Methanoculleus hydrogenitrophicus sp. nov., a methanogenic archaeon isolated from wetland soil

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An obligately anaerobic, methanogenic archaeon, strain HC^T, was isolated from soil of the Zoige wetland on the Tibetan plateau, China. The strain was isolated through construction of an artificial butyrate-degrading consortium in co-culture with a syntrophic bacterium, '*Syntrophomonas erecta* subsp. *sporosyntropha*' JCM 13344. Cells of strain HC^T were irregular coccoids, 0.8–2 µm in diameter, that occurred singly and utilized only H₂/CO₂ for growth and methane production. Growth occurred at 18–45 °C (optimum around 37 °C). The pH for growth was 5.0–8.5 (optimal growth around pH 6.6). The G + C content of the genomic DNA was 60.2 mol%. 16S rRNA gene sequence analysis indicated that strain HC^T was affiliated to the genus *Methanoculleus*, with sequence similarities of 94.8–97.2% to existing members. However, strain HC^T was distinguished from described *Methanoculleus* species by not using formate for growth or methane formation and not requiring acetate as a growth factor. On the basis of phylogenetic analysis and phenotypic characteristics, the novel species *Methanoculleus hydrogenitrophicus* sp. nov. is proposed, with strain HC^T (=CGMCC 1.5146^T =JCM 16311^T) as the type strain.

Natural wetlands are an important methane source inhabited by abundant methanogenic archaea (Matthews & Fung, 1987). Our previous study showed a wide diversity of methanogens in the Zoige wetland of the Tibetan plateau (Zhang *et al.*, 2008b), and phylogenetic analyses based on both 16S rRNA and *mcrA* gene sequences indicated that methanogens of the families *Methanosarcinaceae* and *Methanomicrobiales* were prevalent in the Zoige wetland. Furthermore, a novel psychrophilic, methylotrophic member of the *Methanosarcinaceae*, '*Methanolobus psychrophilus*', was isolated and described (Zhang *et al.*, 2008a). Other studies have shown that members of the *Methanomicrobiales* are likely to be the prevalent hydrogenotrophic methanogens in cold wetlands (Ganzert *et al.*, 2007; Grabowski *et al.*, 2005), and many uncultured members of the *Methanomicrobiales* were enriched by using low levels of H₂ (Sakai *et al.*, 2007). These studies together imply that members of the *Methanomicrobiales* seemed to predominate under low H₂, as found *in situ* in wetlands. In this work, by constructing an artificial butyrate-degrading consortium to create an environment with a low level of H₂, we isolated a novel strain of the *Methanomicrobiales* from Zoige wetland soil.

'*Syntrophomonas erecta* subsp. *sporosyntropha*' JCM 13344 was preserved in our laboratory and cultivated in a medium containing 20 mM crotonate (Wu *et al.*, 2006). *Methanoculleus chikugoensis* DSM 13459^T, *Methanoculleus marisnigri* DSM 1498^T, *Methanoculleus submarinus* DSM 15122^T and *Methanoculleus thermophilus* DSM 2373^T were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and cultivated under a gas phase of 1.01 × 10⁵ Pa H₂/CO₂ (80 : 20). The sampling site, the Zoige wetland, is located on the Tibetan plateau (33° 56' N 102° 52' E). The soil, covered by growth of *Carex muliensis*, was collected at the depth of 20–30 cm at the end of May 2007 and placed in sterile serum bottles sealed with butyl rubber stoppers and kept in a cool box during transportation.

The basal medium used in this study was prepared as described previously (Ma *et al.*, 2005). The medium was dispensed into screw-capped tubes sealed with butyl rubber stoppers, with a headspace of 1.01 × 10⁵ Pa H₂/CO₂ (80 : 20) or N₂ for routine cultivation, as indicated. To isolate low H₂-preferring methanogens from the soil, 0.5 ml each of soil slurry and pre-grown culture of '*S. erecta* subsp. *sporosyntropha*' JCM 13344 were inoculated into 5 ml medium with 20 mM butyrate as sole carbon source and incubated anaerobically at 37 °C. Butyrate degradation and methane production were monitored by gas chromatography as described previously (Ma *et al.*, 2005; Wu *et al.*, 2006). A butyrate-degrading consortium

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HC^T is FJ977567.

Graphs showing effects of temperature, pH and NaCl on growth of strain HC^T are available as supplementary material with the online version of this paper.

was obtained by subculturing the enrichment in the same medium for eight to ten transfers, and the enrichment was then serially diluted in basal medium with H₂/CO₂ (80:20) as headspace; 0.5 g vancomycin l⁻¹ (final concentration) was added to inhibit bacterial growth (Kotelnikova *et al.*, 1998). After several subcultures in H₂/CO₂ (80:20) medium, cultivation was performed by the Hungate roll-tube technique (Hungate, 1969) and the purified strain HC^T was obtained from a single colony in a Hungate tube. Strain HC^T was further purified by repeated culture in roll tubes. The purity of the strain was confirmed by light microscope examination, the absence of growth in rich medium such as peptone/yeast extract/glucose (PYG) broth and identity of 16S rRNA gene sequences in a generated clone library. The bacteria-specific primer 27F [5'-AGAGTTTGATC(AC)TGGCTCAG-3'], the archaea-specific primer Arch4F (5'-TCCGGTTGATCCTGCCRG-3') and the prokaryotic universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA gene.

Cell morphology of strain HC^T was examined under a light microscope (BH-2; Olympus) and transmission electron microscope (H-600A; Hitachi). Before observation, cells were coated with palladium/iridium alloy by using a high-vacuum evaporator (HUS-5GB; Hitachi). Cells were negatively stained with uranyl acetate and lead citrate according to Reynolds (1963). Cell motility was observed by phase-contrast microscopy (BH-2; Olympus) by following the method of Boone & Whitman (1988) and Ma *et al.* (2005). Both examinations showed that cells of strain HC^T were irregular coccoids, around 0.8–2 µm in diameter (Fig. 1), and not motile. Cells stained Gram-negative and resisted disruption by 1% SDS (w/v) or hypotonic solution, tested as described by Boone & Whitman (1988). Strain HC^T grew strictly anaerobically; growth was inhibited completely in the presence of air.

Fig. 1. Electron micrograph of a cell of strain HC^T. Bar, 0.5 µm.

Growth and substrate utilization were determined by monitoring the OD₆₀₀ and methane production. All inoculations and transfers were done with syringes and needles, and incubations for growth/substrate utilization tests were performed in triplicate at 37 °C for over 35 days. Basal medium containing 0.2% yeast extract was used for growth tests. The temperature profile of strain HC^T was determined in H₂/CO₂ (80:20) medium in a water bath with a temperature gradient from 8 to 55 °C at 1 °C intervals. Growth of strain HC^T was observed at 18–45 °C, with optimum growth at 37 °C. To determine the pH range for growth, H₂/CO₂ (80:20) medium was adjusted to pH 3.0–9.0 with 1 M HCl or NaOH; growth was observed at pH 5.0–8.5, with optimum growth around pH 6.6. According to the method of Lai *et al.* (2000) and Boone & Whitman (1988), the specific growth rate of strain HC^T during exponential growth was determined as 0.031 h⁻¹ when grown in H₂/CO₂ (80:20) medium at 37 °C and pH 6.6, calculated by linear regression of the logarithm of the total amount of methane accumulated. The salinity range was determined in media with 0.05–0.5 M NaCl; optimum growth was observed with around 0.2 M NaCl. Effects of temperature, pH and NaCl concentration on growth of strain HC^T are shown in Supplementary Fig. S1, available in IJSEM Online.

The substrate spectrum of strain HC^T was determined by addition of each carbon source to the basal medium and then determining methane production after incubation at 37 °C for 35 days. It was determined that H₂/CO₂ was the only substrate that supported growth and methane production; formate, acetate, methanol, ethanol, trimethylamine, isobutanol and 2-propanol (each at 20 mM) did not. Requirements for growth factors were determined by measuring growth in the H₂/CO₂ medium and omitting one of the components in each test, including vitamins, yeast extract, peptone and acetate. Strain HC^T grew poorly without peptone and vitamins. Yeast extract (0.1–2.5%) was not necessary but stimulated growth.

DNA extraction was carried out as described previously (Ma *et al.*, 2005). The G + C content was determined using the thermal denaturation method (Marmur & Doty, 1962; Owen & Pitcher, 1985) using DNA from *Escherichia coli* K-12 as a reference; the G + C content of genomic DNA of strain HC^T was determined to be 60.2 mol%.

PCR amplification and sequencing of the 16S rRNA gene were performed as described previously (Ma *et al.*, 2005) using primers Arch4F and 1492R (Furlong *et al.*, 2002). The 16S rRNA gene of strain HC^T was sequenced by SinoGenoMax Company (Beijing, China) and the sequence was submitted to GenBank to search for similar sequences using the BLAST algorithm. The best matching sequences were retrieved from the database and aligned and similarity analysis was performed using CLUSTAL_X (Thompson *et al.*, 1994). Based on a consensus length of 1378 bp of the 16S rRNA gene sequence, a phylogenetic tree (Fig. 2) rooted with *Methanomicrobium mobile* DSM 1539^T and

Fig. 2. Phylogenetic tree showing the position of strain HC^T among species of the genus of *Methanoculleus*. 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 *Methanomicrobium mobile* DSM 1539^T and *Methanogenium cariaci* DSM 1497^T. The stability of the tree topology was estimated by bootstrap analysis based on 1000 replications. Numbers at branch points are percentages of bootstrap support. Accession numbers are shown in parentheses. Bar, 2% sequence divergence.

Methanogenium cariaci DSM 1497^T was constructed using neighbour-joining method implemented in the program MEGA4 (Tamura *et al.*, 2007). The robustness of the tree was evaluated by bootstrap analysis (Felsenstein, 1985) with 1000 resamplings. Phylogenetic analysis showed that strain HC^T and type strains of other species of *Methanoculleus* displayed 16S rRNA gene sequence similarities of 94.8–97.2%, indicating that it could represent a novel species of this genus.

DNA–DNA relatedness was determined based on the initial reassociation rate according to the method of Owen & Pitcher (1985) at 61–65 °C by using a UV800 spectrophotometer (Beckman). The DNA–DNA relatedness between strain HC^T and its phylogenetic relatives *Methanoculleus chikugoensis* DSM 13459^T, *Methanoculleus marisnigri* DSM 1498^T, *Methanoculleus submarinus* DSM 15122^T and *Methanoculleus thermophilus* DSM 2373^T was determined

to be 42.0, 39.8, 23.5 and 45.7%, respectively; these values are lower than the threshold of 70% DNA–DNA hybridization recommended for species delineation.

On the basis of phenotypic and phylogenetic characteristics, strain HC^T was determined to belong to the genus *Methanoculleus*. However, some phenotypic features distinguished strain HC^T from the other species listed in Table 1. The major phenotypic differences were as follows: (i) strain HC^T differed from all members of the genus *Methanoculleus* by using H₂/CO₂ only as the substrate for growth and methane production; (ii) acetate did not support growth of strain HC^T nor was it required, while acetate was a growth factor for other members of the genus; (iii) the optimum NaCl concentration for growth differed between strain HC^T and *Methanoculleus bourgensis* (Asakawa & Nagaoka, 2003); (iv) the optimum pH and temperature for growth of strain HC^T were distinct from

Table 1. Differential characteristics between strain HC^T and type strains of phylogenetically related *Methanoculleus* species

Strains: 1, HC^T; 2, *Methanoculleus thermophilus* DSM 2373^T (unless indicated, data from Rivard & Smith, 1982; Spring *et al.*, 2005); 3, *Methanoculleus submarinus* DSM 15122^T (Mikucki *et al.*, 2003); 4, *Methanoculleus marisnigri* DSM 1498^T (Maestrojuán *et al.*, 1990); 5, *Methanoculleus chikugoensis* DSM 13459^T (Dianou *et al.*, 2001). All strains stain Gram-negative and use H₂/CO₂ as a substrate for growth and methane production (verified for reference strains in this study).

Characteristic	1	2	3	4	5
Cell morphology*	IC	IC	IC	C	IC
DNA G + C content (mol%)	60.2 ± 1.2	59.1	58	61.7 ± 1	62.2
Temperature for growth (°C)					
Optimum	~37	55†	45†	~40†	25–30†
Range	18–45	37–65†	10–55†	18–60†	15–40†
Optimum pH	~6.6	7	4.8–7.7	7–8	6.7–7.2
Substrate utilized					
Formate	–	+†	+†	+†	+†
Secondary alcohols	–	–	+	+	+
Growth factor required					
Yeast extract	–	–†	–†	+†	–†
Acetate	–	+†	+†	+†	+†
Optimum NaCl concentration (M)	0.2	0.2	0.2	0.2	0.1

*C, Coccoid; IC, irregular coccoid.

†Data obtained in this study.

those of *Methanoculleus receptaculi* (Cheng *et al.*, 2008) and *Methanoculleus oldenburgensis* (Asakawa & Nagaoka, 2003).

According to the minimal standards for the description of new taxa of methanogens (Boone & Whitman, 1988; Stackebrandt & Goebel, 1994) and based on phylogenetic and phenotypic characteristics, a novel species of the genus *Methanoculleus*, *Methanoculleus hydrogenitrophicus* sp. nov., is proposed.

Description of *Methanoculleus hydrogenitrophicus* sp. nov.

Methanoculleus hydrogenitrophicus [hy.dro.ge.ni.tro'phi.cus. N.L. n. *hydrogenum* hydrogen (from Gr. n. *hudôr* water; Gr. v. *gennaô* to produce); N.L. masc. adj. *trophicus* nursing, tending or feeding (from Gr. adj. *trophikos*); N.L. masc. adj. *hydrogenitrophicus* using hydrogen as electron donor].

Cells are non-motile, Gram-negative-staining, irregular coccoids. Cells resist lysis by 1% (w/v) SDS. Produces methane only from H₂ and CO₂. Acetate is not required for growth; yeast extract stimulates growth. Growth occurs at 18–45 °C with optimum growth around 37 °C. The pH range is 5.0–8.5, with optimum growth around pH 6.6. The DNA G+C content of the type strain is 60.2 mol%.

The type strain, HC^T (=CGMCC 1.5146^T =JCM 16311^T), was isolated from the Zoige wetland, China.

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