Methanocella paludicola gen. nov., sp. nov., a methane-producing archaeon, the first isolate of the lineage ‘Rice Cluster I’, and proposal of the new archaeal order Methanocellales ord. nov.

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A novel mesophilic, hydrogenotrophic methanogen, strain SANAET T, was isolated from an anaerobic, propionate-degrading enrichment culture, which was originally established from rice paddy soil. The cells were non-motile, Gram-negative and rod-shaped (1.8–2.4 μm long by 0.3–0.6 μm wide). Growth of strain SANAET T was observed at 25–40 °C, with an optimum temperature range for growth of 35–37 °C. The pH range for growth was 6.5–7.8, with an optimum at pH 7.0. The salinity range for growth was 0–1 g NaCl l−1 (0–17 mM). The isolate was able to utilize H2/CO2 and formate for growth and methane production. The G+C content of the genomic DNA was 56.6 mol%. Based on comparative 16S rRNA gene sequence analysis, strain SANAET T was affiliated with a clone lineage of the Archaea, Rice Cluster I (RC-I), placing it between the orders Methanosarcinales and Methanomicrobiales within the class ‘Methanomicrobia’. 16S rRNA gene sequence similarities between strain SANAET T and members of Methanosarcinales were in the range 80.0–82.8 %, and those between the strain and members of Methanomicrobiales ranged from 77.5 to 82.4 %. In addition to 16S rRNA gene analysis, sequence analysis of the mcrA gene (encoding the α subunit of methyl-coenzyme M reductase, a key enzyme in the methane production pathway) also showed that strain SANAET T was affiliated with the RC-I lineage. Here, we propose the name Methanocella paludicola gen. nov., sp. nov. for the isolate, the first of the RC-I lineage. The type strain is SANAET T (= JCM 13418 T = NBRC 101707 T = DSM 17711 T). In addition, we also propose the status of order for the RC-I lineage, for which we propose the name Methanocellales ord. nov.
Recent results have shown that RC-I methanogens have so far escaped isolation. We have successfully isolated a novel methane-utilizing methanogen, designated strain SANAET, belonging to the RC-I lineage from a Japanese rice paddy field. Enrichment- and isolation of strain SANAET was achieved by application of the co-culture approach with *Syntrophobacter fumaroxidans* as the hydrogen-producing syntrophic partner (Sakai *et al.*, 2007). In a previous publication we described the isolation of strain SANAET<sup>T</sup>. Here we give a detailed description of strain SANAET<sup>T</sup>, including morphological, physiological and genetic properties. We propose that strain SANAET<sup>T</sup> is the type strain of a novel species within a novel genus with the name *Methanocella paludicola* gen. nov., sp. nov. In addition, we propose the order *Methanocellales* ord. nov., with the newly isolated methanogen as a representative of the new order.

The basal medium used in this study was prepared as described previously (Sekiguchi *et al.*, 2000). All cultivations were performed at 37°C in 50 ml serum vials containing 20 ml medium (pH at 25°C, 7.0) under an atmosphere of N<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) without shaking, unless otherwise mentioned. Growth and substrate utilization was determined by monitoring the optical density of the cultures (at 600 nm) and the concentration of methane. Incubations were stopped after 3 months. The pH, temperature and salinity range of strain SANAET were determined in standard media containing 0.01% yeast extract and 1 mM acetate. The gas phase contained H<sub>2</sub> at approximately 150 kPa. Temperature tests were carried out between 18 and 60°C, pH tests between pH 5.5 and 8.0, and salinity tests between 1 and 30 g NaCl l<sup>-1</sup> (17–513 mM). During the pH test, the medium was routinely monitored to observe if the initial pH conditions were changed or not. Salinity tests were performed using the same medium described above (which already contains 33 mM Na<sup>+</sup> and 14 mM Cl<sup>-</sup>). Antibiotics were evaluated with cultures supplemented at final concentrations of 100 μg antibiotic ml<sup>-1</sup>. Antibiotic susceptibility tests were carried out at 37°C for a period of 2 months. All measurements were performed in triplicate.

Cell morphology and motility were examined by a phase-contrast microscope (Olympus model BX50F). Gram staining was carried out according to the method of Hucker (Doetsch, 1981). The susceptibility of the isolate to detergents was tested with SDS at concentrations ranging from 0.01 to 2.5%, and cell lysis was determined by microscopic observation of cell integrity. Transmission electron microscopy was performed as described previously (Sekiguchi *et al.*, 2003). Short-chain fatty acids, alcohols, methane, H<sub>2</sub> and carbon dioxide were measured as described previously (Imachi *et al.*, 2000, 2002, 2006). The G+C content of the genomic DNA was determined by the method of Kamagata & Mikami (1991).

All procedures for DNA extraction, PCR amplification and sequencing have been reported previously (Imachi *et al.*, 2006). PCR was carried out using primer pairs Arch21F (DeLong, 1992) and 1490R (Weisburg *et al.*, 1991), and ME1 and ME2 (Hales *et al.*, 1996), for amplification of the 16S rRNA and *mcrA* genes, respectively. Phylogenetic analyses were performed using the *ARB* program (Ludwig *et al.*, 2004). A 16S rRNA gene-based tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) for sequences having >1000 nt. Subsequently, shorter sequences were inserted into the tree without changing the tree topology by using the parsimony insertion tool of the *ARB* program. 16S rRNA gene sequence similarity values were calculated using the Calculate Matrix function of the *ARB* program with Jukes and Cantor correction (Jukes & Cantor, 1969; Ludwig *et al.*, 2004). A deduced McrA amino acid sequence-based phylogenetic tree was constructed by the neighbour-joining method implemented in the *ARB* program with 265 amino acid positions and the percentage acceptance mutations (PAM) distance correction. For both phylogenetic trees, the tree topologies were evaluated by using bootstrap resampling analysis (Felsenstein, 1985) for 1000 replicates with the neighbour-joining, maximum-parsimony and maximum-likelihood methods as described previously (Sekiguchi *et al.*, 2006). Fluorescence in situ hybridization was performed as described by Sekiguchi *et al.* (1998). To detect strain SANAET<sup>T</sup> cells, a 16S rRNA-targeted oligonucleotide probe specific for strain SANAET<sup>T</sup>, SANAE1136 (Sakai *et al.*, 2007), was used.

Cells of strain SANAET<sup>T</sup> were non-motile, rod-shaped, 1.8–2.4 μm long and 0.3–0.6 μm wide, and occurred singly (Fig. 1). Coccolid cells were observed in a late-exponential phase culture with H<sub>2</sub> or formate (Fig. 2). Fluorescence in situ hybridization probe targeting of SANAE<sup>T</sup> cells demonstrated that both cell types belong to strain SANAET<sup>T</sup> (Fig. 2). Cells of strain SANAET<sup>T</sup> autofluoresced in epifluorescence microscopy, indicating the presence of the methanogen-specific coenzyme F<sub>420</sub>. The cells stained
Strain SANAE\textsuperscript{T} grew between 25 and 40\textdegree C dimethylamine (10 mM) and propionate (20 mM). (20 mM), methylamine (10 mM), trimethylamine (10 mM), 2-butanol (5 mM), cyclopentanol (5 mM), methanol 2-propanol (5 mM), ethanol (5 mM), 1-butanol (5 mM), lactate (20 mM), acetate (20 mM), 1-propanol (5 mM), growth and/or methane production: pyruvate (20 mM), production. The following substrates did not support (w/v) SDS. Colonies of strain SANAE\textsuperscript{T} in a deep agar Gram-negative and resisted disruption by less than 2 % (w/v) SDS. Colonies of strain SANAE\textsuperscript{T} in a deep agar medium were white to cream, reaching a diameter of 1–1.5 mm after 6 months incubation with H\textsubscript{2} as a substrate.

Strain SANAE\textsuperscript{T} required acetate as carbon source. Yeast extract supported growth; however, it was not required. H\textsubscript{2} and formate (40 mM) supported growth and methane production. The following substrates did not support growth and/or methane production: pyruvate (20 mM), lactate (20 mM), acetate (20 mM), 1-propanol (5 mM), 2-propanol (5 mM), ethanol (5 mM), 1-butanol (5 mM), 2-butanol (5 mM), cyclopentanol (5 mM), methanol (20 mM), methylamine (10 mM), trimethylamine (10 mM), dimethylamine (10 mM) and propionate (20 mM).

Strain SANAE\textsuperscript{T} grew between 25 and 40 \textdegree C with an optimum at 35–37 \textdegree C. The pH range for growth was between 6.5–7.8, with an optimum at around pH 7.0. The strain could grow at NaCl concentrations ranging from 0–1 g NaCl l\textsuperscript{-1} (0–17 mM). In medium containing 2–4 g NaCl l\textsuperscript{-1} (34–68 mM), a small amount of methane was detected; however, growth was inhibited because methane production was not increased. Methane production and growth were completely inhibited by 5 g NaCl l\textsuperscript{-1} (86 mM). Under optimal conditions (pH 7.0, 37 \textdegree C), the doubling time, calculated from methane production rates, was 4.2 days. The strain tolerated ampicillin, penicillin G, vancomycin, kanamycin, bacitracin and streptomycin. Growth was inhibited by rifampicin, tetracycline and chloramphenicol.

The DNA G + C content of strain SANAE\textsuperscript{T} was 56.6 mol\%. Analysis of its 16S rRNA gene sequence affiliates strain SANAE\textsuperscript{T} with sequences in the RC-I lineage (previously published in Sakai \textit{et al.}, 2007). Hitherto, this lineage, which falls between the orders Methanosarcinales and Methanomicrobiales (Fig. 3), was exclusively composed of sequences retrieved from clone libraries. The 16S rRNA gene sequence similarities between strain SANAE\textsuperscript{T} and members of Methanosarcinales were in the range 80.0–82.8\%, and those between the strain and members of Methanomicrobiales ranged from 77.5 to 82.4\%. The closest cultured relative of strain SANAE\textsuperscript{T} was Methanomethylovorans hollandica (82.8\% similarity). In addition to the 16S rRNA gene-based analysis, we also determined the partial sequence of the mcrA gene in strain SANAE\textsuperscript{T} (681 bp) and constructed a phylogenetic tree based on the deduced amino acid sequence of mcrA genes (Fig. 4). In this tree also, strain SANAE\textsuperscript{T} affiliates with a member of the RC-I lineage. The closest relative based on McrA amino acid sequence was Methanosalsum zhilinae (71.8\% similarity).

Phenotypic and genetic analyses in this study showed that strain SANAE\textsuperscript{T} is an H\textsubscript{2}-utilizing methanogen and that it should be considered to be a member of the RC-I lineage. Previous culture-dependent and -independent investigations have provided evidence that members of the RC-I lineage utilize H\textsubscript{2} (Lueders \textit{et al.}, 2001; Sizova \textit{et al.}, 2003; Erkel \textit{et al.}, 2005, 2006; Lehmann-Richter \textit{et al.}, 1999). Erkel \textit{et al.} (2006) constructed a complete genome sequence of an RC-I methanogen from an enrichment culture growing on H\textsubscript{2} and revealed that the RC-I methanogen had a full set of genes involved in methanogenesis from H\textsubscript{2}/CO\textsubscript{2}. Abundant populations of RC-I Archaea were detected in an acidophilic methanogenic consortium supplemented with H\textsubscript{2} as sole energy source (Sizova \textit{et al.}, 2003). The 16S rRNA gene sequences from those RC-I enrichments were determined and are available from the Genbank/EMBL/ DDBJ databases as AM114193 for the metagenome (the genome contained three \textit{rrn} operons) and AF524853 for the acidophilic consortium. The sequence similarities between these RC-I enrichments and strain SANAE\textsuperscript{T} were in the range 92.2–94.2\%, indicating that strain SANAE\textsuperscript{T} and these RC-I organisms are phylogenetically distinct from each other, at least at the species or genus level.

Since the discovery of RC-I, it has been recognized as a different genotypic group from the orders Methanosarcinales and Methanomicrobiales because of the low sequence similarity values with those of the two aforementioned orders (Großkopf \textit{et al.}, 1998). In fact, as described above, the 16S rRNA gene sequence similarities between strain SANAE\textsuperscript{T} and members of the Methanosarcinales and Methanomicrobiales were also low. Additionally, the bootstrap values of both 16S RNA gene-based and McrA amino acid sequence-based phylogenetic trees solidly supported the three lineages, Methanosarcinales, Methanomicrobiales and RC-I (Figs 3 and 4). These results suggest that RC-I, containing strain SANAE\textsuperscript{T}, is certainly a monophyletic taxon at order level within the class ‘Methanomicrobia’ (Garrity \textit{et al.}, 2003). Additionally, the physiological properties of strain SANAE\textsuperscript{T} distinguish it clearly from members of the order Methanosarcinales, which utilize acetate and simple methylated compounds in contrast to

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Photomicrographs of rod and coccoid cells of strain SANAE\textsuperscript{T}. Phase-contrast (a) and fluorescence (b) micrographs of the cells in the same field of view, stained with a Cy-3-labelled 16S rRNA probe, SANAE1136. Arrows indicate coccoid cells. Bars, 10 \textmu m.}
\end{figure}
Fig. 4. Phylogenetic tree based on deduced McrA amino acid sequences, indicating the relationship between strain SANAET and related methanogenic Archaea. The tree was constructed based on a distance matrix (265 amino acid positions; PAM distance correction) by using the neighbour-joining method. The sequence of Methanopyrus kandleri (AE009439) was used as the outgroup. Bootstrap support (>50% indicated only) was obtained from neighbour-joining (first value), maximum-parsonomy (second value) and maximum-likelihood methods (third value) based on 1000 replicates. The accession number of each reference sequence is shown after each strain or clone name. The bar represents 10% estimated sequence divergence.
Table 1. Comparison of morphological and physiological characteristics of strain SANAET, and the families and a genus within the orders Methanomicrobiales and Methanosarcinales

Data for strain SANAET is from this study. Data for other taxa were taken from the following studies: Methanomicrobiaceae (Boone et al., 1993, 2001a; Chong et al., 2002; Dianou et al., 2001; Mikucki et al., 2003; Lai & Chen, 2001; Wu et al., 2005); Methanospirillaceae (Boone et al., 2001a); Methanocorpusculaceae (Boone et al., 1993, 2001a); Methanocalculus (Boone et al., 2001a; Lai et al., 2002, 2004; Mori et al., 2000); Methanosetaeaceae (Boone et al., 2001b; Ma et al., 2006); Methanosarcinaceae (Boone et al., 1993, 2001b; Jiang et al., 2005; Lomans et al., 1999; Lyimo et al., 2000; Simankova et al., 2001; Singh et al., 2005; Sprenger et al., 2000; von Klein et al., 2002). Abbreviations, −, Negative; +, positive; ; ±, dependent on the species; ND, not determined.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SANAET</th>
<th>Methanomicrobiales</th>
<th>Methanospirillaceae</th>
<th>Methanocorpusculaceae</th>
<th>Genus</th>
<th>Methanocalculus</th>
<th>Methanosetaeaceae</th>
<th>Methanosarcinaceae</th>
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<tr>
<td>Cell morphology</td>
<td>Rod, coccus*</td>
<td>Curved or irregular rod, irregular coccus, or irregular plate or disk</td>
<td>Curved rod</td>
<td>Coccus or irregular coccus</td>
<td>Irregular coccus</td>
<td>Rod</td>
<td>Coccus, irregular coccus, or flat polygons</td>
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<td>Gram reaction</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>49.0–55.7</td>
<td>36.3–55</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>56.6</td>
<td>38.7–62.2</td>
<td>45</td>
<td>48.5–52</td>
<td>50.8–55</td>
<td>±</td>
<td>49.0–55.7</td>
<td>36.3–55</td>
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<td>Motility</td>
<td>−</td>
<td>± (weakly)</td>
<td>+</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>±</td>
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<td>Optimum growth temperature (°C)</td>
<td>35–37</td>
<td>20–55</td>
<td>30–37</td>
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<td>35–38</td>
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<td>Optimum pH</td>
<td>7</td>
<td>6.0–7.9</td>
<td>6.6–7.4</td>
<td>6.5–7.0</td>
<td>6.5–7.6</td>
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<td>Substrate utilization</td>
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<td>H₂/CO₂</td>
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<td>Formate</td>
<td>+</td>
<td>+</td>
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<td>Acetate</td>
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<td>Secondary alcohols</td>
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<td>Methanol or methylamines</td>
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<td>Growth requirements</td>
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<td>Yeast extract</td>
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<td>Acetate</td>
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<td>±</td>
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*Cocci are observed in the late-exponential culture.*
strain SANAET. However, the phenotypic distinction between strain SANAET and members of the Methanomicrobiales is not so straightforward as both consume hydrogen and formate exclusively and have similar responses to pH, temperature and salinity. They differ, however, in cell shape (Table 1).

Based on these phenotypic and phylogenetic data, we propose that strain SANAET is the type strain of a novel species within a novel genus, for which we propose the name Methanocella paludicola gen. nov., sp. nov. We also propose the status of order for the RC-I lineage and propose the name Methanocellales ord. nov.

**Description of Methanocella gen. nov.**

*Methanocella* [Me.tha.no cel.la] N.Gr. n. *methane* (from N.Gr. n. *meth(y)l* and chemical suffix -*one*) methane; L. fem. n. *cella* a room, and in biology a cell; N. L. fem. n. *Methanocella* a methane-producing cell.

Cells are rod-shaped and non-motile. Gram-negative. Mesophilic. Produces methane from H₂ or formate.

The type species is *Methanocella paludicola* sp. nov.

**Description of Methanocella paludicola sp. nov.**


Cells occur singly and almost all of the cells are rod-shaped; however, coccolid cells were observed in late-exponential culture. Cells stain Gram-negative, are non-motile and are resistant to lysis by 2% (w/v) SDS. Methane produced from hydrogen and formate. Acetate is required for growth and methane production. The organism uses H₂ or formate for growth and methane production.

The type strain, isolated from a rice paddy soil at Nagaoka, from H₂ and formate. Acetate is required for growth and resistant to lysis by 2% (w/v) SDS. Methane produced culture. Cells stain Gram-negative, are non-motile and are however, coccoid cells were observed in late-exponential culture. Cells occur singly and almost all of the cells are rod-shaped; however, coccolid cells were observed in late-exponential culture. Cells stain Gram-negative, are non-motile and are resistant to lysis by 2% (w/v) SDS. Methane produced from hydrogen and formate. Acetate is required for growth and methane production. The organism uses H₂ or formate for growth and methane production.

The type genus is *Methanocella* gen. nov.

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**References**


