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

Yuto Yashiro,[1,2] Sanae Sakai,[1] Masayuki Ehara,[1,2] Masayuki Miyazaki,[1] Takashi Yamaguchi[2] and Hiroyuki Imachi[1]

Correspondence
Hiroyuki Imachi
imachi@jamstec.go.jp

[1]Subsurface Geobiology Advanced Research (SUGAR) Team, Extremobiosphere Research Program, Institute of Biogeosciences, Japan Agency for Marine-Earth Science & Technology (JAMSTEC), Yokosuka, Kanagawa 237-0061, Japan

[2]Department of Environmental Systems Engineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188, Japan

A novel methane-producing archaeon, strain SMSP^T, 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 SMSP^T 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* 6A8^T (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 SMSP^T and *Methanoregula boonei* 6A8^T 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 SMSP^T represents a novel species of the genus *Methanoregula*, for which we propose the name *Methanoregula formicica* sp. nov., with the type strain SMSP^T (=NBRC 105244^T =DSM 22288^T).

The group E1/E2, originally called the R10 group or fen cluster, is a family-level clade within the order *Methanomicrobiales*, which comprises H_2/CO_2-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* 6A8^T (Bräuer *et al.*, 2006a, 2011), *Methanosphaerula palustris* E1-9c^T (Cadillo-Quiroz *et al.*, 2008, 2009) and *Methanolinea tarda* NOBI-1^T (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 SMSP^T 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 N_2 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 N_2/CO_2 (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 OD_{400} 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 % N_2 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 (3130*xl* 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′-TATCCCCTTCCATAGGGTAGATT-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 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

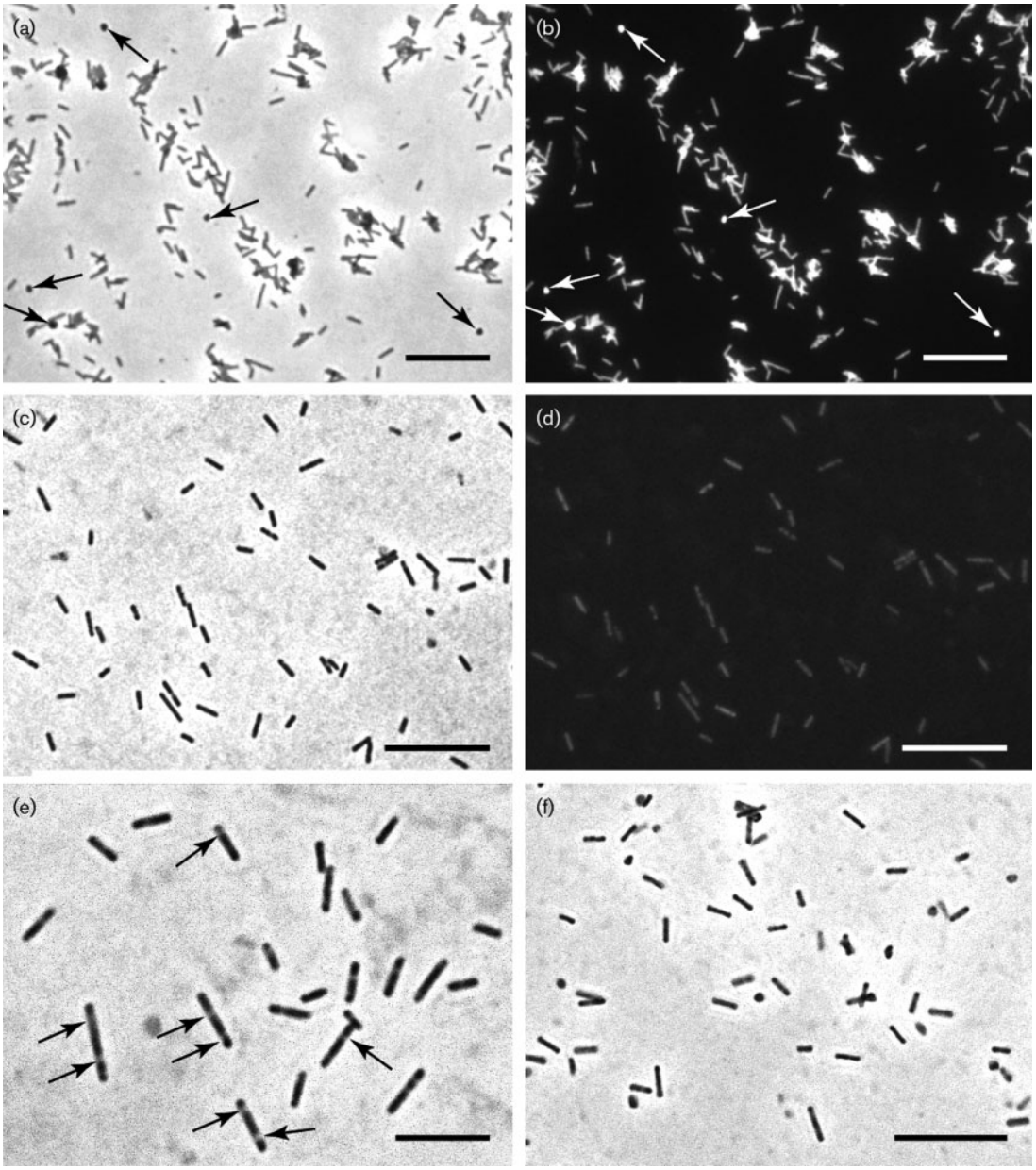

Fig. 1. Photomicrographs of strain SMSP^T grown on formate (40 mM) medium supplemented with acetate (1 mM), yeast extract (0.01 %) and coenzyme M (0.5 mM). (a, b) FISH of SMSP^T cells. Phase-contrast (a) and fluorescence (b) images of cells of strain SMSP^T stained with probe SMSP129. Arrows indicate 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) (Cadillo-Quiroz *et al.*, 2006) and with the genus *Methanosaeta*, containing aceticlastic 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 H_2/CO_2 (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 F_{420} (Fig. 1d). An SDS-resistance test revealed that cell disruption occurred at SDS concentrations $\geqslant$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) 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^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 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 μg ml^{-1}).

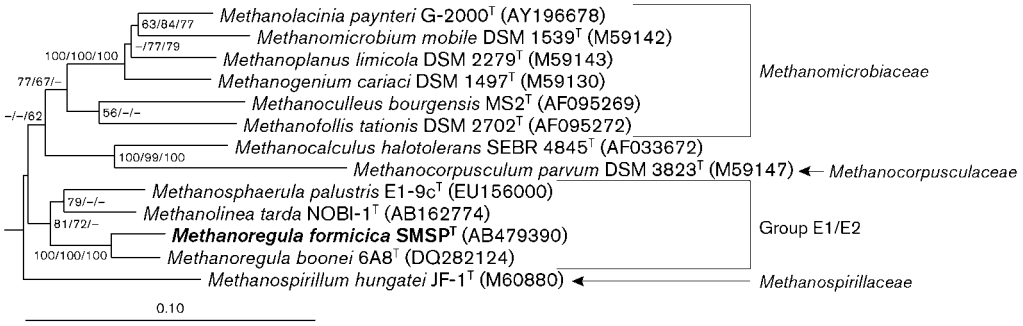

Fig. 2. Phylogenetic tree of members of the order *Methanomicrobiales* based on comparative analysis of 16S rRNA gene sequences, showing the placement of strain SMSP^T. The tree was calculated based on a distance-matrix analysis of 16S rRNA gene sequences (neighbour-joining tree). Three 16S rRNA gene 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.

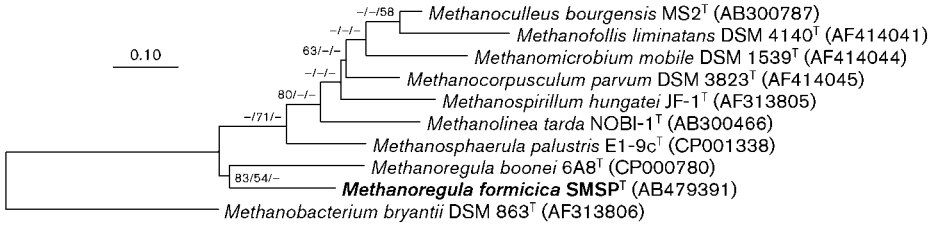

Fig. 3. Phylogenetic tree based on deduced McrA amino acid sequences, indicating the relationship of strain SMSP^T with related methanogenic archaea. The tree was constructed based on a distance matrix (256 amino acid positions; PAM distance correction) by the neighbour-joining method. The sequence of *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.

Acknowledgements

We thank Hideki Inaba at Sumitomo Heavy Industries, Ltd, for his assistance with the sampling of the methanogenic sludge and Dr Eiji Tasumi and Ken Aoi at JAMSTEC for their technical assistance. We also thank Drs Stephen H. Zinder, Hinsby Cadillo-Quiroz and Suzanna L. Bräuer for valuable discussion, critical reading of the manuscript and providing information on *Methanoregula boonei* and *Methanosphaerula palustris*. This study was supported partly 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.

References

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**, 1919–1925.

Bräuer, S. L., Cadillo-Quiroz, H., Yashiro, E., Yavitt, J. B. & Zinder, S. H. (2006a). Isolation of a novel acidiphilic methanogen from an acidic peat bog. *Nature* **442**, 192–194.

Bräuer, S. L., Yashiro, E., Ueno, N. G., Yavitt, J. B. & Zinder, S. H. (2006b). Characterization of acid-tolerant H_2/CO_2-utilizing methanogenic enrichment cultures from an acidic peat bog in New York State. *FEMS Microbiol Ecol* **57**, 206–216.

Bräuer, S. L., Cadillo-Quiroz, H., Ward, R. J., Yavitt, J. B. & Zinder, S. H. (2011). *Methanoregula boonei* gen. nov., sp. nov., an acidiphilic methanogen isolated from an acidic peat bog. *Int J Syst Evol Microbiol* **61**, 45–52.

Cadillo-Quiroz, H., Bräuer, S., Yashiro, E., Sun, C., Yavitt, J. & Zinder, S. (2006). Vertical profiles of methanogenesis and methanogens in two contrasting acidic peatlands in central New York State, USA. *Environ Microbiol* **8**, 1428–1440.

Cadillo-Quiroz, H., Yashiro, E., Yavitt, J. B. & Zinder, S. H. (2008). Characterization of the archaeal community in a minerotrophic fen and terminal restriction fragment length polymorphism-directed isolation of a novel hydrogenotrophic methanogen. *Appl Environ Microbiol* **74**, 2059–2068.

Cadillo-Quiroz, H., Yavitt, J. B. & Zinder, S. H. (2009). *Methanosphaerula palustris* gen. nov., sp. nov., a hydrogenotrophic methanogen isolated from a minerotrophic fen peatland. *Int J Syst Evol Microbiol* **59**, 928–935.

Chan, O. C., Wolf, M., Hepperle, D. & Casper, P. (2002). Methanogenic archaeal community in the sediment of an artificially partitioned acidic bog lake. *FEMS Microbiol Ecol* **42**, 119–129.

Chan, O. C., Claus, P., Casper, P., Ulrich, A., Lueders, T. & Conrad, R. (2005). Vertical distribution of structure and function of the methanogenic archaeal community in Lake Dagow sediment. *Environ Microbiol* **7**, 1139–1149.

Chen, C.-L., Macarie, H., Ramirez, I., Olmos, A., Ong, S. L., Monroy, O. & Liu, W.-T. (2004). Microbial community structure in a thermophilic anaerobic hybrid reactor degrading terephthalate. *Microbiology* **150**, 3429–3440.

Chen, C.-L., Wu, J.-H., Tseng, I.-C., Liang, T.-M. & Liu, W.-T. (2009). Characterization of active microbes in a full-scale anaerobic fluidized bed reactor treating phenolic wastewater. *Microbes Environ* **24**, 144–153.

Daims, H., Brühl, A., Amann, R., Schleifer, K. H. & Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**, 434–444.

DeLong, E. F. (1992). Archaea in coastal marine environments. *Proc Natl Acad Sci U S A* **89**, 5685–5689.

Dojka, M. A., Hugenholtz, P., Haack, S. K. & Pace, N. R. (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* **64**, 3869–3877.

Ficker, M., Krastel, K., Orlicky, S. & Edwards, E. (1999). Molecular characterization of a toluene-degrading methanogenic consortium. *Appl Environ Microbiol* **65**, 5576–5585.

Galand, P. E., Saarnio, S., Fritze, H. & Yrjälä, K. (2002). Depth related diversity of methanogen Archaea in Finnish oligotrophic fen. *FEMS Microbiol Ecol* **42**, 441–449.

Großkopf, R., Janssen, P. H. & Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S RNA gene sequence retrieval. *Appl Environ Microbiol* **64**, 960–969.

Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W. & Saunders, J. R. (1996). Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl Environ Microbiol* **62**, 668–675.

Hatamoto, M., Imachi, H., Ohashi, A. & Harada, H. (2007). Identification and cultivation of anaerobic, syntrophic long-chain fatty acid-degrading microbes from mesophilic and thermophilic methanogenic sludges. *Appl Environ Microbiol* **73**, 1332–1340.

Imachi, H., Sekiguchi, Y., Kamagata, Y., Loy, A., Qiu, Y.-L., Hugenholtz, P., Kimura, N., Wagner, M., Ohashi, A. & Harada, H. (2006). Non-sulfate-reducing, syntrophic bacteria affiliated with *Desulfotomaculum* cluster I are widely distributed in methanogenic environments. *Appl Environ Microbiol* **72**, 2080–2091.

Imachi, H., Sakai, S., Sekiguchi, Y., Hanada, S., Kamagata, Y., Ohashi, A. & Harada, H. (2008). *Methanolinea tarda* gen. nov., sp. nov., a methane-producing archaeon isolated from a methanogenic digester sludge. *Int J Syst Evol Microbiol* **58**, 294–301.

Imachi, H., Sakai, S., Nagai, H., Yamaguchi, T. & Takai, K. (2009). *Methanofollis ethanolicus* sp. nov., an ethanol-utilizing methanogen isolated from a lotus field. *Int J Syst Evol Microbiol* **59**, 800–805.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Jurgens, G., Glöckner, F., Amann, R., Saano, A., Montonen, L., Likolammi, M. & Münster, U. (2000). Identification of novel archaea in bacterioplankton of a boreal forest lake by phylogenetic analysis and fluorescent in situ hybridization. *FEMS Microbiol Ecol* **34**, 45–56.

Keswani, J. & Whitman, W. B. (2001). Relationship of 16S rRNA sequence similarity to DNA hybridization in prokaryotes. *Int J Syst Evol Microbiol* **51**, 667–678.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

Lueders, T. & Friedrich, M. (2000). Archaeal population dynamics during sequential reduction processes in rice field soil. *Appl Environ Microbiol* **66**, 2732–2742.

Macbeth, T. W., Cummings, D. E., Spring, S., Petzke, L. M. & Sorenson, K. S. (2004). Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture. *Appl Environ Microbiol* **70**, 7329–7341.

Nakagawa, S., Takai, K., Horikoshi, K. & Sako, Y. (2003). *Persephonella hydrogeniphila* sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium from a deep-sea hydrothermal vent chimney. *Int J Syst Evol Microbiol* **53**, 863–869.

Narihiro, T., Terada, T., Ohashi, A., Wu, J.-H., Liu, W.-T., Araki, N., Kamagata, Y., Nakamura, K. & Sekiguchi, Y. (2009). Quantitative detection of culturable methanogenic archaea abundance in anaerobic treatment systems using the sequence-specific rRNA cleavage method. *ISME J* **3**, 522–535.

Nüsslein, B., Chin, K.-J., Eckert, W. & Conrad, R. (2001). Evidence for anaerobic syntrophic acetate oxidation during methane production in the profundal sediment of subtropical Lake Kinneret (Israel). *Environ Microbiol* **3**, 460–470.

Purdy, K. J., Munson, M. A., Nedwell, D. B. & Embley, T. M. (2002). Comparison of the molecular diversity of the methanogenic community at the brackish and marine ends of a UK estuary. *FEMS Microbiol Ecol* **39**, 17–21.

Sakai, S., Imachi, H., Sekiguchi, Y., Ohashi, A., Harada, H. & Kamagata, Y. (2007). Isolation of key methanogens for global methane emission from rice paddy fields: a novel isolate affiliated with the clone cluster rice cluster I. *Appl Environ Microbiol* **73**, 4326–4331.

Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H. & Nakamura, K. (1998). Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. *Microbiology* **144**, 2655–2665.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

van Hoek, A. H. A. M., van Alen, T. A., Sprakel, V. S. I., Leunissen, J. A. M., Brigge, T., Vogels, G. D. & Hackstein, J. H. P. (2000). Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. *Mol Biol Evol* **17**, 251–258.

Watanabe, K., Kodama, Y., Hamamura, N. & Kaku, N. (2002). Diversity, abundance, and activity of archaeal populations in oil-contaminated groundwater accumulated at the bottom of an underground crude oil storage cavity. *Appl Environ Microbiol* **68**, 3899–3907.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Wu, X. L., Friedrich, M. W. & Conrad, R. (2006). Diversity and ubiquity of thermophilic methanogenic archaea in temperate anoxic soils. *Environ Microbiol* **8**, 394–404.