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

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Two methanogenic strains, 8-2^T 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 H_2/CO_2 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 8-2^T and 6·8–8·6 for strain 4-1, with the fastest growth at pH 7·2 for strain 8-2^T and pH 7·5–7·7 for strain 4-1. The G+C content of genomic DNA for strain 8-2^T was 38·9 mol%. The similarity levels of the 16S rRNA sequence of strain 8-2^T 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 8-2^T (=DSM 15999^T=CGMCC 1.5011^T).

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 H_2/CO_2 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 H_2/CO_2. 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 formicicum DSM 1535^T, *Methanobacterium congolense* DSM 7095^T and *Methanobacterium oryzae* DSM 11106^T were purchased from the DSMZ (Braunschweig, Germany). Strains 8-2^T 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 H_2/CO_2 (80 : 20, $1·01 \times 10^5$ 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 N_2/CO_2 (80 : 20, $1·01 \times 10^5$ Pa) was used instead of H_2/CO_2 as the gas phase. Requirement for growth factors was determined by measuring growth in the H_2/CO_2 medium omitting one of the components in each test, which included vitamins, yeast extract, peptone, acetate, etc. The pH range

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Abbreviation: UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 8-2^T and 4-1 are AY350742 and AY552778.

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for growth was estimated by cultivating the strains in the H_2/CO_2 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 H_2/CO_2 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 H_2/CO_2 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 8-2^T 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 8-2^T 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 H_2/CO_2 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. H_2/CO_2 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 8-2^T. Strains 8-2^T and 4-1 grew well without peptone and vitamins, whereas yeast

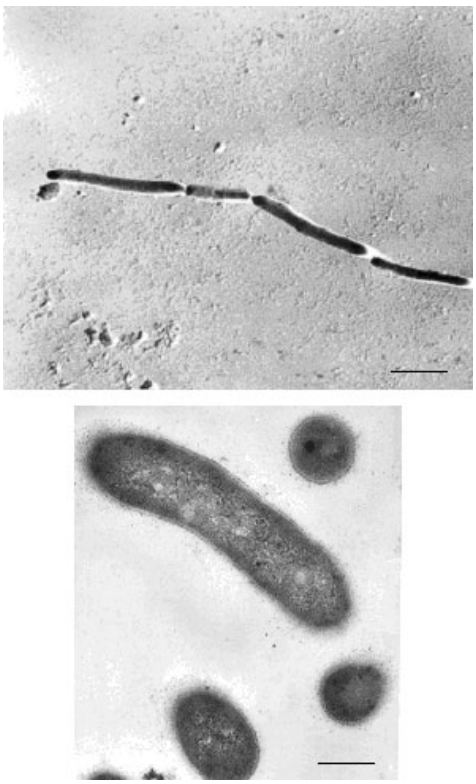

Fig. 1. Electron micrographs of cells of strain 8-2^T. (top) Transmission electron micrograph; bar, 1 μm. (bottom) Ultrathin section micrograph; bar, 0·2 μm.

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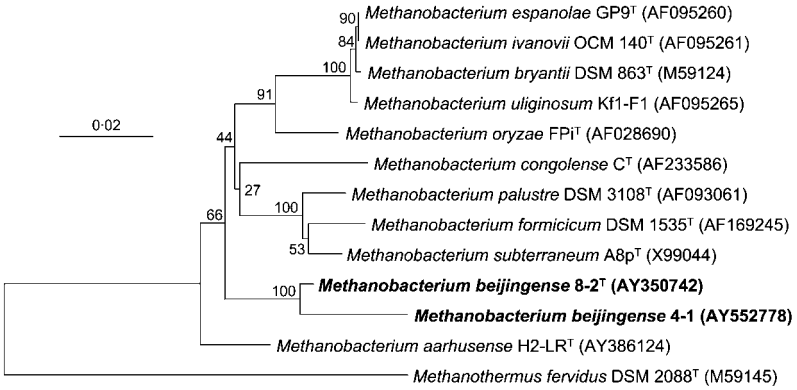

Fig. 2. Phylogenetic tree showing the position of strain 8-2^T amongst other species of the genus *Methanobacterium*. Based on a consensus length of 1378 bp of 16S rRNA gene sequences, the tree was constructed by the neighbour-joining method and rooted with *Methanothermus fervidus* DSM 2088^T. The topology 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.

extract (0·1–2 % w/v) was indispensable. Growth of strains 8-2^T 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 8-2^T and 6·8–8·6 for strain 4-1 and the optimum pH for growth was 7·2 for strain 8-2^T and 7·5–7·7 for strain 4-1. The specific growth rate of strain 8-2^T was 0·049 h^{-1} when grown in the H_2/CO_2 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 8-2^T was 38·9 mol%.

Phylogenetic analysis (Fig. 2) showed 98·2 % 16S rRNA gene sequence similarity between strains 8-2^T and 4-1; however, the similarity between 8-2^T and other species of *Methanobacterium* ranged from 93·8 to 96 %, indicating that strain 8-2^T could represent a novel species of this genus.

DNA–DNA relatedness values between strain 8-2^T and its phylogenetic relatives *Methanobacterium oryzae* DSM 11106^T, *Methanobacterium congolense* DSM 7095^T and *Methanobacterium formicicum* DSM 1535^T 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 8-2^T.

All the phenotypic and phylogenetic characteristics of strains 8-2^T 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 ivanovii* (Belyaev *et al.*, 1986), *Methanobacterium uliginosum* (König, 1984), *Methanobacterium congolense* (Cuzin *et al.*, 2001), *Methanobacterium bryantii* (Zellner & Winter, 1987) and *Methanobacterium aarhusense* (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 *et al.*, 1989); (iii) they differed from *Methanobacterium subterraneum* and *Methanobacterium alcaliphilum* 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).

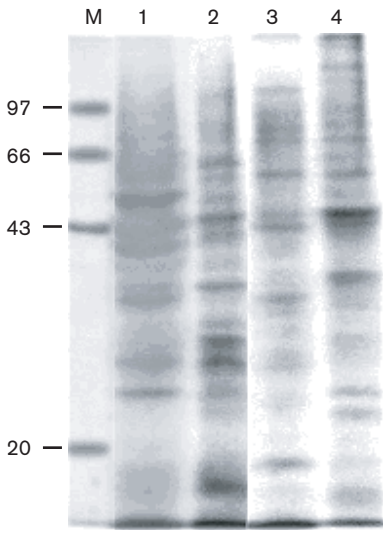

Fig. 3. Cell-protein SDS-PAGE profiles of strain 8-2^T (lane 1), *Methanobacterium oryzae* DSM 11116^T (2), *Methanobacterium congolense* DSM 7095^T (3) and *Methanobacterium formicicum* DSM 1535^T (4). Lane M, molecular mass markers (sizes in kDa).

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Table 1. Differential characteristics between *Methanobacterium beijingense* sp. nov. and other species of *Methanobacterium*

Strains: 1, *M. beijingense* sp. nov. 8-2^T; 2, *M. beijingense* sp. nov. 4-1; 3, *M. formicicum* DSM 1535^T (data from Bryant & Boone, 1987); 4, *M. oryzae* DSM 11106^T (Joulian *et al.*, 2000); 5, *M. congolense* DSM 7095^T (Cuzin *et al.*, 2001); 6, *M. palustre* DSM 3108^T (Zellner & Winter, 1987); 7, *M. subterraneum* DSM 11074^T (Kotelnikova *et al.*, 1998); 8, *M. alcaliphilum* DSM 3387^T (Worakit *et al.*, 1986); 9, *M. bryantii* DSM 863^T (Boone, 1987); 10, *M. espanolae* OCM 178^T (Patel *et al.*, 1990); 11, *M. ivanovii* DSM 2611^T (Belyaev *et al.*, 1986); 12, *M. uliginosum* DSM 2956^T (König, 1984); 13, *M. aarhusense* DSM 15219^T (Shlimon *et al.*, 2004). Abbreviations: ND, not determined; NG, alcohols are oxidized, but do not result in growth; iP, isopropanol; iB, isobutanol.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Source	Anaerobic digester	Anaerobic digester	Sewage sludge digester	Rice field	Anaerobic digester	Peat bog	Deep granitic groundwater	Alkaline lake	Anaerobic digester	Sludge	Rock core	Marshy soil	Marine sediment
Cell size	0·4–0·5 × 3–5	0·4–0·5 × 3–5	0·4–0·8 × 2–15	0·3–0·4 × 3–10	0·4–0·5 × 2–10	0·5 × 2·5 –5	0·1–0·15 × 0·6–1·2	0·5–0·6 × 2–25	0·5–1·0 × 10–1·5	0·8 × 3–22	0·5–0·8 × 1–15	0·2–0·6 × 1·9–3·8	0·7 × 5–18
Colony size (mm)	0·5–1·0	0·5–1·0	Up to 5	1–2	Up to 1	ND	1·0–2·0	0·2	1–5	0·5–1·0	3–6	ND	ND
Substrates used:													
Formate	+	+	+*	+	−*	+	−	−	−	−	−	−	−
iP, iB	−	−	−	−	NG	+	−	ND	NG	NG	−	−	−
Growth temperature (°C):													
Range	25–50	25–50	ND	20–42	25–50	20–45	3·6–45	ND	ND	15–50	15–55	15–45	5–48
Optimum	37	37	37–45	40	37–42	33–37	20–40	37	37–39	35	45	40	45
pH for growth:													
Range	6·5–8·0	6·8–8·6	6·6–7·8	6·0–8·5	5·9–8·2	ND	6·5–9·2	7·0–9·9	ND	4·6–7·0	6·5–8·5	6·0–8·5	5–9
Optimum	7·2	7·5–7·7	ND	7·0	7·2	7·0	7·8–8·8	8·1–9·1	6·9–7·2	5·6–6·2	7·0–7·4	ND	7·5–8
NaCl range (M)	0–0·5	ND	ND	0–0·4	ND	0–0·3	0·2–1·2	ND	ND	ND	ND	ND	ND
G+C content (mol%)†	38·9 (T_m)	ND	41–42 (Bd)	31 (Lc)	39·5 (Lc), 44·8 (T_m)*	34 (T_m)	54·5 (T_m)	57 (Bd)	33–38 (Bd)	34 (T_m)	36·6 (T_m)	33·8 (T_m)	34·9 (Lc)

*Checked in this study.

†Determined by buoyant density analysis (Bd), HPLC analysis (Lc) or melting point analysis (T_m).

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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 H_2/CO_2 and formate. No growth on acetate, methanol, ethanol, trimethylamine, isobutanol or isopropanol. Yeast extract is indispensable; 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, 8-2^T (=DSM 15999^T=CGMCC 1.5011^T), was isolated from an anaerobic digester for the treatment of beer-manufacture wastewater.

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