Methanocella arvoryzae sp. nov., a hydrogenotrophic methanogen isolated from rice field soil

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A novel hydrogenotrophic methanogen, designated strain MRE50T, was isolated from a methanogenic consortium, which was originally established from an Italian rice field soil. Cells were non-motile rods, 1.3–2.8 μm long and 0.4–0.7 μm wide. Coccoid cells were also observed in cultures at the late-exponential phase of growth. Strain MRE50T grew at 37–55 °C (optimally at 45 °C), at pH 6–7.8 (optimally at pH 7.0) and in the presence of 0–20 g NaCl l⁻¹. The isolate utilized H₂/CO₂ and formate for growth and methane production. Phylogenetic analyses of the 16S rRNA gene and the methanogen-specific marker gene mcrA showed that strain MRE50T is affiliated with the order Methanocellales, previously known as uncultured archaeal group Rice Cluster I. Based on both 16S rRNA gene and mcrA gene sequences, strain MRE50T was related most closely to Methanocella paludicola SANAE. Levels of sequence similarity were 92.5 and 86.1 %, respectively, indicating that strains MRE50T and Methanocella paludicola SANAE represent different species within the genus Methanocella. In addition, although these strains shared phenotypic properties including cell morphology and substrate utilization, they differed with respect to susceptibility to antibiotics, and temperature and NaCl ranges for growth. Given the phenotypic differences and the distinct phylogenetic placement of the new isolate relative to the type species of the genus Methanocella, strain MRE50T is considered to represent a novel species of the genus Methanocella, for which the name Methanocella arvoryzae sp. nov. is proposed. The type strain is MRE50T (=NBRC 105507T =DSM 22066T).

The order Methanocellales is a recently proposed euryarchaeal order within the class ‘Methanomicrobia’ (Sakai et al., 2008). The order comprises just a single isolate, Methanocella paludicola SANAE, a mesophilic hydrogenotrophic methanogen isolated from a rice field soil in Japan (Sakai et al., 2007). Before the taxonomic description of Methanocella paludicola and proposal of the order Methanocellales, this euryarchaeal lineage had been recognized as uncultured archaeal group ‘rice cluster I’ (RC-I). Members of RC-I are known to play a key role in methane production in rice paddy fields (Conrad et al., 2006; Lu & Conrad, 2005). However, despite their ubiquity and abundance in rice paddy field environments, only one isolate, representing Methanocella paludicola, has been described so far. Here we report the isolation of a second member of the order Methanocellales, designated strain MRE50T. We describe the physiology and phylogeny of this methanogen and propose that it represents a novel species of the genus Methanocella.

Basal medium was prepared as described previously with the slight modification that both 1.5 mM Na₂S, 9H₂O and 1.5 mM cysteine hydrochloride were added as reducing agents (Chin et al., 1998; Lueders et al., 2001). The standard medium normally contains both 1 mM acetate and 0.01 % w/v yeast extract (Difco). However, we isolated strain MRE50T and performed growth tests by adding either acetate or yeast extract to the medium. After isolation, all incubations were performed at 45 °C in 50 ml...
serum vials containing 20 ml medium (pH 7.0 at 25 °C) under an atmosphere of H₂/CO₂ (80:20, v/v) or N₂/CO₂ (80:20, v/v) without shaking, unless stated otherwise. Growth and substrate utilization were determined by monitoring the concentration of methane by using a GC-3200 gas chromatograph with a thermal conductivity detector (GL Science). Tests for growth temperature, pH and salinity ranges were carried out at 15–60 °C, pH 5.0–8.0 and 0–30 g NaCl l⁻¹. The pH was adjusted at room temperature by adding HCl or NaOH solution. By using a portable pH meter (HORIBA Twin pH B-212), the medium was routinely monitored to see whether the initial pH conditions had changed during incubation, and the pH was readjusted by using HCl and NaOH solution if the initial pH had changed significantly. Antibiotic susceptibility was evaluated by using cultures supplemented with antibiotics at a final concentration of 100 μg ml⁻¹. All measurements were performed in triplicate, and all incubations were terminated after 3 months.

Cell morphology and motility were examined by using a phase-contrast microscope (Olympus BX51F) with a colour CCD camera (Olympus DP71). Transmission electron microscopy of negatively stained cells and thin sections of cells were performed as described by Zillig et al. (1990). For freeze-etched preparations, cells were frozen with slush nitrogen and fractured in a freeze-fracture device (JFD-9010; JEOL). Photomicrographs of freeze-etched cell preparations were taken with a JEOL JEM-1011 electron microscope at 100 kV. The G+C content of the genomic DNA was determined by HPLC as described by Nakagawa et al. (2003). The procedures used for DNA extraction, PCR amplification, cloning and sequencing were as reported elsewhere (Imachi et al., 2006, 2008; Yashiro et al., 2011). Because the partial sequences of the 16S rRNA and mcrA genes obtained by PCR from strain MRE50T matched those of the complete genome sequence of RC-I_MRE50 perfectly (GenBank/EMBL/DDBJ accession no. AM114193; Erkel et al., 2006), we used the latter for further phylogenetic analysis. Sequence similarity values were calculated by using the Calculate Matrix function of the ARB program with similarity correction (Ludwig et al., 2004). Phylogenetic trees based on 16S rRNA and mcrA gene sequences were constructed by using the neighbour-joining method in the ARB program package. To estimate the confidence of the tree topologies, bootstrap resampling analysis (Felsenstein, 1985) with 1000 replicates was performed for the neighbour-joining, maximum-parsimony and maximum-likelihood methods as described previously (Sekiguchi et al., 2006). Fluorescence in situ hybridization (FISH) was performed according to the method described by Sekiguchi et al. (1998). To detect strain MRE50T by FISH, we used probe MRE50_426 (5’-GACGATGGGAGCACCGACC-3’), corresponding to positions 426–444 of the Escherichia coli 16S rRNA gene), which was developed by using the automated probe design tool of the ARB program. Hybridization stringency of probe MRE50_426 was adjusted by changing the formamide concentration in the hybridization buffer. The stringent condition was determined to be 20 % (v/v) formamide in the hybridization buffer. The oligonucleotide probe was labelled with Cy-3.

Strain MRE50T was isolated from a methanogenic consortium named MRE50, which was originally established in the year 2000, by using Italian rice field soil as inoculum (Lueders et al., 2001). This consortium had been used to determine the complete genome sequence of RC-I_MRE50 prior to isolation of strain MRE50T (Erkel et al., 2006). The culture was stably maintained by consecutive transfers under a gas phase of H₂/CO₂ and anaerobic incubation at 50 °C. Molecular community analysis had previously shown that the MRE50 consortium was a mixed culture of bacterial and archaean cells, and that the archaean population was represented entirely by a single RC-I phylotype, which amounted to more than 50 % of total cells (Erkel et al., 2005). To obtain the RC-I archaean in pure culture, a serial dilution method in liquid medium with H₂/CO₂ and 0.01 % (w/v) yeast extract was used. These were the same cultivation conditions as used for the initial establishment and maintenance of the methanogenic consortium MRE50. After 3–10 days of cultivation, cell growth and methane production were observed from the 10⁻¹ to 10⁻⁴ dilution cultures. Microscopic observation showed that the 10⁻⁴ dilution culture contained two morphologically distinct organisms: (i) methanogen-like rod-shaped cells with F₄₃₅ autofluorescence and (ii) curved rod-shaped cells. This culture was used for subcultivation. In the second dilution series under the same cultivation conditions, however, the curved rod-shaped cells dominated in all dilution steps. To determine how to remove the curved rods from the enrichment culture, we identified them using 16S rRNA gene clone library analysis. The universal bacterial primer pair 9f/1490R (Lane, 1991; Weisburg et al., 1991) was used for PCR amplification. Twenty 16S rRNA gene clones were analysed. All had identical sequences and were closely related to the 16S rRNA gene sequence of Graciliceticum thermotolerans JW/YJJ-S1T (99 % similarity; GenBank accession no. AY684082). G. thermotolerans is an anaerobic, thermotolerant, heterotrophic bacterium that is able to utilize yeast extract, Casamino acids, tryptone, peptone and some sugars as substrate (Lee et al., 2006). Therefore, the yeast extract in the medium used to culture strain MRE50T probably supported growth of G. thermotolerans. Based on this result, subsequent cultivation was performed without addition of yeast extract. Instead, antibiotics known to inhibit growth of G. thermotolerans JW/YJJ-S1T (streptomycin and vancomycin, each at 100 μg ml⁻¹) and acetate (1 mM) were added to the culture. Acetate (1 mM) had previously been found to be essential for growth of Methanocella paludicola SANAE². After about 1 week of incubation under these modified conditions, a small amount of methane was produced. Microscopic observation showed that only methanogen-like rods with F₄₃₅ autofluorescence were present, although these cells were frequently damaged,
presumably by the antibiotic treatment. The culture was then transferred to fresh liquid medium supplemented with H₂/CO₂ and 1 mM acetate. Thereafter, we used deep agar and roll-tube methods under H₂/CO₂ growth conditions. Colony formation was observed only via the deep agar method after 2–3 weeks of incubation. Colonies of strain MRE50ᵀ were white to cream and had a diameter of 0.1–1 mm. The deep agar isolation procedure was repeated twice to obtain strain MRE50ᵀ.

The purity of the culture was demonstrated by the failure to grow in the following media at 37 and 55 °C: (i) thioglycolate medium (Difco) containing approximately 150 kPa H₂/CO₂ (in the head space) and 10 mM sulphate; (ii) thioglycolate medium containing 20 mM lactate and 10 mM sulphate; (iii) thioglycolate medium containing 10 mM sucrose, 10 mM glucose, 10 mM cellobiose and 10 mM xylose; and (iv) AC medium (Difco). Moreover, PCR performed with the universal bacterial primer pairs 9f/1490R and EUB338F/1490R (Hatamoto et al., 2007) failed to detect 16S rRNA genes. We also generated an archaeal 16S rRNA gene clone library, by using the universal archaeal primer pair Ar109f/1490R (Großkopf et al., 1998) for PCR amplification. All 30 archaeal clone sequences examined matched the 16S rRNA gene sequence of the RC-I_MRE50 genome perfectly. Finally, we performed FISH by using the 16S rRNA-targeted oligonucleotide probe MRE50_426 specific for strain MRE50ᵀ. All cells in the culture were detected by this probe. The combined results of our purity tests thus indicated that the MRE50ᵀ culture was axenic.

Cells of strain MRE50ᵀ were rod-shaped, 1.3–2.8 μm long and 0.4–0.7 μm wide and occurred singly (Fig. 1). The cells autofluoresced under epifluorescence microscopy when excited with light near 420 nm in wavelength. This indicated the presence of a high concentration of coenzyme F₄₂₀, which is typical of many methanogens. Cocccoid cells were often observed in the late exponential phase. FISH analysis using the strain-specific probe MRE50_426 identified both rod-shaped and coccoid cells as belonging to strain MRE50ᵀ (Fig. 1b, c). Negative staining indicated that cells of strain MRE50ᵀ may possess flagella (Fig. 1d). In good agreement with this finding, genes encoding archaeal flagella had been identified in the previously reported genome sequence of RC-I_MRE50 [e.g. flaB (YP_

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**Fig. 1.** Photomicrographs of cells of strain MRE50ᵀ. (a) Transmission electron micrograph of a thin section. (b, c) Phase-contrast (b) and fluorescence (c) micrographs of rod-shaped and coccoid cells in the same field of view; cells were hybridized with the Cy-3-labelled probe MRE50_426; arrows and circles indicate coccoid-type cells. (d) Transmission electron micrograph of negatively stained cells showing flagellum-like structure. (e) Electron micrograph of a freeze-etched preparation showing a whole cell with a hexagonally ordered S-layer lattice. Bars, 200 nm (a), 10 μm (b, c) and 500 nm (d, e).
684781) and flaJ (YP_684769). However, motility was not observed. Freeze-etched preparations indicated that cells of strain MRE50T are surrounded by an S-layer with hexagonal symmetry (Fig. 1e).

Strain MRE50T used H2/CO2 and formate (40 mM) as energy and carbon sources, and these substrates supported growth and methane production. The following substrates did not support growth and/or methane production: 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) and dimethylamine (10 mM). Acetate (1 mM) was required as a carbon source for growth. Yeast extract (0.01 %, w/v) was not required but supported growth.

Strain MRE50T grew at 37–55 °C with optimum growth at 45 °C, at pH 6–7.8 with an optimum around pH 7.0 and in the presence of 0–20 g NaCl l⁻¹ (growth was inhibited completely at 25 g l⁻¹). Under optimal conditions (pH 7.0, 45 °C), the doubling time on H2/CO2 medium was approximately 8 h, as calculated from the methane production rate. The strain was resistant to ampicillin, vancomycin, kanamycin, rifampicin, tetracycline and streptomycin, but not to chloramphenicol.

The DNA G + C content of strain MRE50T, as determined by HPLC, was 56.7 mol%, which is close to the value of 54.6 mol% that was determined previously by genome sequencing (Erkel et al., 2006). Phylogenetic analyses based on 16S rRNA and mcrA genes showed that strain MRE50T is affiliated with the order Methanocellales (Fig. 2). Strain MRE50T was related most closely to Methanocella paludicola SANAET, with 16S rRNA and mcrA gene sequence similarities of 92.5 and 86.1 %, respectively.

Based on its morphological, physiological and molecular phylogenetic traits, strain MRE50T is a member of the order Methanocellales. Differential characteristics between strain MRE50T and Methanocella paludicola SANAET are listed in Table 1. They share several phenotypic features, including cell morphology and substrate usage. However, they differ in the following phenotypic traits: (i) possession of flagellum-like material, although cells of both strains were non-motile (Fig. 1d, e; electron micrographs of Methanocella paludicola SANAET are available as Supplementary Fig. S1 in IJSEM Online); (ii) rifampicin and tetracycline (each at 100 μg ml⁻¹) do not inhibit growth of strain MRE50T, but these antibiotics completely inhibit growth of Methanocella paludicola SANAET; (iii) Methanocella paludicola SANAET cannot grow above 40 °C, whereas strain MRE50T shows growth up to 55 °C; and (iv) strain MRE50T is able to grow...
Table 1. Differential characteristics between strain MRE50T and Methanocella paludicola SANAET

<table>
<thead>
<tr>
<th>Characteristic</th>
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<tr>
<td>Cell width (μm)</td>
<td>0.4–0.7</td>
<td>0.3–0.6</td>
</tr>
<tr>
<td>Cell length (μm)</td>
<td>1.3–2.8</td>
<td>1.8–2.4</td>
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<tr>
<td>DNA G+C content (mol%)*</td>
<td>56.7 (54.6)</td>
<td>56.6</td>
</tr>
<tr>
<td>Flagellum-like structures</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Temperature for growth (°C)</td>
<td>Range</td>
<td>37–55</td>
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<tr>
<td></td>
<td>Optimum</td>
<td>45</td>
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<td>pH range for growth</td>
<td>Range</td>
<td>6.0–7.8</td>
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<tr>
<td>NaCl concentration for growth (g L⁻¹)</td>
<td>Range</td>
<td>0–20</td>
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<td></td>
<td>Optimum</td>
<td>0–2</td>
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<tr>
<td>Antibiotic tolerance†</td>
<td>Rifampicin</td>
<td>+</td>
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<td></td>
<td>Tetracycline</td>
<td>+</td>
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*Obtained by HPLC; the value in parentheses was obtained by genome sequencing.
†Antibiotic tests were performed with each antibiotic at 100 μg ml⁻¹.

in the presence of 20 g NaCl L⁻¹, whereas Methanocella paludicola SANAET can only grow in the presence of 0–1 g NaCl L⁻¹. In addition, the 16S rRNA gene sequences of strain MRE50T and Methanocella paludicola SANAET show divergence of 7.5%. This level of divergence indicates that strain MRE50T and Methanocella paludicola SANAET are distinct at least at the species level.

According to suggestions made by Whitman et al. (2001) regarding the taxonomy of methanogenic archaea, a 16S rRNA gene sequence divergence of more than 5–7% is sufficient to delineate different genera. Thus, strain MRE50T could represent the type species of a new genus within the order Methanocellales. However, the aforementioned minor phenotypic differences between strain MRE50T and Methanocella paludicola SANAET may not warrant the description of a new genus, because different temperature ranges or salt requirements for growth are known to occur among methanogenic species of the same genus. Indeed, some genera of methanogens contain mesophilic and thermophilic species, such as Methanoculleus bourgensis and Methanoculleus thermophilus within the genus Methanoculleus and Methanomethylovorans hollandica and Methanomethylovorans thermophilus within the genus Methanomethylovorans (Ollivier et al., 1986; Rivard & Smith, 1982; Lonmans et al., 1999; Jiang et al., 2005). Similarly, some species within the same genus differ in the NaCl range for growth; for example, Methanocalculus halotolerans and Methanocalculus taiwanensis can grow in the ranges 0–12.5 and 0–4% NaCl, respectively (Ollivier et al., 1998; Lai et al., 2002). At the time of writing, therefore, knowledge of the physiological diversity among members of the order Methanocellales is not sufficient to propose distinctive genus-level characteristics with certainty. Therefore, we think that the description of a new genus would be premature and instead suggest that strain MRE50T represents a novel species of the genus Methanocella, for which the name Methanocella arvoryzae sp. nov. is proposed.

**Description of Methanocella arvoryzae sp. nov.**

Methanocella arvoryzae (ar.vo.ry.za.e. L. n. arvum an arable field, cultivated land; L. n. oryzae; N.L. gen. n. arvoryzae of a rice paddy field)

Cells occur singly and most are rod-shaped. Coccolid cells are also observed, especially in late-explosive phase cultures. Methane is produced from H₂/CO₂ and formate. Acetate is required for growth and yeast extract enhances growth. Growth occurs at 37–55 °C (optimum, 45 °C), at pH 6–7.8 (optimum, pH 7.0) and in the presence of NaCl concentrations below 20 g L⁻¹. The DNA G+C content of the type strain is 56.7 mol% (HPLC).

The type strain, MRE50T (=NBRC 105507T =DSM 22066T), was isolated from a thermophilic methanogenic consortium, which was originally established from a rice field soil in Italy.

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