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 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;...
Recently, we successfully isolated a novel methanogenic archaeon, strain SMSPT, 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 SMSPT. Because the genetic and phenotypic properties of strain SMSPT 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 SMSPT 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 OD490 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 SMSPT were determined in basal medium containing 40 mM formate plus 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 archaean 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., 1993). 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 archaean 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* NOB1-1T (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 $H_2$ 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, $F_{420}$-autofluorescent cells, (ii) *Methanosaeta*-like thick rods and (iii) oval rods. These results suggested that a syntrophic association of propionate-degrading $H_2$-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

![Figure 1](http://ijs.sgmjournals.org)

**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 $F_{420}$ 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; 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

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

Strain SMSP\(\text{T}\) was strictly anaerobic, as no growth occurred under trace quantities of oxygen [0.1 and \(0.2\%\) (v/v) \(O_2\)]. \(H_2\) and formate (40 \(mM\)) supported growth and methane production. Yeast extract and acetate were required for growth. Coenzyme M stimulated growth of strain SMSP\(\text{T}\) 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\(\text{T}\) were observed between 10 and 40 \(^\circ\)C (optimum, 30–33 \(^\circ\)C). The pH range for growth was pH 7.0–7.6 (optimum, pH 7.4). Under optimum conditions (pH 7.4, 33 \(^\circ\)C), the specific growth rate on formate medium was approx. 1.0 \(d^{-1}\), which was calculated based on measurement of OD\(_{400}\). 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 \(\mu\)g 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\(\text{T}\). 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 (AF056016) 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.
The DNA G+C content of strain SMSPT was 56.2 mol%. Comparative 16S rRNA gene sequence analysis using 1397 bp of the 16S rRNA gene sequence of strain SMSPT showed that strain SMSPT was affiliated with group E1/E2 with the order Methanomicrobiales (Fig. 2). The 16S rRNA gene sequence of strain SMSPT 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 SMSPT (97.0% 16S rRNA gene sequence identity; Supplementary Fig. S1). The discrepancy between the sequence of SMSPT and the clonal sequences suggests that strain SMSPT was a minor archaeal component in the original methanogenic sludge and the propionate enrichment, and thus the sequence of strain SMSPT could not be retrieved from the archaeal clone libraries. The closest cultured relative of strain SMSPT was Methanoregula boonei 6A8T (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 SMSPT (722 bp) and constructed a phylogenetic tree based on deduced amino acid sequences of the mcrA gene (Fig. 3).

Table 1. Differential characteristics between strain SMSPT and other members of group E1/E2 within the order Methanomicrobiales

<table>
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<td>Rods†</td>
<td>Coci</td>
<td>Rods</td>
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<td>Cell width (µm)</td>
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<td>Coenzyme M required for growth</td>
<td>–♀</td>
<td>+</td>
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*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).

Fig. 3. Phylogenetic tree based on deduced McrA amino acid sequences, indicating the relationship of strain SMSPT 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 863T 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.

Table 1. Differential characteristics between strain SMSPT and other members of group E1/E2 within the order Methanomicrobiales

Strains: 1, strain SMSPT (data from this study); 2, Methanoregula boonei 6A8T (data from Brauer et al., 2006a, 2011); 3, Methanosphaera palustris E1-9c (Cadillo-Quiroz et al., 2008, 2009); 4, Methanolinae tarda NOBI-1T (Imachi et al., 2008). ND, No data available.

Characteristics: Cell morphology, Cell width (µm), Cell length (µm), DNA G+C content (mol%), Temperature for growth (°C), pH for growth, NaCl concentration for growth (g l⁻¹), Formate utilized for growth and methane production, Coenzyme M required for growth.
(Fig. 3). The MrA tree also supported the conclusion that strain SMSPT was a member of the order Methanomicrobiales, and the closest relative on the basis of deduced MrA amino acid sequences was also Methanoregula boonei 6A8T (85.4 % sequence identity).

The phenotypic and genetic data obtained in this study showed that strain SMSPT and Methanoregula boonei 6A8T 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 SMSPT and Methanoregula boonei 6A8T 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 (Brauer et al., 2006a) (Fig. 1e). Moreover, their optimum growth temperatures were similar (strain SMSPT, 30–33 °C; Methanoregula boonei 6A8T, 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 SMSPT is pH 7.4, whereas that for Methanoregula boonei 6A8T is pH 5.1. Additionally, substrate usage was different, in that strain SMSPT can utilize formate whereas Methanoregula boonei 6A8T cannot. Based on these phenotypic and phylogenetic properties, we assign strain SMSPT 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 type strain, SMSPT (=NBRC 105244T =DSM 22288T), was isolated from a mesophilic, granular sludge in a UASB reactor used to treat a beer brewery effluent.

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References


