Methanosaeta harundinacea sp. nov., a novel acetate-scavenging methanogen isolated from a UASB reactor

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Two methanogenic strains, 8Ac^T and 6Ac, were isolated from an upflow anaerobic sludge blanket reactor treating beer-manufacture wastewater in Beijing, China. Cells of strains 8Ac^T and 6Ac were rod-shaped (0.8–1.0 × 3–5 µm) and non-motile, occurring singly or in pairs; however, at high cell density the cells were arranged in long chains within a common sheath. The two strains used acetate exclusively for growth and methane production. The specific growth rate of strain 8Ac^T was 0.030 h⁻¹ when growing in acetate (20 mM) at 37 °C. The temperature range for growth was 25–45 °C, with the fastest growth at 34–37 °C. The pH range for growth and methane production was 6.5–9.0, with the fastest growth at pH 7.2–7.6. The G + C content of genomic DNA of strain 8Ac^T was 55.7 mol%. Phylogenetic analysis based on 16S rRNA gene sequence similarity showed that the novel strains clustered with *Methanosaeta* species; the 16S rRNA gene sequence similarities between strain 8Ac^T and *Methanosaeta concilii* DSM 3013 and '*Methanosaeta thermophila*' DSM 6194 were 92.5 and 87.3 %, respectively. The sequence similarity levels of *mcrA*, the gene encoding the α -subunit of methyl-coenzyme M reductase, and of the deduced amino acids of *mcrA*, between strain 8Ac^T and *Methanosaeta concilii* DSM 3671^T were 36 and 78.9 %, respectively. Based on the phylogenetic and phenotypic analyses, the novel species *Methanosaeta harundinacea* sp. nov. is proposed, with strain 8Ac^T (=JCM 13211^T =CGMCC 1.5026^T) as the type strain.

Acetate is an important substrate for methanogenesis in anaerobic bioreactors and two-thirds or more of the methane produced in anaerobic bioreactors is derived from acetate (Zinder, 1993). All described acetate-using methanogens belong to the order *Methanosarcinales*. Among these, *Methanosaeta* species can be distinguished from members of the other genera by their exclusive use of acetate as a substrate for producing methane and by their rod-shaped cells, instead of coccoid or pseudosarcinal cells such as those of *Methanosarcina* species. In addition, in contrast to *Methanosarcina* species, *Methanosaeta* strains are favoured in environments with low concentrations of acetate (Huser *et al.*, 1982). To date, only two species have been described in the genus *Methanosaeta*, *Methanosaeta concilii* (Patel &

Sprott, 1990) and '*Methanosaeta thermophila*' (Kamagata *et al.*, 1992; Boone & Kamagata, 1998), although investigations based on culture-independent approaches have indicated that *Methanosaeta* strains occur widely (Lueders *et al.*, 2001; Zengler *et al.*, 1999). Ultrastructural analysis of granules from upflow anaerobic sludge blanket (UASB) reactors using scanning and transmission electron microscopy has also shown numerous *Methanosaeta*-like cells, implying that they could play important roles in methanogenesis (Guiot *et al.*, 1992; MacLeod *et al.*, 1990; Gonzalez-Gil *et al.*, 2001).

During a survey of the microbial community of a mesophilic methane-producing UASB reactor in Beijing, China, we isolated two strains of rod-shaped methanogens that produced methane exclusively from acetate. Phylogenetically, the strains were affiliated to the genus *Methanosaeta*; however, they were distantly related to the existing species of the genus. Moreover, their cells were rod-shaped instead of in a filament. Based on the phylogenetic and phenotypic data, a novel species of *Methanosaeta* is proposed.

Pre-reduced basal medium was prepared as described by Huser *et al.* (1982) and sodium acetate (20 mM), yeast

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Abbreviation: UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Methanosaeta harundinacea* 8Ac^T and 6Ac are AY817738 and AY970347, respectively, and those for the partial sequences of *mcrA* of *Methanosaeta harundinacea* 8Ac^T and 6Ac are AY970348 and AY970349, respectively.

extract (0.05 %, w/v) and peptone (0.05 %, w/v) were added to form complete medium. The medium was dispensed in screw-capped tubes sealed with butyl rubber stoppers and the gas phase was N_2/CO_2 (80:20, 1.01×10^5 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.

For enrichment of the acetate-using methanogenic culture, UASB granules for treating beer-manufacture wastewater in Beijing were inoculated and suspended with a vortex, and then serially diluted in complete medium containing 0.5 g penicillin l^{-1} (final concentration) to inhibit bacterial growth. Methanogenic dilution cultures were verified by methane production and acetate consumption after cultivation for a few weeks. This manipulation was repeated several times until pure cultures were obtained. Culture purity was confirmed periodically by monitoring cell morphology using normal bright-field microscopy, as well as by lack of growth in a rich medium, such as peptone/yeast extract/glucose liquid.

Cells of strain 8Ac^T in exponential growth phase were used for morphological examination under a transmission electron microscope (H-600A; Hitachi), after being coated with palladium/iridium alloy with a high vacuum evaporator (HUS-5GB; Hitachi). Cell motility was observed with a phase-contrast microscope (BH-2; Olympus). Cells from an exponential-phase culture were incubated in 1 % SDS and distilled water as a hypotonic solution to check the susceptibility to lysis (Boone & Whitman, 1988) and with proteinase K (50 $\mu g\ ml^{-1}$) to break the cell-enclosure sheath. Cell lysis or sheath breakage was determined microscopically, with untreated cells as a control. Physiological studies, such as substrate utilization, growth factor requirement, pH range, growth temperature range and NaCl tolerance were performed as described previously (Ma *et al.*, 2005). Methane production and substrate degradation were measured as described by Zhang *et al.* (2004). Formate cleavage was determined by measuring formate after culture for 20 days in basal medium containing formate (10 mM). Formate was detected at 242 nm by HPLC (Waters model 1525). A reverse-phase HPLC (ODS Hypersil; Hewlett Packard) mobile phase of acetonitrile/water (30:70, v/v) was used at a flow rate of 0.7 $ml\ min^{-1}$.

Genomic DNA extraction and purification were performed according to Jarrell *et al.* (1992), with a 1 l culture. The G+C content was determined by using the thermal denaturation method (Owen & Pitcher, 1985), with *Escherichia coli* K-12 as the reference, with a UV800 spectrophotometer (Beckman).

The 16S rRNA gene was amplified from the genomic DNA as described previously (Furlong *et al.*, 2002). A partial fragment of the gene encoding the α -subunit of methyl-coenzyme M reductase (*mcrA*) was amplified according to Lueders *et al.* (2001). Purified PCR products of the 16S rRNA gene of 1400 bp in length and a partial fragment of

mcrA of 500 bp in length were cloned into the vector pUCm-T, and sequenced by Bioasia Company (Beijing).

Sequences in GenBank similar to those of the 16S rRNA gene and *mcrA* partial fragment were determined using the BLASTN algorithm. The best matching sequences were retrieved from the database and aligned and similarity analysis was performed with CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed using MEGA 2.1 software (Kumar *et al.*, 2001).

Cells of strains 8Ac^T and 6Ac were rod-shaped (0.8–1.0 $\times$ 3–5 μm ; Fig. 1) and occurred singly or in pairs; however, at high cell density when grown in high concentrations of acetate (>100 mM), the cells were arranged as a chain within a common sheath. Cells of strains 8Ac^T and 6Ac were non-motile, Gram-variable and resisted disruption by 1 % SDS (w/v) or hypotonic solution. However, cell-chain sheaths were partly destroyed by proteinase K (50 $\mu g\ ml^{-1}$), indicating a proteinaceous composition. The two strains were strictly anaerobic and growth was completely inhibited in air. Acetate was the only substrate tested to support growth and methane production, at concentrations of acetate of 2–350 mM. None of the following substrates tested was used for growth or methane production: H_2/CO_2 , methanol, ethanol, trimethylamine, isobutanol and isopropanol (each 20 mM). Moreover, formate was not cleaved to hydrogen and carbon dioxide. Yeast extract (0.05–1 %, w/v) or peptone (0.05–1 %, w/v) was required for growth, whereas vitamins were not essential, in contrast to some *Methanosaeta* strains, for which yeast extract might be inhibitory (Touzel *et al.*, 1988). Growth of strains 8Ac^T and 6Ac was observed at 25–45 °C, with the fastest growth occurring at 34–37 °C. The pH range for growth was 6.5–9.0, with the fastest growth at pH 7.2–7.6. The specific

Fig. 1. Transmission electron micrograph of cells of *Methanosaeta harundinacea* strain 8Ac^T grown in 20 mM acetate for 20 days. Bar, 1 μm .

Fig. 2. Phylogenetic tree showing the position of strain 8Ac^T amongst other members of the order *Methanosarcinales*. Based on 16S rRNA gene sequences (consensus length 1350 bp), the tree was constructed by using the neighbour-joining method and rooted with *Methanothermus fervidus* DSM 2088^T. The topology of the tree was estimated by bootstrap analysis based on 1000 replications. Numbers at branch points are percentages supported by bootstrap evaluation. GenBank accession numbers are given in parentheses. Bar, 2% sequence divergence.

growth rate of strain 8Ac^T in complete medium at 37 °C was 0.030 h⁻¹ (doubling time 28 h), whereas the specific growth rate in acetate-containing basal medium with addition of yeast extract, peptone or tryptone (0.05%, w/v) was 0.016, 0.013 and 0.0045 h⁻¹, respectively. The G+C content of the genomic DNA of strain 8Ac^T was 55.7 mol%.

Phylogenetic analysis based on a consensus 1350 bp length of 16S rRNA gene sequences (Fig. 2) showed 99.5% similarity between the two novel strains 8Ac^T and 6Ac, indicating that they represented a single species; however, similarities of only 92.5 and 87.3% were obtained between strain 8Ac^T and *Methanosaeta concilii* DSM 3013 and 'Methanosaeta thermophila' DSM 6194, respectively. In addition to 16S rRNA gene sequence analysis, *mcrA* gene sequences have also been used as a phylogenetic tool for the order *Methanosarcinales*; similarity levels among different species of the same genus range from 87.5 to 93.8%

(Springer *et al.*, 1995). A phylogenetic tree (Fig. 3) was constructed based on deduced amino acid sequences of the *mcrA* gene, including those of strain 8Ac^T and related methanogens. The similarities of the partial fragments of *mcrA* and deduced amino acid sequences between strain 8Ac^T and *Methanosaeta concilii* DSM 3671^T were 36 and 78.9%, respectively. Phylogenetic analysis based on either the 16S rRNA gene or *mcrA* indicated that strain 8Ac^T represented a novel species of the genus *Methanosaeta*. In addition, strain 8Ac^T also differed from other species in cell shape and G+C content. Differential phenotypic characteristics of strain 8Ac^T and other *Methanosaeta* species are shown in Table 1.

Based on morphological, physiological and phylogenetic data, we propose that the newly isolated strains 8Ac^T and 6Ac represent a novel species of the genus *Methanosaeta*, with the name *Methanosaeta harundinacea* sp. nov.

Fig. 3. Phylogenetic tree based on *mcrA* gene sequences showing the position of strain 8Ac^T amongst other members of the order *Methanosarcinales*. Based on consensus lengths of 165 deduced amino acids from 495 bp of *mcrA* sequences, the tree was constructed by using the neighbour-joining method and rooted with *Methanothermus fervidus* DSM 2088^T. The topology of the tree was estimated by bootstrap analysis based on 1000 replications. Numbers at branch points are percentages supported by bootstrap evaluation. GenBank accession numbers are given in parentheses. Bar, 5% sequence divergence.

Table 1. Differential characteristics between *Methanosaeta harundinacea* sp. nov. 8Ac^T and other species of *Methanosaeta*

Species: 1, *Methanosaeta harundinacea* 8Ac^T; 2, *Methanosaeta concilii* DSM 3671^T [data from Patel & Sprott (1990)]; 3, '*Methanosaeta thermophila*' DSM 6194 (Kamagata *et al.*, 1992). Abbreviations: PEP, peptone; SF, sludge fluid; YE, yeast extract; ND, not determined.

Characteristic	1	2	3
Cell width (µm)	0.8–1.0	0.8	0.8–1.3
Cell arrangement in 20 mM acetate (length)	Single or in pairs	Long chain in a sheath (>100 µm)	Short chain (10–100 µm)
Temperature:			
Range (°C)	25–45	10–45	ND
Optimum (°C)	34–37	35–40	55–60
pH:			
Range	6.5–9.0	6.6–7.8	ND
Optimum	7.2–7.6	7.1–7.5	approx. 7.0
Growth factor requirement	YE, PEP	SF, vitamin	SF
Inhibitory factor	ND	YE (0.06 %, w/v)	ND
Doubling time (h)	28	65	ND
G + C content (mol%)	55.7 (<i>T_m</i>)	50.3 (<i>T_m</i>)	52.7 (HPLC)

Description of *Methanosaeta harundinacea* sp. nov.

Methanosaeta harundinacea (ha.run.di.na'ce.a. L. fem. adj. *harundinacea* like a reed, referring to the cell shape of a reed stem).

Cells are rod-shaped (0.8–1.0 × 3–5 µm), occurring singly or in pairs; however, they are arranged as a chain within a common sheath at high cell density when grown in high concentrations of acetate (>100 mM). Gram-variable. Non-motile. Cells are resistant to lysis by 1 % SDS (w/v) and hypotonic solution, whereas the sheath of the long chain is partly destroyed by proteinase K (50 µg ml⁻¹). Methanogenic. Acetate is the exclusive tested substrate for growth and methanogenesis. No growth on or methane production from H₂/CO₂, formate, methanol, ethanol, trimethylamine, isobutanol or isopropanol. Formate is not cleaved to hydrogen and carbon dioxide. Yeast extract or peptone are required for growth, but not vitamins. The temperature range for growth is 25–45 °C, with optimal growth at 34–37 °C. The pH range for growth is 6.5–9.0 and the optimum pH is 7.2–7.6. The DNA G + C content of the type strain is 55.7 mol% (*T_m*).

The type strain, 8Ac^T (=JCM 13211^T = CGMCC 1.5026^T), was isolated from granules from a UASB anaerobic digester treating beer-manufacture wastewater.

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References

Boone, D. R. & Kamagata, Y. (1998). Rejection of the species *Methanoxanthus soehngenii*^{VP} and the genus *Methanoxanthus*^{VP} as *nomina*

confusa, and transfer of *Methanoxanthus thermophila*^{VP} to the genus *Methanosaeta*^{VP} as *Methanosaeta thermophila* comb. nov. Request for an Opinion. *Int J Syst Bacteriol* **48**, 1079–1080.

Boone, D. R. & Whitman, W. B. (1988). Proposal of minimal standards for describing new taxa of methanogenic bacteria. *Int J Syst Bacteriol* **38**, 212–219.

Furlong, M. A., Singleton, D. R., Coleman, D. C. & Whitman, W. B. (2002). Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. *Appl Environ Microbiol* **68**, 1265–1279.

Gonzalez-Gil, G., Lens, P. N. L., Van Aelst, A., Van As, H., Versprille, A. I. & Lettinga, G. (2001). Cluster structure of anaerobic aggregates of an expanded granular sludge bed reactor. *Appl Environ Microbiol* **67**, 3683–3692.

Guiot, S. R., Pauss, A. & Costerton, J. W. (1992). A structured model of the anaerobic granule consortium. *Water Sci Technol* **25** (7–8), 1–10.

Huser, B. A., Wuhrmann, K. & Zehnder, A. J. B. (1982). *Methanoxanthus soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. *Arch Microbiol* **132**, 1–9.

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.

Kamagata, Y., Kawasaki, H., Oyaizu, H., Nakamura, K., Mikami, E., Endo, G., Koga, Y. & Yamasato, K. (1992). Characterization of three thermophilic strains of *Methanoxanthus* ('*Methanosaeta*') *thermophila* sp. nov. and rejection of *Methanoxanthus* ('*Methanosaeta*') *thermoacetophila*. *Int J Syst Bacteriol* **42**, 463–468.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, AZ, USA.

Lueders, T., Chin, K. J., Conrad, R. & Friedrich, M. (2001). Molecular analyses of methyl-coenzyme M reductase α -subunit (*mcrA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environ Microbiol* **3**, 194–204.

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.

- MacLeod, F. A., Guiot, S. R. & Costerton, J. W. (1990).** Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. *Appl Environ Microbiol* **56**, 1598–1607.
- Owen, R. J. & Pitcher, D. (1985).** Current methods for estimating DNA base composition and levels of DNA-DNA hybridization. In *Chemical Methods in Bacterial Systematics*, pp. 67–93. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Patel, G. B. & Sprott, G. D. (1990).** *Methanosaeta concilii* gen. nov., sp. nov. (“*Methanothrix concilii*”) and *Methanosaeta thermoacetophila* nom. rev., comb. nov. *Int J Syst Bacteriol* **40**, 79–82.
- Springer, E., Sachs, M. S., Woese, C. R. & Boone, D. R. (1995).** Partial gene sequences for the a subunit of methyl-coenzyme M reductase (*mcrI*) as a phylogenetic tool for the family *Methanosarcinaceae*. *Int J Syst Bacteriol* **45**, 554–559.
- 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.
- Touzel, J. P., Prensier, G., Roustan, J. L., Thomas, I., Dubourguier, H. C. & Albagnac, G. (1988).** Description of a new strain of *Methanothrix soehngenii* and rejection of *Methanothrix concilii* as a synonym of *Methanothrix soehngenii*. *Int J Syst Bacteriol* **38**, 30–36.
- Zengler, K., Richnow, H. H., Rosselló-Mora, R., Michaelis, W. & Widdel, F. (1999).** Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **401**, 266–269.
- Zhang, C., Liu, X. & Dong, X. (2004).** *Syntrophomonas curvata* sp. nov., an anaerobe that degrades fatty acids in co-culture with methanogens. *Int J Syst Evol Microbiol* **54**, 969–973.
- Zinder, S. H. (1993).** Physiological ecology of methanogens. In *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*, pp. 128–206. Edited by J. G. Ferry. New York: Chapman & Hall.