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

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Two mesophilic methanogenic strains, designated TS-2^T and GH^T, 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-2^T was motile with one or two polar flagella and used only H₂/CO₂ for growth and methanogenesis. Strain GH^T 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-2^T was most closely related to *Methanobacterium alcaliphilum* NBRC 105226^T (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. alcaliphilum* DSM 3387^T was 39–53%. Hence we propose two novel species, *Methanobacterium movens* sp. nov. (type strain TS-2^T=AS 1.5093^T=JCM 15415^T) and *Methanobacterium flexile* sp. nov. (type strain GH^T=AS 1.5092^T=JCM 15416^T).

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-2^T and GH^T, 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-2^T, four were related to strain GH^T and two were identified as members of *Methanobacterium formicicum*. Strains TS-2^T and GH^T 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 & Wuhrmann (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

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

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

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 3387^T and *Methanobacterium formicicum* DSM 1535^T were purchased from the DSMZ and *Methanobacterium beijingense* 8-2^T was obtained from our laboratory collection.

Exponential-phase cells of strains TS-2^T 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-2^T 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-2^T 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-2^T 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-2^T 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

Fig. 1. Transmission electron micrographs of cells of strain TS-2^T (a), strain GH^T under low pressure H₂ (b) and strain GH^T in formate (c), after negative staining with uranyl acetate. Bars, 1 µm (a), 0.5 µm (b), 2.0 µm (c).

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-2^T 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 rRNA 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-2^T was most closely related to *Methanobacterium alcaliphilum* NBRC 105226^T (96 % 16S rRNA gene sequence similarity). The McrA sequence analysis also showed that strains TS-2^T and GH^T were most closely related to *Methanobacterium alcaliphilum* NBRC 105226^T (96 and 91 % MrcA 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-2^T was 39.1 ± 0.8 mol% (T_m) and 39.5 ± 0.6 mol% (HPLC) and of strain GH^T was 36.4 ± 0.6 mol% (T_m) and 37.3 ± 0.3 mol% (HPLC). DNA–DNA relatedness was determined using the initial reassociation 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-2^T and GH^T and *Methanobacterium alcaliphilum* DSM 3387^T was 39–49 % and 41–53 %, respectively.

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

On the basis of phylogenetic, chemotaxonomic and physiological differences, strains TS-2^T and GH^T represent two separate novel species of the genus *Methanobacterium*,

Fig. 2. Neighbour-joining phylogenetic trees showing the positions of strains TS-2^T and GH^T within the genus *Methanobacterium*, based on sequences for the 16S rRNA gene (1294 nucleotides) (a) and McrA (371 amino acids) (b). Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Bars, 0.01 evolutionary distance.

Table 1. Characteristics differentiating strains TS-2^T and GH^T from type strains of species of the genus *Methanobacterium*

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

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Isolation source	Tuosu lake	Gahai lake	Alkaline lake	Marine sediment	Anaerobic digester	Anaerobic digester	Sewage sludge digester	Rice field	Anaerobic digester	Sludge	Rock core
Substrate											
CO ₂	+	+	+	+	+	+	+	+	+	+	+
Formate	−	+	−	−	−	+	+	+	−	−	−
Secondary alcohols	−	−	−*	−	NG	−*	−	−	NG	NG	−
Acetate requirement	−	−	−	−	ND	−	−	ND	−	ND	−
Optimum growth											
Temperature (°C)	35–38	35–38	37	45	37–42	37	37–45	40	37–39	35	45
pH	7.2–7.5	7.0–7.5	8.1–9.1	7.5–8.0	7.2	7.2	7.2*	7	6.9–7.2	5.6–6.2	7.0–7.4
NaCl for growth (M)	0–0.3	0–0.1	0–0.6*	0.05–0.9	ND	0–0.5	0–0.5*	0–0.4	ND	ND	ND
Motility	+	−	−	−	−	−	−	−	−	−	−
DNA G+C content (mol%)†	39.1 ± 0.8 (T _m)	36.4 ± 0.6 (T _m)	57 (Bd)	34.9 (Lc)	39.5 (Lc)	38.9 (Lc)	41–42 (Bd)	31 (Lc)	33–38 (Bd)	34 (T _m)	36.6 (T _m)

*Data were taken from this study.

†Bd, buoyant density; Lc, HPLC; T_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.

Methanobacterium movens (mo'vens. L. part. adj. *movens* movable).

Cells are rods ($0.4\text{--}0.5 \times 2\text{--}5\ \mu\text{m}$), 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 $35\text{--}38\ ^\circ\text{C}$ and pH 7.2–7.5. The DNA G+C content of the type strain is $39.1 \pm 0.8\ \text{mol}\%$ (T_m) and $39.5 \pm 0.6\ \text{mol}\%$ (HPLC).

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

Description of *Methanobacterium flexile* sp. nov.

Methanobacterium flexile (fle'xi.le. L. neut. adj. *flexile* pliable, flexile).

Cells are rods ($0.3\text{--}0.5 \times 2\text{--}5\ \mu\text{m}$). 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 ($\text{H}_2/\text{CO}_2/\text{N}_2$; 40:10:50, v/v; 100–150 kPa). Grows optimally at $35\text{--}38\ ^\circ\text{C}$ and pH 7.0–7.5. Yeast extract is indispensable for growth. The DNA G+C content of the type strain is $36.4 \pm 0.6\ \text{mol}\%$ (T_m) and $37.3 \pm 0.3\ \text{mol}\%$ (HPLC).

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

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References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Boone, D. R. (2001). Genus I. *Methanobacterium* Kluyver and Niel 1936, 399, ^{AL} emend. Balch and Wolfe in Balch, Fox, Magrum, Woese and Wolfe 1979, 284. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 215–218. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.
- Cuzin, N., Ouattara, A. S., Labat, M. & Garcia, J. L. (2001). *Methanobacterium congolense* sp. nov., from a methanogenic fermentation of cassava peel. *Int J Syst Evol Microbiol* **51**, 489–493.
- Embley, T. M., Finlay, B. J., Thomas, R. H. & Dyal, P. L. (1992). The use of rRNA sequences and fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate *Metopus palaeformis* and its archaeobacterial endosymbiont. *J Gen Microbiol* **138**, 1479–1487.
- Garcia, J. (1990). Taxonomy and ecology of methanogens. *FEMS Microbiol Rev* **87**, 297–308.
- Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W. & Saunders, J. R. (1996). Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl Environ Microbiol* **62**, 668–675.
- Hungate, R. (1969). The roll-tube method for cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.
- Jain, M. K., Thompson, T. E., Conway de Macario, E. & Zeikus, J. G. (1987). Speciation of *Methanobacterium* strain Ivanov as *Methanobacterium ivanovii*, sp. nov. *Syst Appl Microbiol* **9**, 77–82.
- Jarrell, K. F., Faguy, D., Hebert, A. M. & Kalmokoff, M. L. (1992). A general method of isolating high molecular weight DNA from methanogenic archaea (archaeobacteria). *Can J Microbiol* **38**, 65–68.
- Jones, W. J., Nagle, D. P., Jr & Whitman, W. B. (1987). Methanogens and the diversity of archaeobacteria. *Microbiol Rev* **51**, 135–177.
- Joulian, C., Patel, B. K., Ollivier, B., Garcia, J. L. & Roger, P. A. (2000). *Methanobacterium oryzae* sp. nov., a novel methanogenic rod isolated from a Philippines ricefield. *Int J Syst Evol Microbiol* **50**, 525–528.
- Kotelnikova, S. V., Obratsova, A. Ya., Gongadze, G. M. & Laurinavichius, K. S. (1993). *Methanobacterium thermoflexum* sp. nov. and *Methanobacterium defluvii* sp. nov., thermophilic rod-shaped methanogens isolated from anaerobic digester sludge. *Syst Appl Microbiol* **16**, 427–435.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Lai, M. C., Shu, C. M., Chen, S. C., Lai, L. J., Chiou, M. S. & Hua, J. J. (2000). *Methanosarcina mazei* strain O1M9704, methanogen with novel tubule isolated from estuarine environment. *Curr Microbiol* **41**, 15–20.
- Ma, K., Liu, X. & Dong, X. (2005). *Methanobacterium beijingense* sp. nov., a novel methanogen isolated from anaerobic digesters. *Int J Syst Evol Microbiol* **55**, 325–329.
- Owen, R. & Pitcher, D. (1985). Current methods for estimating DNA base composition and levels of DNA–DNA hybridization. In *Chemical Methods in Bacterial Systematics*, pp. 63–97. Edited by M. Goodfellow & E. Minnikin. London: Academic Press.
- Patel, G., Sprott, G. & Fein, J. (1990). Isolation and characterization of *Methanobacterium espanolae* sp. nov., a mesophilic, moderately acidiphilic methanogen. *Int J Syst Bacteriol* **40**, 12–18.
- Shlimon, A. G., Friedrich, M. W., Niemann, H., Ramsing, N. B. & Finster, K. (2004). *Methanobacterium aarhusense* sp. nov., a novel methanogen isolated from a marine sediment (Aarhus Bay, Denmark). *Int J Syst Evol Microbiol* **54**, 759–763.
- Sung, Y., Ritalahti, K. M., Apkarian, R. P. & Löffler, F. E. (2006). Quantitative PCR confirms purity of strain GT, a novel trichloroethene-to-ethene-respiring *Dehalococcoides* isolate. *Appl Environ Microbiol* **72**, 1980–1987.
- Thauer, R. K. (1998). Biochemistry of methanogenesis: a tribute to Marjory Stephenson. *Microbiology* **144**, 2377–2406.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Worakit, S., Boone, D. R., Mah, R. A., Abdel-Samie, M.-E. & El-Halwagi, M. M. (1986). *Methanobacterium alcaliphilum* sp. nov., an H_2 -utilizing methanogen that grows at high pH values. *Int J Syst Bacteriol* **36**, 380–382.
- Zehnder, A. & Wuhrmann, K. (1977). Physiology of a *Methanobacterium* strain AZ. *Arch Microbiol* **111**, 199–205.