Methanobacterium movens sp. nov. and Methanobacterium flexile sp. nov., isolated from lake sediment

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Two mesophilic methanogenic strains, designated TS-2T and GHTT, were isolated from sediments of Tuosu lake and Gahai lake, respectively, in the Qaidam basin, Qinghai province, China. Cells of both isolates were rods (about 0.3–0.5 × 2–5 μm) with blunt rounded ends and Gram-staining-positive. Strain TS-2T was motile with one or two polar flagella and used only H2/CO2 for growth and methanogenesis. Strain GHTT was non-motile, used both H2/CO2 and formate and displayed a variable cell arrangement depending on the substrate: long chains when growing in formate (50 mM) or under high pressure H2 and single cells under low pressure H2. Phylogenetic analysis based on 16S rRNA gene sequences placed the two isolates in the genus Methanobacterium. Strain TS-2T was most closely related to Methanobacterium alcalophilum NBRC 105226T (96% 16S rRNA gene sequence similarity). Phylogenetic analysis based on the alpha subunit of methyl-coenzyme M reductase also supported the affiliation of the two isolates with the genus Methanobacterium. DNA–DNA relatedness between the isolates and M. alcalophilum DSM 3387T was 39–53%. Hence we propose two novel species, Methanobacterium movens sp. nov. (type strain TS-2T=AS 1.5093T=JCM 15415T) and Methanobacterium flexile sp. nov. (type strain GHTT=AS 1.5092T=JCM 15416T).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and mcrA sequences of strain TS-2TT are EU366499 and HM802934, respectively, and those for strain GHT are EU333914 and HM802935, respectively.

A supplementary figure is available with the online version of this paper.

Methanogenic archaea are hitherto the only known organisms that generate methane as the final product of energy metabolism (Garcia, 1990), so therefore they play a key role in global methane emission. However, despite their common energy metabolism pathway, methanogens inhabit extremely diverse environments, which range from frozen permafrost and tundra to hot vents with temperatures above 100 °C. They are distributed in almost all anoxic niches, such as freshwater and marine sediments, digestive and intestinal tracts of animals and anaerobic waste digesters (Jones et al., 1987). The majority of rod-shaped methanogens are affiliated with the order Methanobacteriales, which consists of three mesophilic genera (Methanobacterium, Methanobrevibacter and Methanosphaera) and two thermophilic or hyperthermophilic genera (Methanothermobacter and Methanothermus). All methanogens in the order Methanobacteriales are capable of using H2/CO2 to generate methane, except for Methanosphaera stadtmanae, which produces methane by reducing methanol with H2 (Thauer, 1998). In addition, many also utilize formate or a few simple alcohols. During a survey of methanogens in two lakes of the Qaidam basin, China, we isolated 10 rod-shaped methanogenic strains by using H2/CO2 as the carbon substrate. Two strains, designated TS-2T and GHTT, were identified on the basis of phylogenetic and phenotypic data as members of the genus Methanobacterium. Of the remaining eight strains, one was closely related to strain TS-2T, four were related to strain GHT and two were identified as members of Methanobacterium formicum. Strains TS-2TT and GHTT were isolated from sediment samples collected from Tuosu lake and Gahai lake, respectively, in the Qaidam basin, Qinghai province, China.

Hungate anaerobic techniques were used for isolation and culture (Hungate, 1969). Pre-reduced basal medium was prepared as described by Zehnder & Wurhann (1977) except that sodium acetate was omitted. For routine cultivation, the medium was dispensed into screw-capped tubes sealed with butyl rubber stoppers and the gas phase was H2/CO2 (80:20, v/v; 150 kPa). All inoculations and transfers were performed anaerobically with syringes and all cultures were incubated at 37 °C in the dark. Sediment samples (1 ml) were inoculated into 5 ml basal medium containing 0.5 g penicillin l−1 (final concentration) and cultured under H2/CO2 for 4 weeks. After a large amount of...
methane had been produced, the enrichments were serially diluted and the Hungate rolling tube technique was applied. Colonies were observed in the tubes after 14 days and those that produced fluorescence under UV light at a wavelength of 420 nm (model 2071, max. 100 Watts; American Optical) were picked for further purification. Culture purity was examined periodically by monitoring Gram-stained cell morphology with normal bright-field microscopy as well as by the absence of growth in rich media, such as peptone-yeast extract-glucose broth. The production of methane was determined by gas chromatography (GC-14B; Shimadzu) as described by Kotelnikova et al. (1993). Methanobacterium alcaliphilum DSM 3387T and Methanobacterium formicicum DSM 1535T were purchased from the DSMZ and Methanobacterium beijingense 8-2T was obtained from our laboratory collection.

Exponential-phase cells of strains TS-2T and GH T were used for morphological examination by transmission electron microscopy (JEM-1400; JEOL). Motility of cells was observed by phase-contrast microscopy (BH-2; Olympus). Both isolates were Gram-staining-positive rods (about 0.3–0.5 × 2–5 μm). Strain TS-2T was motile with one or two polar flagella (Fig. 1a), while strain GH T had no flagella. Strain GH T grew as single cells under low pressure H2 (H2/CO2/N2; 40:10:50, v/v; 100–150 kPa) (Fig. 1b), but formed long chains with more than 10 cells in formate or under high pressure H2/CO2 (80:20, v/v; 100–250 kPa) (Fig. 1c). Cells of both isolates resisted disruption with 1 % (w/v) SDS or hypotonic solution. Colonies of both isolates were greyish white, opaque and round with entire edges and reached a diameter of 0.5–1.0 mm after 1–2 weeks of cultivation under H2/CO2.

The two isolates grew strictly anaerobically and did not grow in the presence of air. Substrate utilization was tested by measuring methane production in basal medium with each of formate, acetate, methanol, ethanol, trimethylamine, isobutanol and 2-propanol (10 mM), with N2/CO2 (80:20, v/v; 101 kPa) replacing H2/CO2 as the gas phase. Strain TS-2T only used H2/CO2 and strain GH T used both H2/CO2 and formate for growth and methane production. Acetate, methanol, ethanol, trimethylamine, isobutanol and 2-propanol were not used. Growth factors were determined by measuring growth in basal medium by omitting one component in each test (vitamins, yeast extract, peptone, acetate). Strains TS-2T and GH T grew well without peptone and vitamins, whereas yeast extract (0.1–2 %, w/v) was indispensable. The pH range for growth was measured by adjusting the pH of the medium with 10 % (w/v) NaOH or 10 % (w/v) HCl. The temperature for growth was measured in a water bath with temperature controller. Growth with 0–1000 mM NaCl was measured in basal medium with H2/CO2 as the gas phase. Optimal growth was determined by measuring methane production at 8–24 h interval for 10 days. Strains TS-2T and GH T grew at 10–50 °C (optimum 35–38 °C), at pH 6.0–9.0 and 6.5–9.5, respectively, and with 1.7 and 1.0 M NaCl, respectively (optimum 0–0.3 and 0–0.1 M, respectively) (see Supplementary Fig. S1, available in IJSEM Online). The specific growth rate was calculated from the linear part of the methane production curve in basal medium under H2/CO2 at pH 7.2 and 37 °C, according to the method of Lai et al. (2000), and was determined to be 0.027 and 0.032 h⁻¹ for strains TS-2T and GH T, respectively.
Genomic DNA extraction and purification were performed according to Jarrell et al. (1992). For phylogenetic analysis, the almost-complete sequence of the 16S rRNA gene and a partial sequence of mcrA (alpha subunit of methyl-coenzyme M reductase) were determined. The 16S rRNA gene was amplified using the archaea-specific primer 1f (Embley et al., 1992) and the prokaryote primer 1541R (Sung et al., 2006). Purified PCR products (about 1400 bp) were cloned into the pUCm-T vector (Takara) and sequenced by Bioasia. The mcrA gene was amplified using the primer set ME1 and ME2 (Hales et al., 1996) and sequenced as described for the 16S rRNA gene. The 16S RNA gene sequences and the deduced amino acid sequences for McrA were submitted to GenBank to search for related sequences using BLAST (Altschul et al., 1990), which were then aligned using CLUSTAL_X (Thompson et al., 1997). Phylogenetic trees based on 16S rRNA gene (Fig. 2a) and McrA (Fig. 2b) sequences were constructed using the neighbour-joining algorithm in MEGA3.1 (Kumar et al., 2004). The 16S rRNA gene sequence analysis indicated that the two isolates were affiliated with the genus Methanobacterium but represented two novel species. Strain TS-2T was most closely related to Methanobacterium alcaliphilum NBRC 105226T (96 % 16S rRNA gene sequence similarity). The McrA sequence analysis also showed that strains TS-2T and GH2 were most closely related to Methanobacterium alcaliphilum NBRC 105226T (96 and 91 % McrA sequence similarity, respectively).

The DNA G+C content of each isolate was determined using both thermal denaturation and HPLC with Escherichia coli K-12 as the reference strain. The G+C content of strain TS-2T was 39.1 ± 0.8 mol% (Tm) and 39.5 ± 0.6 mol% (HPLC) and of strain GH2 was 36.4 ± 0.6 mol% (Tm) and 37.3 ± 0.3 mol% (HPLC). DNA–DNA relatedness was determined using the initial reassocation rate method of Owen & Pitcher (1985) with a hybridization temperature of 65 °C and a UV800 spectrophotometer (Beckman). DNA–DNA relatedness between the two isolates was determined to be 36–42 % and between strains TS-2T and GH2 and Methanobacterium alcaliphilum DSM 3387T was 39–49 % and 41–53 %, respectively.

The differential phenotypic characteristics of strains TS-2T and GH2 and the type strains of most of the recognized species in the genus Methanobacterium are summarized in Table 1. Strain TS-2T differed from all members of the genus Methanobacterium by its flagellar mobility. Strains TS-2T and GH2 differed from Methanobacterium alcaliphilum by their optimal growth at neutral pH. Strains TS-2T and GH2 differed from Methanobacterium aarhusense and Methanobacterium ivanovii by their inability to grow at 45 °C. Strains TS-2T and GH2 differed from Methanobacterium petrolearium by not requiring acetate for growth. Strain TS-2T differed from strain GH2, Methanobacterium beijingense, Methanobacterium formicicum and Methanobacterium oryzae by not producing methane from formate.

On the basis of phylogenetic, chemotaxonomic and physiological differences, strains TS-2T and GH2 represent two separate novel species of the genus Methanobacterium,
Table 1. Characteristics differentiating strains TS-2\textsuperscript{T} and GH\textsuperscript{T} from type strains of species of the genus *Methanobacterium*

Strains: 1, TS-2\textsuperscript{T}; 2, GH\textsuperscript{T}; 3, *M. alcaliphilum* DSM 3387\textsuperscript{T} (Worakit et al., 1986); 4, *M. aarhusense* H2-LR\textsuperscript{T} (Shlimon et al., 2004); 5, *M. conglobense* C\textsuperscript{T} (Cuzin et al., 2001); 6, *M. beijingense* 8-2\textsuperscript{T} (Ma et al., 2005); 7, *M. formicicum* DSM 1535\textsuperscript{T} (Boone, 2001); 8, *M. oryzae* FP\textsuperscript{T} (Joulian et al., 2000); 9, *M. bryantii* M.o.H.\textsuperscript{T} (Boone, 2001); 10, *M. espanolae* GP9\textsuperscript{T} (Patel et al., 1990); 11, *M. ivanovi* Ivanov\textsuperscript{T} (Jain et al., 1987). NG, No growth but alcohols are oxidized; +, positive; −, negative; ND, no data available.

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
<th>5</th>
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<th>8</th>
<th>9</th>
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<tr>
<td>Isolation source</td>
<td>Tuosu lake</td>
<td>Gahai lake</td>
<td>Alkaline lake</td>
<td>Marine sediment</td>
<td>Anaerobic digester</td>
<td>Anaerobic digester</td>
<td>Sewage sludge digester</td>
<td>Rice field</td>
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<td>Secondary alcohols</td>
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<td>–</td>
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<td>ND</td>
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<td>Optimum growth</td>
<td>Temperature (°C)</td>
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<td>pH</td>
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<td>NaCl for growth (M)</td>
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<td>0–0.6*</td>
<td>0.05–0.9</td>
<td>ND</td>
<td>0–0.5</td>
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<td>0–0.4</td>
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<td>Motility</td>
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<td>–</td>
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<td>–</td>
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<td>DNA G+C content (mol%)*</td>
<td>39.1±0.8 (T\textsubscript{m})</td>
<td>36.4±0.6 (T\textsubscript{m})</td>
<td>57 (Bd)</td>
<td>34.9 (Lc)</td>
<td>39.5 (Lc)</td>
<td>38.9 (Lc)</td>
<td>41–42 (Bd)</td>
<td>31 (Lc)</td>
<td>33–38 (T\textsubscript{m})</td>
<td>34 (T\textsubscript{m})</td>
<td>36.6 (T\textsubscript{m})</td>
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</table>

*Data were taken from this study.
†Bd, buoyant density; Lc, HPLC; T\textsubscript{m}, melting point.
for which the names *Methanobacterium mobile* sp. nov. and *Methanobacterium flexile* sp. nov., respectively, are proposed.

### Description of *Methanobacterium movens* sp. nov.


Cells are rods (0.4–0.5 × 2–5 μm), occurring singly or in pairs and motile by one or two polar flagella. Uses only H$_2$/CO$_2$ for methane production. Acetate, methanol, ethanol, trimethylamine, isobutanol and 2-propanol are not used. Yeast extract is indispensable for growth. Grows optimally at 35–38 °C and pH 7.2–7.5. The DNA G+C content of the type strain is 39.1 ± 0.8 mol% ($T_m$) and 39.5 ± 0.6 mol% (HPLC).

The type strain TS-2$^T$ (= AS 1.5093$^T$ = JCM 15415$^T$), was isolated from the sediment of Tuusu lake, China.

### Description of *Methanobacterium flexile* sp. nov.

*Methanobacterium flexile* (fle’xi.i.e. L. neut. adj. flexile pliable, flexible).

Cells are rods (0.3–0.5 × 2–5 μm). Utilizes both H$_2$/CO$_2$ and formate for methane production. Chains of cells are formed in formate or under high pressure H$_2$/CO$_2$ (80:20, v/v; 100–250 kPa), and single cells are formed under lower pressure H$_2$ (H$_2$/CO$_2$/N$_2$: 40:10:50, v/v; 100–150 kPa). Grows optimally at 35–38 °C and pH 7.0–7.5. Yeast extract is indispensable for growth. The DNA G+C content of the type strain is 36.4 ± 0.6 mol% ($T_m$) and 37.3 ± 0.3 mol% (HPLC).

The type strain is GH$^T$ (= AS 1.5092$^T$ = JCM 15416$^T$), isolated from the sediment of Galhah lake, China.

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### References


