Methanoculleus sediminis sp. nov., a methanogen from sediments near a submarine mud volcano

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A mesophilic, hydrogenotrophic methanogen, strain S3FaT, was isolated from sediments collected by Ocean Researcher I cruise ORI-934 in 2010 near the submarine mud volcano MV4 located at the upper slope of south-west Taiwan. The methanogenic substrates utilized by strain S3FaT were formate and H2/CO2 but not acetate, secondary alcohols, methylamines, methanol or ethanol. Cells of strain S3FaT were non-motile, irregular cocci, 0.5–1.0 μm in diameter. The surface-layer protein showed an Mr of 128 000. The optimum growth conditions were 37°C, pH 7.1 and 0.17 M NaCl. The DNA G+C content of the genome of strain S3FaT was 62.3 mol %. Phylogenetic analysis revealed that strain S3FaT was most closely related to Methanoculleus marisnigri JR1T (99.3 % 16S rRNA gene sequence similarity). Genome relatedness between strain S3FaT and Methanoculleus marisnigri JR1T was computed using both genome-to-genome distance analysis (GGDA) and average nucleotide identity (ANI) with values of 46.3–55.5 % and 93.08 %, respectively. Based on morphological, phenotypic, phylogenetic and genomic relatedness data, it is evident that strain S3FaT represents a novel species of the genus Methanoculleus, for which the name Methanoculleus sediminis sp. nov. is proposed. The type strain is S3FaT (BCRC AR10044T=DSM 29354T).

The genus Methanoculleus comprises hydrogenotrophic methanogens that have been isolated from diverse habitats, including marine and river sediments, wetland, paddy field soil, anaerobic digesters, oilfields and a deep diatomaceous shale formation (Blotevogel et al., 1991; Cheng et al., 2008; Corder et al., 1983; Dianou et al., 2001; Mikucki et al., 2003; Ollivier et al., 1986; Rivard & Smith, 1982; Romesser et al., 1979; Shimizu et al., 2013; Tian et al., 2010; Weng et al., 2015; Zellner et al., 1998). Methanoculleus submarinus Nankai-1T (Mikucki et al., 2003) and Methanoculleus taiwanensis CYW4T (Weng et al., 2015) were isolated from marine sediments associated with gas (methane) hydrates.

The submarine mud volcanoes, gas seeps and mud diapirs were observed at the upper slope domain of the eastern part of an accretionary wedge at the shore of south-western Taiwan (Fig. S1, available in the online Supplementary Material) (Chen et al., 2014). Gassy sediments and submarine mud volcanoes are the most common routes for methane, the dominant gas in marine sediments, to migrate from the seabed into the atmosphere (Dimitrov, 2002). The origin of gassy sediments has been attributed to the upward migration of biogenic and/or thermogenic gases from deeper parts of sedimentary layers (Oung et al., 2006). They have become increasingly important in global studies of methane hydrates and climate change (Chiu et al., 2006). To identify the methanogen involved in deep-sea methane formation that may also be associated with methane seep or methane hydrate environments, methanogens were isolated from deep-sea sediments offshore of south-
western Taiwan. In this study, a novel hydrogenotrophic, mesophilic *Methanoculleus* strain, S3Fa\textsuperscript{T}, was enriched from mud samples from the submarine mud volcano MV4 and was further purified and characterized.

A piston core 227 cm in length was obtained during the ORI-934 cruise on 28 July to 3 Aug 2010 at station S3 (22° 8.63′ N 120° 19.49′ E), which was located near the mud volcano MV4 (Fig. S1; Chen et al., 2014) offshore of south-western Taiwan. The core sediments were mainly composed of mud and carbonates. The water depth of station S3 was 464 m and estimated temperature from nearby heat flow measurement by Dr Hsieh-Tang Chiang (unpublished data) was around 10 °C. The concentration ranges of methane, ethane and CO\textsubscript{2} for this 227 cm core varied from 25–206, 2–4 and 170–430 μl l\textsuperscript{-1}, respectively. Propane was under the detectable range. After the core was sectioned on board the ship, 0.5 g portions of mixed core station S3 sediments were inoculated immediately into 20 ml MB/W enrichment medium with formate, acetate or methanol as the catabolic substrate, respectively in 60 ml serum bottles (Kimble Chase) sealed with butyl rubber stoppers and aluminium caps. These enrichment samples were incubated at room temperature during the cruise and in the laboratory.

For cultivation of the methanogen, a modified anaerobic technique of Hungate was utilized (Balch et al., 1979; Sowers & Noll, 1995). Sterilized media were prepared under an oxygen-free N\textsubscript{2}/CO\textsubscript{2} (4 : 1) atmosphere. MB/W medium was composed of (I:\textsuperscript{-1}): MgCl\textsubscript{2}.6H\textsubscript{2}O, 1.0 g; KCl, 0.5 g; NaCl, 5 g; CaCl\textsubscript{2}.2H\textsubscript{2}O, 0.1 g; K\textsubscript{2}HPO\textsubscript{4}, 0.4 g; NH\textsubscript{4}Cl, 1.0 g; cysteine.HCl, 0.25 g; NaHCO\textsubscript{3}, 4.0 g; yeast extract, 2 g; tryptone, 2 g; and resazurin, 0.001 g. Vitamin (Wolin et al., 1963) and trace element (Ferguson & Mah, 1983) solutions with sodium tungstate (Na\textsubscript{2}WO\textsubscript{4}, 0.3 mg l\textsuperscript{-1}) were each added to final concentrations of 1 % (v/v). The pH of the MB/W medium was 7.0. Minimal MM/W medium was MB/W medium without the addition of yeast extract and tryptone. Both media were prepared according to our previous studies (Lai et al., 2002, 2004; Wu et al., 2005). All of the constituents except NaHCO\textsubscript{3}, yeast extract, tryptone and vitamin solution, were dissolved in boiling water and then added after cooling. The medium was prepared and distributed into serum bottles (Kimble Chase) or Hungate tubes (Bellco Glass) under an oxygen-free N\textsubscript{2}/CO\textsubscript{2} (4 : 1) atmosphere. The anaerobic tubes and tubes were then sealed and autoclaved at 121 °C for 20 min. Sodium sulfide from a sterilized anoxic stock solution was added to a final concentration of 1.0 mM for tests of optimum growth conditions. For solid roll-tube medium, agar was added at 20 g l\textsuperscript{-1}. To measure the effect of pH on growth, the pH of the media were modulated by the partial pressure of CO\textsubscript{2} and the concentration of NaHCO\textsubscript{3} to obtain values between pH 5.6 and pH 8.1.

The enriched cultures, which actively produced methane, were transferred periodically to fresh MB/W medium with the same substrate and vancomycin (100 μg ml\textsuperscript{-1}) to reduce the growth of bacteria. Methane production was determined by GC (G-3000; Hitachi) with flame-ionization detection (Lai et al., 1999). High methane production was detected in the station S3 enrichment with formate as the catabolic substrate. Hungate roll-tubes in MB/W agar medium were inoculated with the enrichment after several sub-transfers with MB/W medium and vancomycin (Wu & Lai, 2011). A single, white, round colony was picked in an anaerobic Coy chamber and inoculated into MB/W medium with formate and vancomycin. Further serial dilutions with MB/W medium with formate and vancomycin were performed to purify this strain. The conclusion that the culture was axenic was based on the presence of a single morphotype upon microscopic examination and the absence of growth in Bacto thioglycollate medium. This strain, designated S3Fa\textsuperscript{T}, was deposited in the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as strain DSM 29354\textsuperscript{T} and in the Taiwan Bioresource Collection and Research Center as strain BCRC AR10044\textsuperscript{T}.

The cell morphology of strain S3Fa\textsuperscript{T} was examined under a phase-contrast microscope (BH-2; Olympus), transmission electron microscopes (EM-1200EXII and JEM-1400; JEOL) (Lai & Shih, 2001) and a model JSM-7401F (JEOL) scanning electron microscope with gold sputter-coated cells (Lai & Chen, 2001). Cells of strain S3Fa\textsuperscript{T} were irregular cocci with diameters of 0.5–1.0 μm (Fig. 1a–c). Under transmission and scanning electron microscopy, dividing cells were observed in exponential-phase cultures (Fig. 1b, d). Cells of strain S3Fa\textsuperscript{T} were not motile, and flagella were not observed. Strain S3Fa\textsuperscript{T} lysed in SDS (0.01 %, w/v), which indicated that the cell envelope was composed by surface-layer proteins and lacked peptidoglycans such as pseudomurien. The surface-layer proteins were then isolated according to the protocol of König (1995), separated by SDS-PAGE (Laemmli, 1970) and stained with Coomassie blue R-250 (Merck). It was found that strain S3Fa\textsuperscript{T} had one surface-layer protein with an Mr of 128 000 (Fig. S2).

The catabolic substrate tests were performed in 22 ml Hungate tubes with 5 ml MB/W medium under N\textsubscript{2}/CO\textsubscript{2} (4 : 1). The substrates used in this study were: sodium formate (100 mM), sodium formate (100 mM) plus sodium acetate (5 mM) as carbon source, sodium acetate (50 mM), dimethyamine (111 mM), trimethylamine (40 mM), methanol (50 mM), ethanol (48 mM), 2-propanol (48 mM), 2-butanol (48 mM), iso-butanol (48 mM) and H\textsubscript{2}/CO\textsubscript{2}, in which H\textsubscript{2} to a partial pressure of ~60 kPa. Utilization of the substrates was determined by monitoring methane production. The sensitivity of strain S3Fa\textsuperscript{T} to ampicillin, kanamycin, penicillin, spectinomycin, tetracycline and chloramphenicol (each at 100 μg ml\textsuperscript{-1}) was tested in MB/W medium with sodium chloride (80 mM), sodium formate (100 mM) and sodium acetate (5 mM) at 37 °C. Cell growth was determined from methane production. Specific growth rates were calculated from the rate of methane production, which was analysed by linear regression of the logarithm of the total amount of methane production.
that accumulated over time (Wu & Lai, 2011). Inocula were grown under experimental conditions as described in the text.

Cells of strain S3FaT used H2/CO2 or formate as methanogenic substrates, but not sodium acetate, trimethylamine, methanol, ethanol, 2-propanol, 2-butanol or iso-butanol (Fig. S3). Acetate was required for the growth of strain S3FaT in minimal MM/W medium. Growth of strain S3FaT was unaffected by the antibiotics ampicillin, penicillin, kanamycin, spectinomycin and vancomycin, but was inhibited by chloramphenicol and tetracycline. Strain S3FaT grew over the temperature range 20–50 °C (Fig. S4a), the NaCl range 0–1.0 M (Fig. S4b), and the pH range of pH 5.6–7.5 (Fig. S4c). The optimal growth conditions for strain S3FaT were 37 °C, 0.17 M NaCl and pH 7.1. Under optimal growth conditions, the specific growth rate in MB/W with formate (100 mM) plus acetate (5 mM) was 0.046 h⁻¹ (doubling time 15.07 h). Therefore, strain S3FaT is a mesophilic, neutrophilic and hydrogenotrophic methanoarchaeon.

Chromosomal DNA from strain S3FaT was isolated by the general procedure of Jarrell et al. (1992). The method for PCR amplification, cloning and sequencing of the 16S rRNA gene of strain S3FaT was similar to that of Weng et al. (2015). The gene sequences of archaea used in this study were obtained from the GenBank database. Phylogenetic trees were reconstructed by the MEGA6 program (Tamura et al., 2013) using the neighbour-joining method with 1000 bootstrap replicates. The phylogenetic tree based on the 1398 bp of 16S rRNA gene sequence, placed strain S3FaT in the genus Methanoculleus and closely related to Methanoculleus marisnigri JR1T (99.28 % 16S rRNA gene sequence similarity), M. submarinus Nankai-1T (98.85 %), Methanoculleus chikugoensis MG62T (98.57 %) and Methanoculleus horonobensis T10T (98.07 %) (Fig. 2). Bootstrap analysis indicated a clear branching of strain S3FaT from the above species (Fig. 2), which suggested that strain S3FaT was a novel species. A draft genome sequence of strain S3FaT was obtained using the Illumina MiSeq platform with paired-end read length of 2 × 300 bp and assembled using CLC Genomics Workbench v7.0. Further genome annotation was processed using NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) and checked by the GenBank curation team. The draft genome sequence was used to analyse G+C content. For analysis of genome relatedness, the average nucleotide identity (ANI) value was calculated at a website (http://enve-omics.ce.gatech.edu/ ani/) by using the method described previously (Goris et al., 2007). In addition, genome-to-genome distance analysis (GGDA) was performed at the genome-to-genome distance calculator (GGDC) website (http://ggdc.dsmz.de/distcalc2.php) using the method described in Meier-Kolthoff et al. (2013). The draft genome comprises 15 contigs with total length of ~2.49 Mb and a G+C content of 62.3 mol %. Gene annotations were performed by NCBI PGAAP and obtained 2459 predicted genes, 3 rRNAs, 48 tRNAs and 1 ncRNA (Table S1). In order to reveal the relatedness of genomes of strain S3FaT and M. marisnigri JR1T,
comparison of both genomes by ANI yielded a value of 93.08 %, which was below the cut-off value of 95 % for species delineation. Furthermore, the analysis of GGDA, which produced the simulation of DNA–DNA Hybridization (DDH) values, showed that the two strains exhibited a DDH-equivalent relatedness ranging from 46.3–55.5 %. Both independent analyses supported that strain S3FaT represents a novel species of the genus *Methanoculleus*.

Strain S3FaT and all recognized species of the genus *Methanoculleus* are hydrogenotrophic methanogens and irregular cocci. Members of the genus *Methanoculleus* can be found in marine sediments, anaerobic digesters, rice fields, oilfields and wetland soils (Table 1). *M. submarinus* Nankai-1T was the first methanogen isolated from methane hydrate-bearing sediment (Mikucki et al., 2003). Strain S3FaT was isolated from sediment at a submarine mud volcano MV4. The major difference between strain S3FaT and other most closely related species of the genus *Methanoculleus* is its inability to utilize 2-propanol/CO2 and 2-butanol/CO2 as catabolic substrates (Table 1). In addition, acetate was required for the growth of strain S3FaT in minimal medium, but not for *M. marisnigri* JR1T. Moreover, the optimum growth temperature of strain S3FaT was 37 °C and distinct from that of *M. marisnigri* JR1T and *M. submarinus* Nankai-1T. Morphologically, *M. marisnigri* JR1T possessed peritrichous flagella, but strain S3FaT did not have flagella. Furthermore, analyses of GGDA and ANI values between both genomes of strain S3FaT and *M. marisnigri* JR1T also strongly suggested that strain S3FaT represented a novel species of the genus *Methanoculleus*. In conclusion, on the basis of phenotypic and phylogenetic data and genome relatedness between strain S3FaT and *M. marisnigri* JR1T, strain S3FaT represents a novel species of the genus *Methanoculleus*, for which the name *Methanoculleus sediminis* sp. nov. is proposed.

**Description of *Methanoculleus sediminis* sp. nov**


Cells are irregular cocci, non-motile and 0.5–1.0 μm in diameter. Cells are lysed easily by SDS (0.01 %, w/v) and the purified S-layer protein possesses an Mr of 128 000. Catabolic substrates are formate and H2/CO2, but not acetate, secondary alcohols, methylamines, methanol or ethanol. Optimum growth occurs at 37 °C, pH 7.1 and 0.17 M NaCl. Acetate is required for the growth of cells in minimal medium. Cell growth is completely inhibited by chloramphenicol and tetracycline, but not by ampicillin, kanamycin, penicillin, spectinomycin or streptomycin.

The type strain is S3FaT (=BCRC AR10044T=DSM 29354T), isolated from submarine mud volcano MV4 located near...
### Table 1. Comparison of physiological characteristics of strain S3FaT and type strains of related species of the genus *Methanoculleus*

Strains: 1, S3FaT (data from this study); 2, *M. marisnigri* JR1T (Romesser et al., 1979); 3, *M. submarinus* Nankai-1T (Mikucki et al., 2003); 4, *M. chikugensis* MG62T (Dianou et al., 2001); 5, *M. thermophilus* CR-1T (Rivard & Smith, 1982); 6, *M. palmolei* INSLUZT (Zellner et al., 1998); 7, *M. bourgensis* MS2T (Ollivier et al., 1986); 8, *M. receptaculi* ZC-2T (Cheng et al., 2008); 9, *M. hydrogenitrophicus* HCT (Tian et al., 2010); 10, *M. horonobensis* T10T (Shimizu et al., 2013); 11, *M. taiwanensis* CYW4T (Weng et al., 2015). All strains have irregular coccus-shaped cells. ND, Not determined.

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>9</th>
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<th>11</th>
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</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
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<td>1.3</td>
<td>0.8–2.0</td>
<td>1.0–2.0</td>
<td>1.0–1.3</td>
<td>1.25–2.0</td>
<td>1.0–2.0</td>
<td>0.8–1.7</td>
<td>0.8–2.0</td>
<td>0.7–1.6</td>
<td>0.6–1.5</td>
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<tr>
<td>NaCl concentration for growth (M)</td>
<td>0–1.0</td>
<td>0–0.69</td>
<td>0–1.3</td>
<td>0–0.3</td>
<td>0.75–0.70</td>
<td>ND</td>
<td>ND</td>
<td>0.17</td>
<td>0–1.3</td>
<td>0.05–0.5</td>
<td>0–1.3</td>
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<tr>
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<td>0.1</td>
<td>0.25</td>
<td>ND</td>
<td>ND</td>
<td>0.2</td>
<td>0.1</td>
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<td>0.08</td>
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<tr>
<td>pH for growth</td>
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<td>5.6–7.6</td>
<td>5.0–8.7</td>
<td>6.7–8.0</td>
<td>6.18–7.82</td>
<td>6.5–8.0</td>
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<td>5.0–8.5</td>
<td>5.8–8.2</td>
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<tr>
<td>Optimum</td>
<td>7.0</td>
<td>6.2–6.6</td>
<td>6.0–7.5</td>
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<td>7.0</td>
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<td>6.7</td>
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<td>6.7–6.8</td>
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<td>S-layer (kDa)</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>112</td>
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<td>DNA G+C content (mol%)†</td>
<td>62.3 (Gs)</td>
<td>61.2 (Bd)</td>
<td>ND</td>
<td>62.2 (Lc)</td>
<td>59 (Bd)</td>
<td>59 (Tm)</td>
<td>59 (Bd)</td>
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<td>61 (Lc)</td>
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<td>TR</td>
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<td>AR, CaR, TR, YR</td>
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<td>AR, TcS, YS</td>
<td>AR</td>
<td>P3, YS</td>
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</table>

*H, H₂/CO₂; F, formate; 2P, 2-propanol/CO₂; 2B, 2-butanol/CO₂.
†Determined by: Lc, HPLC; Bd, buoyant density; Tm, thermal denaturation; Gs, genome sequencing.
‡A, Acetate; Y, yeast extract; Tc, Trypticase; Ca, Casitone; P, Peptone.
south-western Taiwan. The DNA G+C content of the genome of the type strain was 62.3 mol %.

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References


