Methanofollis ethanolicus sp. nov., an ethanol-utilizing methanogen isolated from a lotus field

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A novel methane-producing archaeon, designated strain HASU^T, was isolated from a lotus field. Cells were Gram-negative, non-motile, irregular cocci, 2–3 µm in diameter, and occurred singly. Growth was observed at 15–40 °C (optimum, 37 °C) and pH 6.5–7.5 (optimum, pH 7.0). The G + C content of the genomic DNA was 60.9 mol%. Strain HASU^T utilized ethanol, 1-propanol, 1-butanol, hydrogen and formate for growth and methane production. It converted ethanol to methane and acetate. Based on comparative 16S rRNA gene sequence analysis, strain HASU^T was shown to be affiliated with the genus *Methanofollis*. It was related most closely to the type strain of *Methanofollis liminatans* (96.1 % 16S rRNA gene sequence similarity). Based on phylogenetic analysis and phenotypic characteristics, strain HASU^T is considered to represent a novel species of the genus *Methanofollis*, for which the name *Methanofollis ethanolicus* sp. nov. is proposed. The type strain is HASU^T (=NBRC 104120^T=JCM 15103^T=DSM 21041^T).

Widdel (1986) described the first methane-producing archaea (methanogens) that were able to utilize alcohols other than methanol as substrates for growth and methane production. Two strains that were able to grow on secondary alcohols such as 2-propanol and 2-butanol were isolated, and one of these strains, designated strain CV^T, was also able to utilize ethanol as an energy source (Widdel, 1986). Strain CV^T was subsequently characterized taxonomically and was described as representing *Methanogenium organophilum* (Widdel *et al.*, 1988). At the time of writing, although several different taxonomic groups of methanogens have been shown to be capable of growth on secondary alcohols (Grahame & Stadtman, 1993), *Methanogenium organophilum* is the only methanogen that is known to be able to grow with primary alcohols other than methanol. Recently, we have successfully isolated an ethanol-utilizing methanogen, designated strain HASU^T, from a lotus field. In the present report, we provide details of the morphological, physiological and phylogenetic characteristics of strain HASU^T and propose that this strain represents a novel species of the genus *Methanofollis*. This novel taxon is the second example of a methanogen that is capable of utilizing primary alcohols for growth and methane production.

A lotus field mud sample was obtained at Nagaoka, Niigata, Japan (37° 30' 57" N 138° 52' 34" E). After sampling, the mud was immediately washed with phosphate buffer (10 mM, pH 7.2) under nitrogen gas to remove extra roots and plant debris, and the mud was then collected by centrifugation for 5 min at 9000 g. The mud was resuspended in phosphate buffer and inoculated into an enrichment culture medium. The medium for cultivation of strain HASU^T was based on that of Widdel & Pfennig (1981). The medium consisted of the following components (per litre distilled water): 0.54 g NH₄Cl, 0.14 g KH₂PO₄, 0.2 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, 2.5 g NaHCO₃, 0.3 g Na₂S·9H₂O, 0.3 g cysteine·HCl, 1 ml trace element solution, 1 ml vitamin solution and 1 ml resazurin solution (1 mg ml⁻¹). The trace element solution contained (per litre distilled water): 1.27 g FeCl₂·4H₂O, 0.2 g MnCl₂·4H₂O, 0.13 g CoCl₂, 0.14 g ZnCl₂, 0.001 g CuCl₂·2H₂O, 0.01 g AlCl₃, 0.006 g H₃BO₃, 0.02 g Na₂MoO₄·2H₂O, 0.01 g NiCl₂, 0.002 g Na₂SeO₃ and 0.003 g Na₂WO₄·2H₂O. The vitamin solution was composed of the following vitamins (per litre distilled water): 4.9 mg biotin, 8.8 mg folic acid, 4.1 mg pyridoxine·HCl, 6.7 mg thiamine·HCl, 7.5 mg riboflavin, 2.4 mg nicotinic acid, 9.5 mg D-pantothenate (calcium salt), 0.1 mg vitamin B₁₂, 2.7 mg *p*-aminobenzoic acid and 4.1 mg lipoic acid. Enrichment cultures were incubated anaerobically at 25 °C. After isolation, all incubations were performed at 37 °C in 50-ml serum vials containing 20 ml

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HASU^T is AB371073.

medium (pH 7.2 at 25 °C) under an atmosphere of N₂/CO₂ (80:20, by vol.) without shaking, unless otherwise mentioned. The serum vials were sealed with butyl rubber stoppers and aluminium crimp seals. To monitor whether the media were kept under anaerobic conditions, resazurin was added to the medium as a redox indicator. In the nutritional tests, substrates added to the vials were neutralized prior to inoculation, and the media were supplemented with 0.01 % yeast extract (w/v) and 1 mM acetate. Growth and substrate utilization were determined by monitoring the turbidity of the cultures (OD at 400 nm) and the production of methane. All incubations for growth/substrate utilization tests were performed in triplicate at 37 °C for over 3 months. Effects of temperature, pH, NaCl concentration and antibiotics on the growth of strain HASU^T were determined in media containing 0.01 % yeast extract (w/v) in the presence of 5 mM ethanol in triplicate culture vessels. Tests for growth temperature, pH and salinity ranges were carried out at 4–60 °C, pH 5.5–8.5 and 1–30 g NaCl l⁻¹. For pH growth tests, the pH value was adjusted at room temperature to 5.5–8.5 by adding HCl or NaOH solution under a 100 % N₂ atmosphere prior to inoculation. The pH of the medium was monitored every 5 days by using a handy-type pH meter (HORIBA Twin pH B-212), and the pH was readjusted by using HCl and NaOH solution if it had changed significantly from the initial value. To test for the effect of NaCl concentration on growth, NaCl was added to the medium at concentrations of 1–30 g l⁻¹. Antibiotic susceptibility was examined with cultures supplemented with antibiotics at a final concentration of 100 µg ml⁻¹.

Cell morphology was examined under an epifluorescence microscope (Olympus BX51F) with a colour CCD camera system (Olympus DP71). The Gram-staining reaction was performed according to Hucker's method (Doetsch, 1981). Cell lysis was checked by adding SDS at a final concentration of 0.01–1.0 % (w/v), and was determined by microscopic observation of cell integrity. Short-chain fatty acids, methane, hydrogen and carbon dioxide were measured as described by Imachi *et al.* (2000, 2002). Ethanol was measured by HPLC by using an SCR-101-H column (Shimadzu) and a refractive index detector (Sekiguchi *et al.*, 2001). The G+C content of the genomic DNA was determined by HPLC as described by Nakagawa *et al.* (2003), and represented the result of a single determination.

All procedures for DNA extraction, 16S rRNA gene-based cloning and sequencing were as reported previously (Imachi *et al.*, 2006). For PCR amplification, primers Arch21F (DeLong, 1992) and 1490R (Weisburg *et al.*, 1991) were used to obtain the nearly full-length 16S rRNA gene sequence of strain HASU^T. Comparative 16S rRNA gene sequence phylogenetic analysis was performed as described previously (Imachi *et al.*, 2006). 16S rRNA gene sequence similarity values were calculated by using the Calculate Matrix function of the ARB program with Jukes and Cantor correction (Jukes & Cantor, 1969; Ludwig *et al.*, 2004). Bootstrap resampling analysis was performed

with the neighbour-joining, maximum-parsimony and maximum-likelihood methods to estimate the confidence of tree topologies, as described by Sekiguchi *et al.* (2006).

To cultivate ethanol-utilizing methanogens, the initial enrichment medium containing 5 mM ethanol as an energy source, 0.01 % yeast extract (w/v) and three antibiotics (vancomycin, penicillin G and kanamycin at a concentration of 100 µg ml⁻¹) was used. The enrichment culture was incubated anaerobically at 25 °C. Cell growth and methane formation were observed after approximately 6 months incubation in the initial enrichment culture, but growth and methane production were detected within 14–20 days incubation in subsequent cultures (5 % inoculum, v/v) at 25 °C. Microscopic observation showed that irregularly coccoid, F₄₂₀-autofluorescent cells were dominant in the ethanol enrichment culture. After successive transfers, the roll-tube method was employed three times. However, colony formation was not observed after 3 months incubation. Purification of the methanogen was by a serial dilution-to-extinction technique in liquid medium containing 5 mM ethanol, vancomycin, penicillin G and kanamycin. The purity of strain HASU^T was demonstrated by the failure to grow in the following media at 25, 37 or 55 °C: (i) thioglycollate medium (Difco) containing approximately 150 kPa H₂/CO₂ (in the head space) and 10 mM sulphate; (ii) thioglycollate medium containing 5 mM ethanol and 10 mM sulphate; (iii) thioglycollate medium containing 10 mM sucrose, 10 mM glucose, 10 mM cellobiose and 10 mM xylose; and (iv) AC medium (Difco). Moreover, we also tested purity by using a 16S rRNA gene-based cloning analysis with the archaeal universal primer set Ar109f (Großkopf *et al.*, 1998) and 1490R. Forty-eight clones were selected randomly and sequenced. All 48 clonal sequences were identical and had the same 16S rRNA gene sequence as strain HASU^T. We evaluated purity based on the failure to

Fig. 1. Phase-contrast micrograph of cells of strain HASU^T grown on 5 mM ethanol medium supplemented with 0.01 % yeast extract (w/v). Bar, 10 µm.

Fig. 2. Methane and acetate production from ethanol by strain HASU^T. Cultivation was performed at 37 °C. The experiments were repeated twice and gave similar results.

recover bacterial 16S rRNA gene amplifications by PCR with a universal bacterial primer set EUB338* (Amann *et al.*, 1990; Daims *et al.*, 1999) and 1490R. The results of

these molecular surveys also indicated that the culture of strain HASU^T was axenic.

Cells of strain HASU^T were Gram-negative, non-motile, irregular cocci, 2–3 µm in diameter, that occurred singly (Fig. 1). Cells were susceptible to lysis with 0.1 % SDS (w/v), suggesting the presence of a proteinaceous cell wall (Boone & Whitman, 1988). The strain was strictly anaerobic given that its growth was completely inhibited in the presence of trace quantities of oxygen (0.1 and 0.2 % O₂, v/v). Strain HASU^T required acetate as carbon source for growth on substrates other than ethanol. Yeast extract was not required for growth, but it did stimulate growth. Interestingly, in ethanol medium in the absence of yeast extract, cells of strain HASU^T formed floc-like aggregates. Ethanol (5 mM), 1-propanol (5 mM), 1-butanol (5 mM), H₂ (approximately 150 kPa in the head space) or formate (40 mM) supported growth and methane production as the sole energy source. In ethanol medium, ethanol was converted to methane and acetate stoichiometrically, which is explained by the equation $2\text{CH}_3\text{CH}_2\text{OH} + \text{HCO}_3^- \rightarrow \text{CH}_4 + 2\text{CH}_3\text{COO}^- + \text{H}_2\text{O} + \text{H}^+$ (Fig. 2). The end products from 1-propanol were methane and propionate. The following substrates did not support growth or methane production: pyruvate (20 mM), lactate (20 mM), acetate (20 mM), propionate (20 mM), trimethylamine (10 mM), dimethylamine (10 mM), methylamine (10 mM), cyclopent-

Fig. 3. Phylogenetic tree based on comparative analyses of 16S rRNA gene sequences, showing the position of strain HASU^T among members of the order *Methanomicrobiales*. The tree was constructed based on a distance matrix analysis of 16S rRNA gene sequences (neighbour-joining tree). 16S rRNA gene sequences of three strains belonging to the class *Thermoplasma* [*Picrophilus oshimae* KAW2/2^T (GenBank accession no. X84901), clone WCHD3-02 (AF050616) and clone pMC2A24 (AB019736)] were used to root the tree (not shown). Branching points supported by bootstrap percentages above 90 % by all three analysis methods [based on 1000 replicates, estimated by using the neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods] are indicated by solid circles, and nodes with open circles indicate >70 % bootstrap support by the three analyses. The accession number of each reference sequence is shown in parentheses. Bar, 0.1 nucleotide changes per sequence position.

tanol (20 mM), methanol (20 mM), 2-propanol (5 mM) and 2-butanol (5 mM).

The optimum growth temperature of strain HASU^T was 37 °C, although the first enrichment culture was incubated at 25 °C. No growth occurred below 10 °C or above 42 °C over 3 months incubation. The pH range for growth was estimated to be 6.5–7.5, with optimum growth at pH 7.0. Under optimum conditions (pH 7.0, 37 °C), the doubling time on ethanol medium containing 0.01 % yeast extract (w/v) was 3.0 days, which was calculated based on measurement of the optical density at 400 nm. Strain HASU^T did not require NaCl for growth, but it was able to grow at NaCl concentrations up to 25 g l⁻¹. Strain HASU^T was resistant to ampicillin, vancomycin, penicillin G and kanamycin. Rifampicin, neomycin and chloramphenicol completely inhibited growth.

The G + C content of the total DNA of strain HASU^T was 60.9 mol%. The nearly complete 16S rRNA gene sequence (1396 bp) of strain HASU^T was determined. Comparative 16S rRNA gene sequence analysis showed that strain HASU^T was affiliated with the genus *Methanofollis* (Fig. 3). The closest relative of strain HASU^T was *Methanofollis liminatans* GKZPZ^T (96.1 % 16S rRNA gene sequence similarity; Fig. 3) (Zellner *et al.*, 1990, 1999).

Based on its morphological, physiological and molecular phylogenetic traits, strain HASU^T is considered to belong within the genus *Methanofollis*. As shown in Table 1, strain HASU^T and members of the genus *Methanofollis* share several phenotypic features, for example cell morphology, DNA G + C content, growth temperature and pH range (Table 1). However, strain HASU^T was able to utilize primary alcohols (ethanol, 1-propanol and 1-butanol), in contrast to recognized members of the genus *Methanofollis*. In addition, levels of 16S rRNA gene sequence similarity also suggest that strain HASU^T is distinct from recognized species of the genus *Methanofollis* (Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001).

Strain HASU^T converted ethanol to methane and acetate. This reaction was also observed for *Methanofollis organophilum*, an ethanol-utilizing methanogen. This might imply that strain HASU^T and *Methanofollis organophilum* share a similar metabolic pathway for methanogenesis from ethanol. However, as the pattern of secondary alcohol utilization is different between these two methanogens (Table 1), the metabolic pathway for alcohol degradation may also be different for the two taxa. From the taxonomic point of view, strain HASU^T can be phylogenetically distinguished from the type strain of *Methanofollis organophilum* (16S rRNA gene sequence similarity of 92.1 %; Fig. 3).

Table 1. Differential characteristics between strain HASU^T (*Methanofollis ethanolicus* sp. nov.), other members of the genus *Methanofollis* and *Methanogenium organophilum*

Strains: 1, HASU^T (data from the present study); 2, *Methanofollis tationis* Chile 9^T (Zabel *et al.*, 1984; Zellner *et al.*, 1999; Zellner & Boone, 2001); 3, *Methanofollis liminatans* GKZPZ^T (Zellner *et al.*, 1990, 1999; Zellner & Boone, 2001); 4, *Methanofollis aquaemaris* N2F9704^T (Lai & Chen, 2001; Wu *et al.*, 2005); 5, *Methanofollis formosanus* ML15^T (Wu *et al.*, 2005); 6, *Methanogenium organophilum* CV^T (Widdel, 1986; Widdel *et al.*, 1988). +, Positive; –, negative; +/–, variable; (+), growth and methane production very poor; ND, not determined.

Characteristic	1	2	3	4	5	6
Cell morphology	Irregular cocci	Irregular cocci	Irregular cocci or ring-shaped	Irregular cocci	Irregular cocci	Irregular cocci
Cell diameter (µm)	2–3	1.5–3.0	1.5	1.2–2.0	1.5–2.0	0.5–1.5
DNA G + C content (mol%)	60.9	54*	60	59.1	58.4	46.7*
Optimum temperature (range) (°C)	37 (15–40)	37–40 (25–45)	40 (25–44)	37 (20–43)	37 (20–42)	30–35
Optimum pH (range)	7 (6.5–7.5)	7 (6.3–8.8)	7	6.5 (6.3–8.0)	6.6 (5.6–7.3)	6.4–7.3
Optimum NaCl (range) (g l ⁻¹)	0 (0–25)	8–12	0 (0–35)	5 (0–6)	3 (0–4)	20
Motility	–	+ / –	+	–	–	–
Substrate utilization						
Ethanol	+	–	–	–	–	+
1-Propanol	+	–	–	ND	ND	+
1-Butanol	+	–	–	ND	ND	(+)
2-Propanol	–	–	+	–	–	+
2-Butanol	–	–	+	–	–	+
Growth requirement						
Yeast extract	–	+	–	–	+	–
Acetate	+†	+	ND	–	–	+†

*Determined by thermal denaturation.

†Acetate is required as carbon source for growth on substrates other than ethanol.

Based on these phenotypic and phylogenetic properties, we suggest that strain HASU^T represents a novel species of the genus *Methanofollis*, for which the name *Methanofollis ethanolicus* sp. nov. is proposed.

Description of *Methanofollis ethanolicus* sp. nov.

Methanofollis ethanolicus (e.tha.no'li.cus. N.L. n. *ethanol*; L. suf. *-icus -a -um* suffix used with various meanings; N.L. masc. adj. *ethanolicus* belonging to ethanol, referring to the substrate ethanol, which can be metabolized by the species).

Strictly anaerobic. Cells stain Gram-negative, are non-motile, irregular cocci, 2–3 µm in diameter, and are resistant to lysis with <0.1% SDS (w/v). H₂, formate, ethanol, 1-propanol and 1-butanol can be used for growth and methane production. Secondary alcohols (2-propanol, 2-butanol and cyclopentanol) are not utilized. Acetate is required as carbon source for growth on substrates other than ethanol. Yeast extract enhances growth. Temperature range for growth is 15–40 °C (optimum, 37 °C). pH range for growth is 6.5–7.5 (optimum, pH 7.0). Growth occurs in the presence of 0–2.5% NaCl but does not occur in the presence of 3.0% NaCl. The G + C content of the DNA of the type strain is 60.9 mol% (HPLC).

The type strain, HASU^T (=NBRC 104120^T=JCM 15103^T=DSM 21041^T), was isolated from lotus field mud at Nagaoka, Niigata, Japan. Related most closely to *Methanofollis liminatans* GKZPZ^T (96.1% 16S rRNA gene sequence similarity).

Acknowledgements

We thank Norika Meguro, Eiji Tasumi and Masayuki Miyazaki at JAMSTEC for their technical assistance. We also thank Ryoko Taniguchi at the Nagaoka University of Technology for her assistance with ethanol measurements, and Hidenobu Takahashi at Ohguchi Lotus Productive Society for his assistance with sampling in the field. This research was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan, the Japan Society for the Promotion of Science, and the Institute for Fermentation, Osaka.

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