia paynteri based primarily on a phylogenetic analysis of 16S rRNA gene sequences and substrate utilization (Zellner et al., 1989). Methanomicrobium mobile (Paynter & Hungate, 1968) has, therefore, constituted the sole species of the genus.

The Minami-Kanto gas field, in which natural gases are dissolved in formation water, is located near the centre of the Boso Peninsula, Japan. The reservoir rocks are turbidite sandstones, which were deposited approximately 1 million years ago in a bathyal environment and filled with ancient seawater (Sudo, 1967; Kunisu et al., 2002). The origin of the methane in the natural gas is considered biogenic based on its stable carbon and hydrogen isotopic composition as well as the molar ratio of methane to ethane and propane (Igari & Sakata, 1989; Katayama et al., 2015). Indeed, phylogenetically diverse
methanogens were present and active in the formation water collected at the Minami Kanto gas field (Mochimaru et al., 2007; Katayama et al., 2015). In this subsurface environment, Methanolobus profundi (Mochimaru et al., 2009) and Methanothermobacter hollolyticus (Katayama et al., 2014) have been isolated and proposed as a novel species. In the present study, we describe the isolation and characterization of a hydrogenotrophic methanogen, namely strain MobH, from the Minami-Kanto gas field.

The sediment and formation water samples were obtained from a commercial gas-water-producing well at Mobar, Chiba prefecture, Japan, as described by Mochimaru et al. (2007). The samples came from the reservoir rocks in the screened depth range of 347 to 795 m and were collected at a settling pond placed downstream of the production well to remove suspended sand particles from the formation water. The temperature, pH and the redox potential of the formation water sample were 22°C, pH 8.0 and −232 mV, respectively.

For the enrichment of the methanogens, we used the saline medium WS previously described by Sekiguchi et al. (2000) with slight modifications as follows: the concentration of MgCl2.6H2O was changed to 15 mM and 350 mM of NaCl was added. The water and sediment samples were dispensed in 10 ml aliquots into 50 ml serum vials containing 20 ml WS medium. The culture was supplemented with 20 mM ethanol as a sole catabolic substrate and incubated at 25°C under an atmosphere of N2/CO2 (80 : 20, v/v) without shaking. Ethanol was used for the primary enrichment of methanogens as an indirect substrate because direct substrates, such as H2/CO2, would stimulate the growth of fast-growing but less dominant methanogens, which would result in outcompeting slow-growing and dominant methanogens (Sakai et al., 2009; Kamagata, 2015).

Cultures showing methane production were periodically transferred to a fresh medium. The concentration of methane in the headspace of the bottle was measured by a GC-8A gas chromatograph (Shimadzu) equipped with a thermal conductivity detector and a Unibeads Column with 60/80 mesh (GL Science).

The microscopic observation showed that after seven passages, the ethanol enrichment culture contained at least two morphologically distinct organisms: (i) curved rod-shaped and (ii) thin plate-shaped. F200-autofluorescent methanogen-like cells. To isolate the methanogen cells from the ethanol enrichment culture, we used a serial dilution technique (Katayama & Kamagata, 2016) in liquid medium containing H2/CO2 (80 : 20, v/v; 100 kPa), acetic acid (20 mM), yeast extract (1 g l−1), ampicillin and vancomycin (each 100 µg ml−1). This procedure was repeated five times, and a pure culture of the curved-rod-shaped methanogen, strain MobH, was eventually obtained. Because the strain was not able to form colonies in the solid medium, the purity of the isolate was verified by cultivation in various media and 16S rRNA gene PCR amplification and sequencing. No growth was observed in the WS medium containing various substrates (10 mM lactate plus 10 mM sulfate, 10 mM acetate plus 10 mM sulfate, 0.1 g yeast extract l−1 plus 0.1 g Bacto peptone l−1, 10 mM glucose, 10 mM sucrose or 10 mM xylose). The DNA of the culture was extracted utilizing a Fast DNA kit (MP Biomedicals), and the 16S rRNA gene of strain MobH was amplified and directly and successfully sequenced with the universal archaeal primer pair Ar109f-1490R (Mochimaru et al., 2007). By contrast, no PCR amplification with the universal bacterial primer pair 8F-1490R (Weisburg et al., 1991) was observed. These results indicated that the culture of strain MobH was axenic.

The pure culture of strain MobH was incubated at 35°C in a medium containing H2/CO2 (80 : 20, v/v; 100 kPa), acetate (20 mM) and yeast extract (1 g l−1) without shaking unless indicated otherwise. Cells at the late-exponential phase and grown in a liquid culture were observed by phase-contrast microscopy and epifluorescence microscopy using an Olympus AX80 microscope. Transmission electron microscopy was performed with a Hitachi H7000 transmission electron microscope (Nakamura et al., 2006). The Gram reaction and the susceptibility to lysis by SDS were performed as previously described by Boone & Whitman (1988). The effects of temperature, pH and concentrations of NaCl on growth were examined in medium containing 20 mM acetate and 1 g yeast extract l−1 under an atmosphere of H2/CO2 (80 : 20, v/v). The effects of tungsten, acetate and yeast extract on growth were tested. The sensitivity of strain MobH to antibiotics, i.e. ampicillin, penicillin G, vancomycin, kanamycin and tetracycline (each at a final concentration of 100 µg ml−1) was examined. These experiments were performed in duplicate. Growth was measured by increasing the turbidity of the culture (OD600) using an Ultraspec 500 pro (GE Healthcare UK).

The DNA of strain MobH was extracted using a Fast DNA kit (MP Biomedicals). The G+C content of the genomic DNA was determined by HPLC (Shimadzu LC-10A) with a UV detector (Kamagata & Mikami, 1991). The 16S rRNA genes were amplified and sequenced using the archaeal primer pair Arc10f-1490R (Mochimaru et al., 2007). Cells of strain MobH were curved rods, 2.0 µm long and 0.8 µm wide, and occurred singly or in pairs (Fig. 1a). A few cells were weakly motile under a phase-contrast microscope, and multiple flagella were observed under negative-staining microscopy (Fig. 1b). A thin-section electron microscope showed the absence of an outer membrane and a thick cell wall (Fig. 1c). Cells of strain MobH were Gram-stained negative and lysed with 0.01% (w/v) SDS solution but not with hypotonic solution (distilled water). Strain MobH grew at 15–40°C with the optimum growth temperature of 35°C (Fig. S1a, available in the online Supplementary Material). Growth was observed at 15°C but not at 10°C or 45°C after 4 months of incubation. The isolate grew within a pH range of pH 5.9–7.9 with an optimum of pH 7.0–7.5 (the initial pH of culture media). No growth was observed at pH 5.4 or pH 8.8. Strain MobH grew in the presence of 0–5.8% NaCl (the optimum concentration was 2%) but not 8.7%...
NaCl (Fig. S1b). The doubling time in the culture under optimum conditions was 18 h. Strain MobH\textsuperscript{T} utilized H\textsubscript{2}/CO\textsubscript{2} (80:20, v/v; 100 kPa) or formate (20 mM) for methanogenesis. Growth and methane formation were not observed after 7 weeks of cultivation on the following substrates: methanol (20 mM), mono-, di- or trimethylamine (each 20 mM), dimethyl sulfide (5 mM), acetate (20 mM), ethanol (20 mM), 1-propanol (20 mM), 2-propanol (20 mM), 2-butanol (20 mM) or cyclopentanol (20 mM). Acetate (2 mM) and tungsten (0.01 µM) were required for growth. The addition of yeast extract (0.2 g l\textsuperscript{-1}) to culture stimulated the growth. Strain MobH\textsuperscript{T} was resistant to ampicillin, penicillin G, vancomycin, kanamycin and tetracycline. The G+C content of the genomic DNA was 37.6 mol%.

Strain MobH\textsuperscript{T} showed highest sequence similarities to Methanolacinia petrolearia SEBR 4847\textsuperscript{T} (95.6%), Methanolacinia paynteri G-2000\textsuperscript{T} (95.4%) and Methanomicrobium mobile BP\textsuperscript{T} (95.4%) in the 16S rRNA genes (Table 1). The phylogenetic tree of the 16S rRNA gene sequences showed that strain MobH\textsuperscript{T} clustered together with Methanomicrobium mobile and separated from members of the genus Methanolacinia, which was supported by high bootstrap values (Fig. 2). Consistent with this phylogenetic relationship, the two species of the genus Methanolacinia utilized 2-propanol as a sole carbon source, whereas both strain MobH\textsuperscript{T} and Methanomicrobium mobile did not (Table 1). In addition, strain MobH\textsuperscript{T} clearly differed from Methanomicrobium mobile in the DNA G+C content and the requirement of the growth factors.

On the basis of phenotypic and phylogenetic characteristics, we propose that strain MobH\textsuperscript{T} is classified as a representative of a novel species of the genus Methanomicrobium with the name Methanomicrobium antiquum sp. nov.

### Table 1. Comparison of the characteristics of strain MobH\textsuperscript{T} and related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Saline, deep subsurface</td>
<td>Bovine rumen</td>
<td>Marine sediment</td>
<td>Offshore oil field</td>
<td>Marine ciliate</td>
<td>Swamp</td>
</tr>
<tr>
<td>Shape</td>
<td>Curved rod</td>
<td>Curved rod</td>
<td>Irreg. rod</td>
<td>Irreg. disc</td>
<td>Irreg. disc</td>
<td>Plate</td>
</tr>
<tr>
<td>Cell dimensions (µm)</td>
<td>0.8×2.0</td>
<td>0.7×1.5–2.0</td>
<td>0.6×1.5–2.5</td>
<td>1–3</td>
<td>1.6–3.4</td>
<td>0.07–0.3 thick, 1.6–2.8×1.5</td>
</tr>
<tr>
<td>Optimum temperature (range) (°C)</td>
<td>35 (15–40)</td>
<td>40 (30–45)</td>
<td>40 (20–45)</td>
<td>37 (28–43)</td>
<td>32 (16–36)</td>
<td>40 (17–41)</td>
</tr>
<tr>
<td>Optimum pH (range)</td>
<td>7–7.5 (5.9–7.9)</td>
<td>6.1–6.9 (5.9–7.7)</td>
<td>7</td>
<td>7 (5.3–8.4)</td>
<td>6.8–7.3 (6.1–8.0)</td>
<td>6.5–7.5</td>
</tr>
<tr>
<td>Optimum Na\textsuperscript{+} concentration (range) (mM)</td>
<td>2 (0–5.8)</td>
<td>ND</td>
<td>0.9</td>
<td>1–3 (0–5)</td>
<td>1.5 (0–4.4)</td>
<td>1 (0.4–5.4)</td>
</tr>
<tr>
<td>Catabolic substrates</td>
<td>Formate,</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2-Propanol</td>
<td>–</td>
<td>–</td>
<td>+*</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth requirements</td>
<td>W, AC</td>
<td>AC, YE, RF</td>
<td>AC</td>
<td>AC</td>
<td>p-Cresol, W</td>
<td>AC</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>37.6</td>
<td>48.8†</td>
<td>44.9</td>
<td>50</td>
<td>38.7</td>
<td>47.5</td>
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<tr>
<td>Similarity (%)‡</td>
<td>–</td>
<td>95.4</td>
<td>95.4</td>
<td>95.6</td>
<td>94.8</td>
<td>94.9</td>
</tr>
</tbody>
</table>

*2-Butanol and cyclopentanol were also used.
†Data from Balch et al. (1979).
‡16S rRNA gene sequence similarity (%) with respect to strain MobH\textsuperscript{T}.

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Fig. 1. Phase-contrast photomicrograph (a), an electron micrograph of negatively stained cells (b) and a thin section (c) of strain MobH\textsuperscript{T} in the late-exponential growth phase. Bars: (a) 10 µm, (b) 1 µm and (c) 0.5 µm.
providing the samples and information regarding gas wells. We are grateful to Toshihiro Hoaki in the Technology Center, Taisei Corporation, for his kind help during sample collection. We would also like to thank Xian-Ying Meng, Mizuho Muramatsu and Fumie Nozawa in AIST for technical support. This study was supported financially in part by JSPS KAKENHI, grant numbers JP26709070, JP25289333, JP26710012 and JP26106004.

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Description of *Methanomicrobium antiquum* sp. nov.


The cells are curved rods (0.8×2.0 µm) and occur singly or in pairs. The cells are strictly anaerobic and Gram-stain-negative. In addition, they are lysed by 0.01 % (w/v) SDS solution but not a hypotonic solution (distilled water). The cells produce methane from H2/CO2 and formate, but not methanol, monomethylamine, dimethylamine, trimethylamine, dimethyl sulfide, acetate, ethanol, 1-propanol, 2-propanol, 2-butanol or cyclopentanol. Growth occurs between 35 and 60 °C with an optimum at 37 °C. The pH range for growth is pH 5.5–7.9 with an optimum pH of 7.0–7.5. Sodium chloride range for growth is 0–5.8 % (optimum 2 %). Magnesium ions are not required for growth, but acetate (2 mM) and tungsten (0.01 µM) are required and yeast extract (0.2 g l⁻¹) is stimulatory for growth. The cells are resistant to at least 100 µg ml⁻¹ of ampicillin, penicillin G, vancomycin, kanamycin or tetracycline.

The type strain, MobHᵀ (=DSM 21220ᵀ=NBRC 104160ᵀ), was isolated from the sediment and formation water that was derived from sedimentary aquifers filled with ancient seawater in a natural gas field in Japan. The DNA G+C-content of strain MobHᵀ is 37.6 mol%.

References


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**Fig. 2.** Phylogenetic tree of the family *Methanomicrobiaceae* based on the 16S rRNA gene sequences and showing the phylogenetic position of strain MobHᵀ. The tree was reconstructed by the neighbour-joining method (Saito & Nei, 1987) with the CLUSTAL X software package (Thompson et al., 1997). The tree topologies were evaluated by a bootstrap analysis (based on 1000 replicates) with the neighbour-joining/maximum-parsimony/maximum-likelihood methods, using the PAUP* package (Swofford, 2003). *Methanosaeta thermophila* (AB071701) was used as an outgroup (not shown). Bar, 2 substitutions per 100 nt.


