Methanoregula formicica sp. nov., a methane-producing archaeon isolated from methanogenic sludge

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A novel methane-producing archaeon, strain SMSPT, was isolated from an anaerobic, propionate-degrading enrichment culture that was originally obtained from granular sludge in a mesophilic upflow anaerobic sludge blanket (UASB) reactor used to treat a beer brewery effluent. Cells were non-motile, blunt-ended, straight rods, 1.0–2.6 µm long by 0.5 µm wide; cells were sometimes up to 7 µm long. Asymmetrical cell division was observed in rod-shaped cells. Coccoid cells (0.5–1.0 µm in diameter) were also observed in mid- to late-exponential phase cultures. Growth was observed between 10 and 40 °C (optimum, 30–33 °C) and pH 7.0 and 7.6 (optimum, pH 7.4). The G + C content of the genomic DNA was 56.2 mol%. The strain utilized formate and hydrogen for growth and methane production. Based on comparative sequence analyses of the 16S rRNA and *mcrA* (encoding the alpha subunit of methyl-coenzyme M reductase, a key enzyme in the methane-producing pathway) genes, strain SMSPT was affiliated with group E1/E2 within the order *Methanomicrobiales*. The closest relative based on both 16S rRNA and *mcrA* gene sequences was *Methanoregula boonei* 6A8T (96.3 % 16S rRNA gene sequence similarity, 85.4 % deduced McrA amino acid sequence similarity). The percentage of 16S rRNA gene sequence similarity indicates that strain SMSPT and *Methanoregula boonei* 6A8T represent different species within the same genus. This is supported by our findings of shared phenotypic properties, including cell morphology and growth temperature range, and phenotypic differences in substrate usage and pH range. Based on these genetic and phenotypic properties, we propose that strain SMSPT represents a novel species of the genus *Methanoregula*, for which we propose the name *Methanoregula formicica* sp. nov., with the type strain SMSPT (=NBRC 105244T =DSM 22288T).

The group E1/E2, originally called the R10 group or fen cluster, is a family-level clade within the order *Methanomicrobiales*, which comprises H₂/CO₂-using methanogenic archaea (Hales *et al.*, 1996; Bräuer *et al.*, 2006b; Galand *et al.*, 2002; Cadillo-Quiroz *et al.*, 2006). This clade has long been recognized as an uncultured

group; however, to date, three methanogenic strains have been isolated and characterized: *Methanoregula boonei* 6A8T (Bräuer *et al.*, 2006a, 2011), *Methanosphaerula palustris* E1-9cT (Cadillo-Quiroz *et al.*, 2008, 2009) and *Methanolinea tarda* NOBI-1T (Imachi *et al.*, 2008). *Methanoregula boonei* and *Methanosphaerula palustris* were isolated from peatlands, while *Methanolinea tarda* was isolated from a methanogenic digester. In addition to the these isolates, many 16S rRNA gene clones belonging to group E1/E2 have been retrieved from a wide variety of anoxic environments, such as bogs and fens (Bräuer *et al.*, 2006a, b; Cadillo-Quiroz *et al.*, 2006, 2008; Chan *et al.*, 2002; Galand *et al.*, 2002), methanogenic sludges (Imachi *et al.*, 2008; Chen *et al.*, 2004, 2009; Narihiro *et al.*, 2009), contaminated aquifers/groundwaters (Dojka *et al.*, 1998;

Abbreviations: FISH, fluorescence *in situ* hybridization; UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *mcrA* gene sequences of strain SMSPT are AB479390 and AB479391, respectively. Other 16S rRNA gene clone sequences have been deposited under accession numbers AB479392–AB479411.

A supplementary figure and two supplementary tables are available with the online version of this paper.

Ficker *et al.*, 1999; Watanabe *et al.*, 2002; Macbeth *et al.*, 2004), rice-field soils (Großkopf *et al.*, 1998; Lueders & Friedrich, 2000; Wu *et al.*, 2006), freshwater sediments (Jurgens *et al.*, 2000; Nüsslein *et al.*, 2001; Chan *et al.*, 2005) and brackish sediment (Purdy *et al.*, 2002) and as endosymbionts of anaerobic ciliates (van Hoek *et al.*, 2000) (see Supplementary Fig. S1, available in IJSEM Online), indicating the widespread distribution of E1/E2 methanogens.

Recently, we successfully isolated a novel methanogenic archaeon, strain SMSP^T, belonging to group E1/E2, from methanogenic granular sludge in an upflow anaerobic sludge blanket (UASB) reactor. In this report, we describe the detailed morphological and physiological characteristics and genetic features of strain SMSP^T. Because the genetic and phenotypic properties of strain SMSP^T are generally similar to those of *Methanoregula boonei*, described elsewhere in this issue (Bräuer *et al.*, 2011), we propose that this strain represents a second species of the genus *Methanoregula*.

Methanogenic granular sludge was taken from a full-scale mesophilic (30–35 °C) UASB reactor located in Japan that was treating beer brewery wastewater. The granules were yellowish brown to dark brown and about 2–3 mm in diameter. After sampling, the granular sludge was washed immediately with phosphate buffer (10 mM, pH 7.2) under N₂ and then homogenized briefly before inoculation into primary enrichment culture medium. The medium for cultivation of strain SMSP^T was prepared as described previously (Imachi *et al.*, 2009). Enrichment cultures were incubated anaerobically at 37 °C in 50 ml serum vials containing 20 ml medium (pH at 25 °C, 7.0) under an atmosphere of N₂/CO₂ (80:20, v/v) without shaking, unless indicated otherwise. After isolation of the strain, all incubations were performed at 33 °C. Growth and substrate utilization were determined by monitoring the OD₄₀₀ of the culture and methane production in basal medium containing 0.01 % (w/v) yeast extract, 1 mM acetate and 0.5 mM coenzyme M (2-mercaptoethanesulfonic acid). Effects of pH, temperature, NaCl concentration and antibiotics on growth of strain SMSP^T were determined in basal medium containing 40 mM formate plus 0.01 % (w/v) yeast extract, 1 mM acetate and 0.5 mM coenzyme M in triplicate culture vessels. All incubations for these tests were performed for over 2 months. To evaluate the optimum temperature for growth, the strain was cultivated at 4, 10, 15, 20, 25, 30, 33, 37, 40, 45, 50 and 60 °C. To determine the pH range for growth, the medium was adjusted at room temperature to pH 5.0–8.5 by adding HCl or NaOH solution under a 100 % N₂ atmosphere prior to inoculation. The pH of the medium was monitored every 4 days during growth using a portable pH meter (Horiba Twin pH B-212), and the pH was readjusted by using HCl or NaOH if the initial pH had changed significantly. The pH test was performed twice. To test the effect of NaCl concentration on growth, NaCl was added to the medium at final concentrations of 1–30 g l⁻¹.

Antibiotic susceptibility was examined with cultures supplemented with antibiotics at final concentrations of 100 µg ml⁻¹.

Cell morphology was examined under an epifluorescent microscope (Olympus BX51F) with a colour CCD camera system (Olympus DP71). Susceptibility to lysis was examined by adding SDS to final concentrations of 0.01–1.0 % (w/v), and cell lysis was determined by microscopic observation. The G+C content of the genomic DNA was determined by HPLC, as described previously (Nakagawa *et al.*, 2003). Procedures for DNA extraction and PCR amplification were reported previously (Imachi *et al.*, 2006). In the PCR amplification, we used the primer pair Ar109f (Großkopf *et al.*, 1998) and 1490R (Weisburg *et al.*, 1991) for construction of 16S rRNA gene-based archaeal clone libraries. We also used primer pairs Arch21F (DeLong, 1992)/1490R and ME1/ME2 (Hales *et al.*, 1996) for amplification of the 16S rRNA gene and *mcrA* gene of the isolate, respectively. PCR products were purified with a MinElute PCR purification kit (Qiagen) and subsequently cloned into *Escherichia coli* TOP10 using a TOPO TA cloning kit (Invitrogen). Sequences of the 16S rRNA genes of clones and the pure culture were determined with a Big Dye terminator version 3.1 cycle sequencing kit (Applied Biosystems) and an automated sequence analyser (3130xl Genetic Analyzer; Applied Biosystems). Phylogenetic analyses were performed as described previously (Imachi *et al.*, 2006, 2008). 16S rRNA gene sequence similarity was calculated using the ARB program with Jukes and Cantor correction (Jukes & Cantor, 1969; Ludwig *et al.*, 2004). Fluorescence *in situ* hybridization (FISH) was done according to the method described by Sekiguchi *et al.* (1998). To detect strain SMSP^T by FISH, we used probe SMSP129 (5'-TATCC-CCTTCCATAGGGTAGATT-3'; *E. coli* positions 129–151), which was designed by using the ARB program (Ludwig *et al.*, 2004). Hybridization stringency of the probe was adjusted by changing the formamide concentration in the hybridization buffer; stringent conditions for probe SMSP129 were determined to be 35 % (v/v) formamide in the hybridization buffer. The oligonucleotide probe was labelled with Cy-3.

Our preliminary analysis of the archaeal 16S rRNA gene clone library revealed that the methanogenic granular sludge used as the inoculum contained clones belonging to group E1/E2 within the order *Methanomicrobiales*, accounting for 41.7 % (20 of 48 clones) of the total archaeal population (Supplementary Table S1 and Fig. S1). In our previous study, we used propionate as substrate for enrichment of a methanogen belonging to group E1/E2, and we successfully cultivated and eventually isolated the methanogen *Methanolinea tarda* NOBI-1^T (Imachi *et al.*, 2008; Sakai *et al.*, 2007). Thus, to cultivate E1/E2 organisms thriving in the methanogenic granular sludge, we incubated the sludge anaerobically with 20 mM propionate. Cell growth and methane production accompanied by propionate degradation occurred after 1 month of incubation.

The culture was successively transferred into fresh medium every 30–40 days [10 % (v/v) inoculum]. During cultivation, the partial pressure of H₂ in the cultures remained less than 30 Pa. Microscopic observation showed that, after five transfers, the propionate enrichment culture contained at least three morphologically distinct organisms: (i) blunt-ended rod-shaped, F₄₂₀-autofluorescent

cells, (ii) *Methanosaeta*-like thick rods and (iii) oval rods. These results suggested that a syntrophic association of propionate-degrading H₂-producing syntrophs, hydrogenotrophic methanogens and aceticlastic methanogens carried out propionate degradation. To identify the methanogens thriving in the syntrophic propionate-degrading enrichment culture, we constructed an archaeal 16S rRNA

Fig. 1. Photomicrographs of strain SMSP^T grown on formate (40 mM) medium supplemented with acetate (1 mM), yeast extract (0.01 %) and coenzyme M (0.5 mM). (a, b) FISH of SMSP^T cells. Phase-contrast (a) and fluorescence (b) images of cells of strain SMSP^T stained with probe SMSP129. Arrows indicate coccoid cells. (c, d) Photomicrographs of a mid-exponential phase culture obtained by phase-contrast (c) and fluorescence (d) microscopy indicating the presence of the methanogen-specific coenzyme F₄₂₀ in identical fields. (e) Asymmetrical cell division in rod cells of strain SMSP^T; arrows indicate asymmetrical division points. (f) Phase-contrast micrograph of a late-exponential phase culture contained rods and coccoid cells. Bars, 10 µm (a–d and f) and 5 µm (e).

gene clone library. 16S rRNA gene clone analysis detected many archaeal sequences, which were mostly affiliated with group E1/E2 within the order *Methanomicrobiales* (8 out of 10 clones; phylotypes SMS-T-Pro-1 to -4 in Supplementary Table S2 and Fig. S1). We also retrieved phylotypes affiliated with group E1' within the order *Methanomicrobiales* (phylotype SMS-T-Pro-7 in Supplementary Fig. S1 and Table S2) (Cadillo-Quiroz *et al.*, 2006) and with the genus *Methanosaeta*, containing acetoclastic methanogens (Supplementary Table S2; phylotypes SMS-T-Pro-5 and -6). To obtain the microbes belonging to the E1/E2 phylotype in pure culture from the propionate enrichment, we used a serial dilution method with both liquid and solid media supplemented with formate (40 mM) and hydrogen (approx. 150 kPa in the headspace), with the propionate enrichment as the inoculum. As a result, we obtained a pure culture of the methanogenic archaeon strain SMSP^T in solid medium (deep-agar method) supplemented with formate. Colonies formed in deep agar after 2 months of incubation were brownish, lens-shaped and 0.1 mm in diameter. The purity of strain SMSP^T was demonstrated by failure to grow in the following media at 37 and 55 °C: thioglycolate medium (Difco) containing approx. 150 kPa H₂/CO₂ (in the headspace) and 10 mM sulfate, thioglycolate medium containing 20 mM lactate and 10 mM sulfate, thioglycolate medium containing 10 mM sucrose, 10 mM glucose, 10 mM cellobiose and 10 mM xylose and AC medium (Difco). Moreover, we also confirmed the culture purity by the failure to recover bacterial 16S rRNA gene amplifications by PCR with a universal bacterial primer pair EUB338F (Amann *et al.*, 1990; Daims *et al.*, 1999; Hatamoto *et al.*, 2007)/1490R. In addition, we performed FISH with 16S rRNA-targeted oligonucleotide probe SMSP129 specific for strain SMSP^T, and we confirmed that all cells hybridized with the probe (Fig. 1a, b). These results of molecular surveys also indicated that the culture of strain SMSP^T was axenic.

Cells of strain SMSP^T were non-motile, blunt-ended, straight rods, 1.0–2.6 µm long and 0.5 µm wide; cells up to 7.0 µm long were sometimes observed (Fig. 1c, d). Asymmetrical cell division was observed in rod-shaped cells (Fig. 1e). Coccoid cells (0.5–1.0 µm diameter) were also observed in mid- to late-exponential phase cultures (Fig. 1f). Targeting of cells of strain SMSP^T by FISH probe SMSP129 demonstrated that both cell types belong to strain SMSP^T (Fig. 1a, b). Cells of strain SMSP^T were autofluorescent in epifluorescence microscopy when excited with light with wavelengths near 420 nm, indicating the presence of the methanogen-specific coenzyme F₄₂₀ (Fig. 1d). An SDS-resistance test revealed that cell disruption occurred at SDS concentrations ≥0.5 % (w/v).

Strain SMSP^T was strictly anaerobic, as no growth occurred under trace quantities of oxygen [0.1 and 0.2 % (v/v) O₂]. H₂ and formate (40 mM) supported growth and methane production. Yeast extract and acetate were required for growth. Coenzyme M stimulated growth of strain SMSP^T greatly; however, it was not required. The following substrates did not support growth and/or methane production: acetate (20 mM), pyruvate, trimethylamine, dimethylamine, methylamine, cyclopentanol (all at 10 mM), ethanol (5 mM), methanol (20 mM), 1-propanol, 2-propanol, 1-butanol and 2-butanol (all at 5 mM).

Growth and methanogenesis of strain SMSP^T were observed between 10 and 40 °C (optimum, 30–33 °C). The pH range for growth was pH 7.0–7.6 (optimum, pH 7.4). Under optimum conditions (pH 7.4, 33 °C), the specific growth rate on formate medium was approx. 1.0 day⁻¹, which was calculated based on measurement of OD₄₀₀. The strain could grow with 0–10 g NaCl l⁻¹ (0–171 mM) and could tolerate rifampicin, ampicillin, penicillin, kanamycin, vancomycin and streptomycin. Tetracycline and chloramphenicol inhibited cell growth completely (each antibiotic was tested at 100 µg ml⁻¹).

Fig. 2. Phylogenetic tree of members of the order *Methanomicrobiales* based on comparative analysis of 16S rRNA gene sequences, showing the placement of strain SMSP^T. The tree was calculated based on a distance-matrix analysis of 16S rRNA gene sequences (neighbour-joining tree). Three 16S rRNA gene sequences from members of the class *Thermoplasmata* [*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). Bootstrap percentages were obtained from the neighbour-joining/maximum-parsimony/maximum-likelihood methods based on 1000 replicates; –, <50 %. Accession numbers are shown in parentheses. Bar, 0.1 changes per nucleotide sequence position.

Fig. 3. Phylogenetic tree based on deduced McrA amino acid sequences, indicating the relationship of strain SMSP^T with related methanogenic archaea. The tree was constructed based on a distance matrix (256 amino acid positions; PAM distance correction) by the neighbour-joining method. The sequence of *Methanobacterium bryantii* DSM 863^T was used as the outgroup. Accession numbers are shown in parentheses. Bootstrap percentages were obtained from the neighbour-joining/maximum-parsimony/maximum-likelihood methods based on 1000 replicates; –, <50 %. Bar, 10 % sequence divergence.

The DNA G + C content of strain SMSP^T was 56.2 mol%. Comparative 16S rRNA gene sequence analysis using 1397 bp of the 16S rRNA gene sequence of strain SMSP^T showed that strain SMSP^T was affiliated with group E1/E2 with the order *Methanomicrobiales* (Fig. 2). The 16S rRNA gene sequence of strain SMSP^T was not identical to any clonal sequence retrieved from the propionate enrichment culture or the original methanogenic sludge, although phylotype SMS-T-Pro-4 detected from the propionate enrichment was closely related to strain SMSP^T (97.0 % 16S rRNA gene sequence identity; Supplementary Fig. S1). The

discrepancy between the sequence of SMSP^T and the clonal sequences suggests that strain SMSP^T was a minor archaeal component in the original methanogenic sludge and the propionate enrichment, and thus the sequence of strain SMSP^T could not be retrieved from the archaeal clone libraries. The closest cultured relative of strain SMSP^T was *Methanoregula boonei* 6A8^T (96.3 % sequence identity). In addition to the 16S rRNA gene sequence-based analysis, we also determined the partial sequence of the *mcrA* gene of strain SMSP^T (722 bp) and constructed a phylogenetic tree based on deduced amino acid sequences of the *mcrA* gene

Table 1. Differential characteristics between strain SMSP^T and other members of group E1/E2 within the order *Methanomicrobiales*

Strains: 1, strain SMSP^T (data from this study); 2, *Methanoregula boonei* 6A8^T (data from Bräuer *et al.*, 2006a, 2011); 3, *Methanosphaerula palustris* E1-9c^T (Cadillo-Quiroz *et al.*, 2008, 2009); 4, *Methanolinea tarda* NOBI-1^T (Imachi *et al.*, 2008). ND, No data available.

Characteristic	1	2	3	4
Cell morphology	Rods*	Rods†	Cocci	Rods
Cell width (µm)	0.5	0.2–0.3	0.5–0.8	0.7–1.0
Cell length (µm)	1.0–2.6‡	0.8–3.0	0.5–0.8	2–8§
DNA G + C content (mol%)	56.2	54.5	55.4	56.3
Temperature for growth (°C)				
Optimum	30–33	35–37	30	50
Range	10–40	10–40	14–35	35–55
pH for growth				
Optimum	7.4	5.1	5.3–5.5	7
Range	7.0–7.6	4.5–5.5	4.8–6.5	6.7–8.0
NaCl concentration for growth (g l ⁻¹)				
Optimum	0	<1	<2	0
Range	0–10	0–3	0–6	0–15
Formate utilized for growth and methane production	+	–	+	+
Coenzyme M required for growth	–¶	+	+	ND

*Irregular coccoid cells, 0.5–1.0 µm in diameter, observed in mid- to late-exponential phase.

†Cells sometimes become spherical, diameter 0.3–0.8 µm.

‡Cells up to 7 µm long sometimes observed.

§Cells often form multicellular filaments longer than 8 µm in syntrophic propionate-degrading enrichment culture.

||DNA G + C content determined from full genome sequence (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>).

¶Growth is greatly stimulated.

(Fig. 3). The McrA tree also supported the conclusion that strain SMSP^T was a member of the order *Methanomicrobiales*, and the closest relative on the basis of deduced McrA amino acid sequences was also *Methanoregula boonei* 6A8^T (85.4% sequence identity).

The phenotypic and genetic data obtained in this study showed that strain SMSP^T and *Methanoregula boonei* 6A8^T have very similar features. The 16S rRNA gene sequence similarity of 96.3% is in the range of species-level differences (Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001) and is similar to species-level differences among described species in genera belonging to the order *Methanomicrobiales*, e.g. *Methanoculleus* (96.3–96.9%) and *Methanofollis* (95.2–95.6%). Thus, strain SMSP^T and *Methanoregula boonei* 6A8^T should be members of the same genus. In addition to the 16S rRNA gene sequence similarity, their cell morphologies are similar, i.e. both organisms have blunt-ended, straight rod-shaped and coccoid-shaped cells (Table 1). In addition, their most striking feature is their asymmetrical cell division (Bräuer *et al.*, 2006a) (Fig. 1e). Moreover, their optimum growth temperatures were similar (strain SMSP^T, 30–33 °C; *Methanoregula boonei* 6A8^T, 35–37 °C) and their growth temperature ranges were the same (10–40 °C). However, the pH ranges for growth differ markedly; the optimum pH for strain SMSP^T is pH 7.4, whereas that for *Methanoregula boonei* 6A8^T is pH 5.1. Additionally, substrate usage was different, in that strain SMSP^T can utilize formate whereas *Methanoregula boonei* 6A8^T cannot. Based on these phenotypic and phylogenetic properties, we assign strain SMSP^T to a novel species of the genus *Methanoregula*, for which we propose the name *Methanoregula formicica* sp. nov.

Description of *Methanoregula formicica* sp. nov.

Methanoregula formicica (for.mi'ci.ca. N.L. n. *acidum* formicum formic acid; L. fem. suff. *-ica* suffix used with the sense of pertaining to; N.L. fem. adj. *formicica* pertaining to formic acid, referring to the ability of the type strain to utilize formate).

Strictly anaerobic. Cells are non-motile and rod-shaped, 1.0–7 µm long and 0.5 µm wide. In mid- to late-exponential phase cultures, coccoid cells are observed (0.5–1.0 µm diameter). Asymmetrical cell division is observed. Hydrogen and formate are used for growth and methane production. Yeast extract and acetate are required for growth. Coenzyme M stimulates growth greatly. The temperature range for growth is 10–40 °C (optimum, 30–33 °C). The pH range for growth is 7.0–7.6 (optimum, pH 7.4). Growth occurs in the presence of 0–10 g NaCl l⁻¹, but does not occur in the presence of 20 g NaCl l⁻¹. The G+C content of the DNA of the type strain is 56.2 mol%.

The type strain, SMSP^T (=NBRC 105244^T =DSM 22288^T), was isolated from a mesophilic, granular sludge in a UASB reactor used to treat a beer brewery effluent.

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