Methanobacterium beijingense sp. nov., a novel methanogen isolated from anaerobic digesters

Kai Ma, Xiaoli Liu and Xiuzhu Dong

State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, PR China

Two methanogenic strains, 8-2T and 4-1, with rod-shaped (0.4–0.5 × 3–5 μm), non-motile cells, sometimes observed in chains, were isolated from two anaerobic digesters in Beijing, China. The two strains used H2/CO2 and formate for growth and produced methane. The temperature range for growth was 25–50 °C, with fastest growth at 37 °C. The pH ranges for growth and methane production were 6.5–8.0 for strain 8-2T and 6.8–8.6 for strain 4-1, with the fastest growth at pH 7.2 for strain 8-2T and pH 7.5–7.7 for strain 4-1. The G+C content of genomic DNA for strain 8-2T was 38.9 mol%. The similarity levels of the 16S rRNA sequence of strain 8-2T with other species of the genus Methanobacterium ranged from 93.8 to 96.0%. Based on the phylogenetic analysis and phenotypic characteristics, the novel species Methanobacterium beijingense sp. nov. is proposed, with the type strain 8-2T (=DSM 15999T = CGMCC 1.5011T).

Methanogens share primarily two common physiological characteristics, namely growing strictly anaerobically and producing methane as the exclusive final product of energy metabolism (Garcia, 1990). In contrast to their significantly similar energy metabolism, methanogens inhabit extremely diverse environments, including freshwater and marine sediments, the digestive and intestinal tracts of animals and anaerobic waste digesters (Jones et al., 1987). So far, 28 genera of methanogens have been described. The majority of rod-shaped methanogens are affiliated to the order Methanobacteriales, which consists of three mesophilic genera (Methanobacterium, Methanobrevibacter and Methanosphaera) and two thermophilic or hyperthermophilic genera (Methanothermobacter and Methanothermus). All methanogens grow on a H2/CO2 gas mixture; in addition, many of them utilize formate and some grow on a few other simple alcohols. The anaerobic digester is a compatible surrounding for the growth of mesophilic methanogens and Methanobacterium strains constitute the main microbial flora, which play an important role in the anaerobic degradation of organic compounds as the terminal metabolic groups (Hobson & Shaw, 1973).

When surveying the microbial communities of two mesophilic methane-producing up-flow anaerobic sludge blanket (UASB) reactors, we isolated 11 strains of rod-shaped methanogens that produced methane from H2/CO2. Two strains from two different reactors showed high similarity of 16S rRNA gene sequences and phenotypic characters; however, they were distantly related to all existing species of the genus Methanobacterium. Based on phylogenetic and phenotypic data, a novel species of Methanobacterium is proposed.

Methanobacterium formicum DSM 1535T, Methanobacterium congolense DSM 7095T and Methanobacterium oryzae DSM 1106T were purchased from the DSMZ (Braunschweig, Germany). Strains 8-2T and 4-1 were isolated respectively from the granular sludge of a mesophilic UASB reactor treating beer-manufacture wastewater in Tsinghua University and one treating wastewater of bean-curd manufacture in Beijing.

The pre-reduced basal medium was prepared as described previously (Zehnder & Wuhermann, 1977), but omitting rumen fluid and titanium solution. The medium was dispensed in screw-capped tubes sealed with butyl rubber stoppers and the gas phase was H2/CO2 (80:20, 1·01 × 105 Pa) for routine cultivation unless indicated. All inoculations and transfers were done with syringes and needles and all cultures were incubated at 37 °C in the dark. Substrate utilization was tested by measuring methane production from basal medium with the addition of each tested compound, and N2/CO2 (80:20, 1·01 × 105 Pa) was used instead of H2/CO2 as the gas phase. Requirement for growth factors was determined by measuring growth in the H2/CO2 medium omitting one of the components in each test, which included vitamins, yeast extract, peptone, acetate, etc. The pH range...
for growth was estimated by cultivating the strains in the H₂/CO₂ medium with various pH values adjusted with 10% (w/v) NaOH or HCl. The growth temperature range was measured by cultivating the strains in a water bath with a temperature controller. To determine NaCl tolerance, 0–1000 mM NaCl was added to the H₂/CO₂ medium. The fastest growth was determined by measuring methane production after 6 days cultivation. Specific growth rates were calculated from the linear part of methane production curves determined from the amount of methane at 24 h intervals according to the method of Lai et al. (2000). Methane production was measured by gas chromatograph GC-14B (Shimadzu).

Hungate anaerobic techniques were used for isolation and culture of the strains (Hungate, 1969). During enrichment, 0·5 g vancomycin l⁻¹ (final concentration) (Kotelnikova et al., 1998) was added to the H₂/CO₂ medium to inhibit bacterial growth. The enrichments were serially diluted and single colonies were obtained by the Hungate roll-tube method after cultivation at 37 °C for 14 days. Colonies that produced fluorescence under UV light at a wavelength of 420 nm (model 2071 Max. Watts 100; American Optical) were picked for further purification. The purity of cultures was examined periodically by monitoring the cell morphology, under the normal bright-field microscope, and colonies, as well as the absence of growth in rich media like peptone/yeast extract/glucose (PYG) broth.

Exponential-phase cells of strain 8-2T were used for morphological examination under a transmission electron microscope (H-600A; Hitachi). Before observation, cells were coated with palladium/iridium alloy with a high vacuum evaporator (HUS-5GB; Hitachi). Ultrathin sections were stained with uranyl acetate and lead citrate according to Reynolds (1963). The motility of cells was observed by phase-contrast microscope (BH-2; Olympus).

Cells from an exponentially growing culture were used to check susceptibility to lysis by 1% SDS and distilled water as a hypotonic solution (Boone & Whitman, 1988). Cell lysis was determined by microscopic observation of cell integrity.

Genomic DNA extraction and purification were performed according to Marmur (1961) and Jarrell et al. (1992). The G+C content was determined using the thermal denaturation method (Marmur & Doty, 1962; Owen & Pitcher, 1985) using Escherichia coli K-12 as the reference. DNA–DNA relatedness was determined from the initial reassociation rate at 61.5–65.5 °C according to the method of Owen & Pitcher (1985). Both assays were performed by using a UV800 spectrophotometer (Beckman).

The 16S rRNA gene was amplified using the genomic DNA mentioned above as the template as described previously (Furlong et al., 2002). Purified PCR products of ~1400 bp were cloned into pUCm-T vector and sequenced by Bioasia Company. The similarities of the 16S rRNA gene sequences to all sequences in GenBank were determined using the BLASTN algorithm. The best matching sequences were retrieved from the database and aligned and similarity analysis was performed by CLUSTAL X (Thompson et al., 1994). The phylogenetic tree was constructed by using MEGA 2.1 software (Sudhir et al., 2001).

Soluble cell protein was extracted from the sonicated cell pellet of 50 ml exponential cultures. The protein profile was determined by running an SDS-PAGE gel and visualized by silver staining.

Cells of the two strains were rod-shaped, 0·4–0·5 × 3–5 μm (Fig. 1), stained Gram-negative and were non-motile. The cells resisted disruption by 1% SDS (w/v) or hypotonic solution. Colonies of strains 8-2T and 4-1 were greyish-white, opaque and rounded with entire edges, and the diameter reached 0·5–1·0 mm after 2–3 weeks cultivation at 37 °C on H₂/CO₂ medium. The colonies produced bright fluorescence under UV light at 420 nm. The two strains grew strictly anaerobically and growth was inhibited completely in the presence of air. H₂/CO₂ and formate supported growth and methane production. Acetate, methanol, ethanol, trimethylamine, isobutanol and isopropanol (each at 10 mM) were not used; however, 0·025% acetate (w/v) could stimulate growth of strain 8-2T. Strains 8-2T and 4-1 grew well without peptone and vitamins, whereas yeast
extract (0.1–2 % w/v) was indispensable. Growth of strains 8-2T and 4-1 was observed in the temperature range 25–50 °C, with fastest growth at 37 °C. The pH range for growth was 6.5–8.0 for strain 8-2T and 6.8–8.6 for strain 4-1 and the optimum pH for growth was 7.2 for strain 8-2T and 7.5–7.7 for strain 4-1. The specific growth rate of strain 8-2T was 0.049 h⁻¹ when grown in the H₂/CO₂ medium at 37 °C and 0.030, 0.023 and 0.021 h⁻¹ in the absence of acetate, yeast extract and both, respectively. The G+C content of the genomic DNA of strain 8-2T was 38.9 mol%.

Phylogenetic analysis (Fig. 2) showed 98.2 % 16S rRNA gene sequence similarity between strains 8-2T and 4-1; however, the similarity between 8-2T and other species of Methanobacterium ranged from 93.8 to 96 %, indicating that strain 8-2T could represent a novel species of this genus.

DNA–DNA relatedness values between strain 8-2T and its phylogenetic relatives Methanobacterium oryzae DSM 11106T, Methanobacterium congoles DSM 7095T and Methanobacterium formicicum DSM 1535T were respectively 29.5, 25.2 and 7 %, SDS-PAGE profiles of whole-cell proteins (Fig. 3) of the three phylogenetic relatives also showed distinct protein patterns from strain 8-2T.

All the phenotypic and phylogenetic characteristics of strains 8-2T and 4-1 indicated their membership of the genus Methanobacterium; however, some phenotypic features distinguished them from others as follows: (i) they differed from Methanobacterium espanolae (Patel et al., 1990), Methanobacterium ivanovi (Belyaev et al., 1986), Methanobacterium uliginosum (König, 1984), Methanobacterium congoles (Cuzin et al., 2001), Methanobacterium bryantii (Zellner & Winter, 1987) and Methanobacterium aarhense (Shlimon et al., 2004) in their ability to produce methane from formate; (ii) they differed from Methanobacterium palustre in the latter’s capacity to use secondary alcohols as sole carbon and energy sources (Zellner & Winter, 1987); (iii) they differed from Methanobacterium subterraneum and Methanobacterium alcalophilum in their optimum pH for growth (Kotelnikova et al., 1998; Worakit et al., 1986); (iv) they differed from Methanobacterium oryzae in their higher growth temperature (8 °C difference) (Joulian et al., 2000); and (v) they differed from Methanobacterium formicicum in colony size and shape (Bryant & Boone, 1987). The characteristics that differentiate the novel strains from all other Methanobacterium species are shown in Table 1.

It had been proposed that strains with >3 % 16S rRNA gene sequence divergence could be regarded as different species (Stackebrandt & Goebel, 1994). According to the minimal standards for new taxa of methanogens (Boone & Whitman, 1987) and based on phylogenetic and phenotypic characters, a novel species of the genus Methanobacterium is proposed, Methanobacterium beijingense sp. nov.

**Description of Methanobacterium beijingense sp. nov.**

*Methanobacterium beijingense* (bei.jing.en’se. N.L. neut. adj. beijingense from Beijing, where the type strain was isolated).

**Fig. 2.** Phylogenetic tree showing the position of strain 8-2T amongst other species of the genus Methanobacterium. Based on a consensus length of 1378 bp of 16S rRNA gene sequences, the tree was constructed by the neighbour-joining method and rooted with Methanothermus fervidus DSM 2088T. The topology of the tree was estimated by bootstraps based on 1000 replications. Numbers at branch points are percentages supported by bootstrap evaluation. Numbers in parentheses are GenBank accession numbers. Bar, 2 % sequence divergence.

**Fig. 3.** Cell-protein SDS-PAGE profiles of strain 8-2T (lane 1), Methanobacterium oryzae DSM 11116T (2), Methanobacterium congoles DSM 7095T (3) and Methanobacterium formicicum DSM 1535T (4). Lane M, molecular mass markers (sizes in kDa).
Table 1. Differential characteristics between *Methanobacterium beijingense* sp. nov. and other species of *Methanobacterium*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
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</thead>
<tbody>
<tr>
<td><strong>Source</strong></td>
<td>Anaerobic digester</td>
<td>Anaerobic digester</td>
<td>Sewage sludge digester</td>
<td>Rice field</td>
<td>Anaerobic digester</td>
<td>Peat bog</td>
<td>Deep granitic groundwater</td>
<td>Alkaline lake</td>
<td>Anaerobic digester</td>
<td>Sludge</td>
<td>Rock core</td>
<td>Marshy soil</td>
<td>Marine sediment</td>
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<tr>
<td><strong>Cell size</strong></td>
<td>0-4-0.5</td>
<td>0-4-0.5</td>
<td>0-4-0.8</td>
<td>0-3-0.4</td>
<td>0-4-0.5</td>
<td>0.5-2-5</td>
<td>0.1-0-15</td>
<td>0.5-0-6</td>
<td>0.5-1-0</td>
<td>0.8 x</td>
<td>0.5-0.8</td>
<td>0.2-0.6 x</td>
<td>0.7 x</td>
</tr>
<tr>
<td><strong>Colony size (mm)</strong></td>
<td>0.5-1-0</td>
<td>0.5-1-0</td>
<td>Up to 5</td>
<td>1-2</td>
<td>Up to 1</td>
<td>ND</td>
<td>1-2-2-0</td>
<td>0-2</td>
<td>1-5</td>
<td>0-5-1-0</td>
<td>3-6</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td><strong>Substrates used:</strong></td>
<td>Formate</td>
<td>+</td>
<td>+</td>
<td>+ *</td>
<td>+</td>
<td>- *</td>
<td>+</td>
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<td><strong>Growth temperature (°C):</strong></td>
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<tr>
<td><strong>Optimum</strong></td>
<td>37</td>
<td>37</td>
<td>37-45</td>
<td>40</td>
<td>37-42</td>
<td>33-37</td>
<td>20-40</td>
<td>37</td>
<td>37-39</td>
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<td>45</td>
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<td><strong>pH for growth:</strong></td>
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<td><strong>Range</strong></td>
<td>6.5-8.0</td>
<td>6.8-8.6</td>
<td>6.6-7.8</td>
<td>6.0-8.5</td>
<td>5.9-8.2</td>
<td>ND</td>
<td>6.5-9.2</td>
<td>7.0-9.9</td>
<td>ND</td>
<td>4.6-7.0</td>
<td>6.5-8.5</td>
<td>6.0-8.5</td>
<td>5-9</td>
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<tr>
<td><strong>Optimum</strong></td>
<td>7.2</td>
<td>7.5-7.7</td>
<td>ND</td>
<td>7.0</td>
<td>7.2</td>
<td>7.0</td>
<td>7.8-8.8</td>
<td>8.1-9-1</td>
<td>6.9-7.2</td>
<td>5.6-6.2</td>
<td>7.0-7-4</td>
<td>ND</td>
<td>7.5-8</td>
</tr>
<tr>
<td><strong>NaCl range (M)</strong></td>
<td>0-0.5</td>
<td>ND</td>
<td>ND</td>
<td>0-0.4</td>
<td>0-0.3</td>
<td>0-2-1.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td><strong>G+C content (mol%)</strong></td>
<td>38-9 (Tm)</td>
<td>ND</td>
<td>41-42 (Bd)</td>
<td>31 (Lc)</td>
<td>39-5 (Lc)</td>
<td>34 (Tm)</td>
<td>54.5 (Tm)</td>
<td>57 (Bd)</td>
<td>33-38 (Bd)</td>
<td>34 (Tm)</td>
<td>36-6 (Tm)</td>
<td>33-8 (Tm)</td>
<td>34-9 (Lc)</td>
</tr>
</tbody>
</table>

*Checked in this study.
†Determined by buoyant density analysis (Bd), HPLC analysis (Lc) or melting point analysis (Tm).
Cells are rod-shaped and non-motile and stain Gram-negative. Cells are resistant to lysis by 1% (w/v) SDS and hypotonic solution. Colonies are greyish-white, opaque and rounded with entire edges and up to 1 mm in diameter. Methanogenic. Growth substrates include H2/CO2 and formate. No growth on acetate, methanol, ethanol, tri-methylamine, isobutanol or isopropanol. Yeast extract is indispensible; however, peptone, vitamins and acetate are not required. Acetate stimulates growth. The temperature for growth ranges from 25 to 50°C, with optimal growth at 37°C. The pH value range for growth is 6.5–8.6 and the optimum pH is 7.2–7.7. The DNA base composition of the type strain is 38.9 mol% G+C ($T_m$).

The type strain, 8-2T ($=\text{DSM 15999}^T = \text{CGMCC 1.5011}^T$), was isolated from an anaerobic digester for the treatment of beer-manufacture wastewater.

**Acknowledgements**

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**References**


