Methanobacterium petrolearium sp. nov. and Methanobacterium ferruginis sp. nov., mesophilic methanogens isolated from salty environments

Koji Mori¹ and Shigeaki Harayama¹,²

¹NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan
²Department of Biological Sciences, Faculty of Science and Engineering, Chuo University, 1-13-27 Kasuga, Bunkyo-ku, Tokyo 112-8551, Japan

Two methane-producing archaea, designated Mic5c12T and Mic6c05T, were isolated from sludge deposited in a crude oil storage tank and a tubercle on the interior of a pipe transporting natural gas-containing brine, respectively. The isolates were Gram-staining-variable, non-motile rods and grew only on H₂/CO₂. Strain Mic6c05T produced methane from some alcohols without showing any growth; strain Mic5c12T did not utilize alcohols. The optimum growth conditions for strain Mic5c12T were 35°C, pH 6.5 and 0–0.68 M NaCl and for strain Mic6c05T were 40°C, pH 6.0–7.5 and 0.34 M NaCl. Strain Mic5c12T was halotolerant and strain Mic6c05T was halophilic. Comparative 16S rRNA gene sequence analysis revealed that strains Mic5c12T and Mic6c05T belonged to the genus Methanobacterium and their closest relative was Methanobacterium subterraneum A8pT (97.3 and 97.9 % 16S rRNA gene sequence similarity, respectively). The findings from the 16S rRNA gene sequence analyses were supported by analysis of McrA, the alpha subunit of methyl-coenzyme M reductase. On the basis of phylogenetic analyses and phenotypic characteristics, two novel species are proposed, Methanobacterium petrolearium sp. nov. and Methanobacterium ferruginis sp. nov., with type strains Mic5c12T (¼ NBRC 105198T = DSM 22353T) and Mic6c05T (¼ NBRC 105197T = DSM 21974T), respectively.

The genus Methanobacterium contains some of the earliest-known hydrogenotrophic methanogens and 24 species have been described thus far (Boone, 2000; Cuzin et al., 2001; Joulian et al., 2000; Ma et al., 2005; Shlimon et al., 2004), however some have subsequently been transferred to other genera. Members of the genus Methanobacterium have been isolated from mesophilic, neutral and freshwater environments such as those found in anaerobic digesters; the exceptions are Methanobacterium aarhusense and Methanobacterium alcaliphilum, which were isolated from marine sediment (Shlimon et al., 2004) and alkaline lake sediment (Worakit et al., 1986), respectively. Recently, we isolated two hydrogenotrophic methanogens, strains Mic5c12T and Mic6c05T, both belonging to the genus Methanobacterium.

Two samples were used as isolation sources: a sludge sample from the bottom of a crude oil storage tank in Japan, collected in June 2005, and a tubercule sample from the interior surface of a pipe transporting natural gas-containing brine in the southern Kanto gas field in Japan, collected in July 2005. In order to enrich and isolate hydrogen-consuming methanogens, we used a basal medium [containing 1⁻¹: 0.26 g KH₂PO₄, 0.53 g K₂HPO₄, 3.05 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, 0.54 g NH₄Cl, 30 g NaCl, 1.42 g sodium sulfate, 0.82 g sodium acetate, 0.02 g Bacto yeast extract (Difco), 1.5 g Na₂CO₃, 2 ml each of trace element solution and vitamin solution of NBRC medium 377 (NBRC, 2005), 1 mg resazurin, 0.5 g Na₂S·9H₂O, 0.5 g cystine-HCl; about pH 7]. To enrich methanogens, a sample of approximately 1 g was used to inoculate 20 ml basal medium in a 50 ml serum bottle sealed with a butyl rubber stopper and an aluminium cap. The bottle was incubated at 25°C for 4 weeks under a H₂/CO₂ atmosphere (4:1, v/v; 150 kPa). The cultures were serially diluted in the same medium and incubated again at 25°C. The highest dilution in which microbial growth was observed was subsequently diluted and incubated in fresh medium at least three more times. Subsequently, colonies were isolated under H₂/CO₂ on basal medium solidified with 1.5 % (w/v) agar. Thus, pure cultures of strains

The GenBank/EMBL/DDBJ accession numbers for strains Mic5c12T and Mic6c05T for the 16S rRNA gene sequences are AB542742 and AB542743 and for the mcrA sequences are AB542744 and AB542745, respectively.

Supplementary figures are available with the online version of this paper.
Mic5c12T and Mic6c05T were obtained from the sludge and tubercle samples, respectively.

Cells of strains Mic5c12T and Mic6c05T were non-motile rods (0.3 × 2.4–4.7 and 0.3 × 2.3–3.7 μm, respectively; Supplementary Fig. S1, available in IJSEM Online) and showed autofluorescence under a AX70 epifluorescence microscope with U-MWU2 mirror unit (Olympus). The two isolates formed white colonies (approximately 0.5 mm in diameter after 3 weeks) on solid basal medium. Gram-staining and tests for susceptibility to SDS were performed as described by Boone & Whitman (1988). The isolates were Gram-staining-variable and were not lysed in 0.01 % (w/v) SDS or hypotonic (distilled) water.

Strains Mic5c12T and Mic6c05T were strictly anaerobic and grew with H2/CO2 (80 : 20, v/v), Formate (20 and 40 mM), acetate (20 and 40 mM), methanol (20 mM), ethanol (5 mM) and methylamine (10 mM) did not support growth or methane production under a N2/CO2 (80 : 20, v/v) atmosphere. Strain Mic6c05T showed slight methane production from 2-propanol (5 mM), isobutanol (5 mM) and cyclopentanol (5 mM) under N2/CO2, but growth was not observed. Methane production from these alcohols was not observed in strain Mic5c12T. Strain Mic5c12T required yeast extract (0.02 g l⁻¹) and acetate (10 mM) for growth. In contrast, although strain Mic6c05T required vitamins for growth, yeast extract and acetate were not required and did not stimulate growth.

The optimum temperature, pH and NaCl concentration for growth were determined by examining the time-course of methane production. The gas phase of the cultures was analysed by GC using a thermal conductivity detector and a Molecular Sieve 60/80 column (Shimadzu). Methane production with varying NaCl concentration is shown in Fig. 1 and with varying temperature and pH is shown in Supplementary Fig. S2. Strain Mic5c12T was able to grow at 20–40 °C (optimum 35 °C), at pH 5.5–9.0 (optimum pH 6.5) and with 0–1.2 M NaCl (optimum 0–0.68 M NaCl) and doubling time under optimum conditions was 39.5 h. Strain Mic6c05T was able to grow at 20–45 °C (optimum 40 °C), at pH 5.5–9.0 (optimum pH 6.0–7.5) and with 0–1.2 M NaCl (optimum 0.34 M NaCl) and doubling time under optimum conditions was 18.5 h. While both strains were able to grow with 1.2 M NaCl, strain Mic5c12T showed optimal growth with 0–0.68 M NaCl, which indicated that it was halotolerant. To date, M. aarhusense and M. subterraneum have been reported as halotolerant species of the genus Methanobacterium (Kotelnikova et al., 1998; Shlimon et al., 2004) and the effects of NaCl on their growth are identical to those observed for strain Mic5c12T. In contrast, strain Mic6c05T showed optimal growth with 0.34 M NaCl, which indicated that it was halophilic. To the authors’ knowledge, this is the first study to report a strain of the genus Methanobacterium that is acclimated to marine environments.

Genomic DNA was extracted and purified (Mori et al., 2000) and G+C content was analysed using HPLC with a reversed-phase column (Mesbah et al., 1989). The G+C contents of strains Mic5c12T and Mic6c05T were 38.3 and 37.6 mol%, respectively.

For phylogenetic analysis, partial sequences of the 16S rRNA gene and mcrA (alpha subunit of methyl-coenzyme M reductase) were used. The 16S rRNA genes for strains Mic5c12T and Mic6c05T were amplified using primers Ar0023mFL and Ar1530R (Mori et al., 2008b) and amplification products were sequenced using the method described previously (Mori et al., 2008a). Thus, almost-complete 16S rRNA gene sequences were determined for strains Mic5c12T (1392 bp) and Mic6c05T (1412 bp). The mcrA genes for strains Mic5c12T and Mic6c05T were amplified using primers MR1 (Simankova et al., 2003) and ME2 (Hales et al., 1996) and the amplification products were cloned using the TOPO TA Cloning kit (Invitrogen). Cloned mcrA sequences were amplified using the M13 primer set and sequences of amplification products were determined using M13 Forward, M13 Reverse, ME1, ME2 (Hales et al., 1996), MCRf (Springer et al., 1995) and MCRfr (5'-ARCCADATYTGTRCTTA-3'). Thus, deduced amino acid sequences were obtained from mcrA sequences for strains Mic5c12T (1148 bp) and Mic6c05T (1148 bp). Because the mcrA sequences for strains of species with validly published names for the genus Methanobacterium that are available in public databases are short except for M. aarhusense H2-LK3, the above method was used to determine mcrA sequences for M. alcaliphilum NBRC 105226T (1145 bp), M. congolense NBRC 105227T (1148 bp), M. formicum NBRC 100475T (1145 bp), M. oryzae NBRC 105229T (1145 bp) and M. subterraneum NBRC 105231T (1148 bp). However, the combination of MR1 and ME2 generated sequences of mrtA, which encodes an isoenzyme of MrcA, for M. bryantii NBRC 104951T (1139 bp, AB542756), M. espanolae NBRC
M. ivanovii NBRC 104952^T (1139 bp, AB542759), M. palustre NBRC 105230^T (1139 bp, AB542760) and M. uliginosum NBRC 105232^T (1134 bp). Therefore, a new primer, MR1, was designed using the newly obtained mcrA sequences for the genus Methanobacterium and was used to substitute MR1 to determine mcrA sequences for M. bryantii NBRC 104951^T (1133 bp), M. espanolae NBRC 105228^T (1133 bp), M. ivanovii NBRC 104952^T (1133 bp), M. palustre NBRC 105230^T (1134 bp) and M. uliginosum NBRC 105232^T (1134 bp).

A phylogenetic tree based on almost-complete (1240 bp) 16S rRNA gene sequences was constructed with the neighbour-joining method using CLUSTAL_X (Saitou & Nei, 1987; Thompson et al., 1997) after alignment using ARB (Ludwig et al., 2004; Fig. 2a). Strains Mic5c12^T and Mic6c05^T were closely related and exhibited 97.9% 16S rRNA gene sequence similarity. The strains were closely affiliated with M. subterraneum NBRC 105231^T (1133 bp), M. espanolae NBRC 105228^T (1133 bp), M. ivanovii NBRC 104952^T (1133 bp), M. palustre NBRC 105230^T (1134 bp) and M. uliginosum NBRC 105232^T (1134 bp).

Fig. 2. Neighbour-joining phylogenetic trees showing the relationships between strains Mic6c05^T and Mic5c12^T and type strains of species of the genus Methanobacterium, based on (a) 16S RNA gene sequences (1240 bp) and (b) deduced McrA sequences (365 amino acids). Bootstrap values (≥50%) are shown at branch nodes. Bars, 0.01 substitutions per position.

105228^T (1139 bp, AB542757), M. ivanovii NBRC 104952^T (1139 bp, AB542759), M. palustre NBRC 105230^T (1139 bp, AB542760) and M. uliginosum NBRC 105232^T (1139 bp, AB542761). Therefore, a new primer, MR1, was designed using the newly obtained mcrA sequences for the genus Methanobacterium and was used to substitute MR1 to determine mcrA sequences for M. bryantii NBRC 104951^T (1133 bp), M. espanolae NBRC 105228^T (1133 bp), M. ivanovii NBRC 104952^T (1133 bp), M. palustre NBRC 105230^T (1134 bp) and M. uliginosum NBRC 105232^T (1134 bp).

The differential characteristics of strains Mic5c12^T and Mic6c05^T and type strains of species of the genus Methanobacterium are summarized in Table 1 and as follows: (i) strains Mic5c12^T and Mic6c05^T differed from M. beijingense, M. formicicum, M. oryzae and M. palustre with regard to their inability to produce methane from formate; (ii) strains Mic5c12^T and Mic6c05^T differed from M. aarhusense and M. ivanovii with regard to their not requiring a moderately high temperature for growth. Additionally, strain Mic5c12^T differed from all type strains of species of the genus Methanobacterium because it required acetate for growth.

On the basis of the phylogenetic and physiological findings, we propose two novel species to accommodate strains Mic5c12^T and Mic6c05^T, with the names Methanobacterium petrolearium sp. nov. and Methanobacterium ferruginis sp. nov., respectively.

**Description of Methanobacterium petrolearium sp. nov.**

*Methanobacterium petrolearium* (pe.tro.le.a’ri.um. L. fem. n. petra rock; L. adj. olearius -a -um related to oil; N.L. neut. adj. petrolearium related to mineral oil).
Table 1. Characteristics of strains Mic5c12\textsuperscript{T} and Mic6c05\textsuperscript{T} and type strains of species of the genus Methanobacterium

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Crude oil storage tank sludge</td>
<td>Corroded pipe sediment</td>
<td>Marine sediment</td>
<td>Alkaline lake sediment</td>
<td>Anaerobic digester</td>
<td>Anaerobic digester</td>
<td>Anaerobic digester</td>
<td>Sludge</td>
<td>Sewage sludge digester</td>
<td>Rock core</td>
<td>Rice field</td>
<td>Peat bog</td>
<td>Deep granitic groundwater</td>
<td>Marshy soil</td>
</tr>
<tr>
<td>Growth with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td>$+$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$+$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$+$</td>
<td>$-$</td>
<td>$+$</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>Secondary alcohols</td>
<td>$-$</td>
<td>NG</td>
<td>$-$</td>
<td>ND</td>
<td>$-$</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>$-$</td>
<td>$-$</td>
<td>$+$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>Acetate requirement</td>
<td>$+$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>ND</td>
<td>ND</td>
<td>$-$</td>
<td>$-$</td>
<td>ND</td>
<td>ND</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>Optimum growth at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>35</td>
<td>40</td>
<td>45</td>
<td>37</td>
<td>37</td>
<td>37–39</td>
<td>37–42</td>
<td>35</td>
<td>37–45</td>
<td>45</td>
<td>40</td>
<td>33–37</td>
<td>20–40</td>
<td>40</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
<td>6.0–8.0</td>
<td>7.5–8.0</td>
<td>8.1–9.1</td>
<td>7.2</td>
<td>6.9–7.2</td>
<td>7.2</td>
<td>5.6–6.2</td>
<td>ND</td>
<td>7.0–7.4</td>
<td>7.0</td>
<td>7.0</td>
<td>7.8–8.8</td>
<td>ND</td>
</tr>
<tr>
<td>NaCl range (M)</td>
<td>0–1.2</td>
<td>0–1.2</td>
<td>0.05–0.9</td>
<td>ND</td>
<td>0–0.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0–0.4</td>
<td>0–0.3</td>
<td>0.2–1.2</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>38.3 (LC)</td>
<td>37.6 (LC)</td>
<td>34.9 (LC)</td>
<td>57 (BD)</td>
<td>38.9 ($T_m$)</td>
<td>33–38 (BD)</td>
<td>39.5 (LC)</td>
<td>34 ($T_m$)</td>
<td>41–42 (BD)</td>
<td>36.6 ($T_m$)</td>
<td>31 (LC)</td>
<td>34 ($T_m$)</td>
<td>54.5 ($T_m$)</td>
<td>33.8 ($T_m$)</td>
</tr>
</tbody>
</table>

*DNA G+C content determined using HPLC (LC), buoyant density (BD) or melting point ($T_m$).
Cells are non-motile, Gram-staining-variable rods, 0.3 μm wide and 2.4–4.7 μm long, and are not lysed by 0.01 % SDS or hypotonic solution (distilled water). White colonies are formed on solid medium. Strictly anaerobic. H₂ is utilized for growth and methane production, but formate, acetate, methanol, methylamine, 2-propanol, isobutanol, cyclopentanol and ethanol are not. Acetate and yeast extract are essential for growth. Grows at 20–40 °C (optimum 35 °C), at pH 5.5–9.0 (optimum pH 6.5) and with 0–1.2 M NaCl (optimum 0–0.68 M NaCl).

The type strain, Mic5c12^T (=NBRC 105198^T=DSM 22353^T), was isolated from sludge deposited in a crude oil storage tank in Japan. The DNA G+C content of the type strain is 38.3 mol%.

**Description of Methanobacterium ferruginis sp. nov.**

*Methanobacterium ferruginis* (fer.ru.gi’nis. L. fem. n. ferrugo iron rust; L. gen. n. ferruginis of iron rust).

Cells are non-motile, Gram-staining-variable rods, 0.3 μm wide and 2.3–3.7 μm long, and are not lysed by 0.01 % SDS or hypotonic solution (distilled water). White colonies are formed on solid medium. Strictly anaerobic. H₂ is used for growth and methane production, but formate, acetate, methanol, methylamine and ethanol are not. Methane is produced from 2-propanol, isobutanol and cyclopentanol, but growth is not observed. Vitamins are essential for growth, but acetate is not. Grows at 20–45 °C (optimum 40 °C), at pH 5.5–9.0 (optimum pH 6.0–8.0) and with 0–1.2 M NaCl (optimum 0.34 M NaCl).

The type strain, Mic6c05^T (=NBRC 105197^T=DSM 21974^T), was isolated from an iron-corrosive tubercle on an interior pipe transporting natural-gas-containing brine in the southern Kanto gas field, Japan. The DNA G+C content of the type strain is 37.6 mol%.

**Acknowledgements**

We thank Kuniko Shimamura and Motoyuki Ouchi for technical support. This work was supported by the New Energy and Industrial Technology Development Organization (grant number NEDO P05032).

**References**


