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

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A novel hydrogenotrophic methanogen, designated strain MRE50^T, 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 MRE50^T 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 MRE50^T 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 MRE50^T was related most closely to *Methanocella paludicola* SANAET. Levels of sequence similarity were 92.5 and 86.1 %, respectively, indicating that strains MRE50^T and *Methanocella paludicola* SANAET 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 MRE50^T 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 MRE50^T (=NBRC 105507^T =DSM 22066^T).

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* SANAET, 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 long 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 MRE50^T. 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 MRE50^T 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

Abbreviations: FISH, fluorescence *in situ* hybridization; RC-I, rice cluster I.

The GenBank/EMBL/DDBJ accession number for the whole genome sequence of strain MRE50^T is AM114193; the 16S rRNA and *mcrA* gene sequences are located between *E. coli* positions 2470125 and 2471601 and between positions 1033346 and 1035016, respectively.

Photomicrographs of cells of *Methanocella paludicola* SANAET are available as supplementary material with the online version of this paper.

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 MRE50^T matched those of the complete genome sequence of RC-I_{MRE50} perfectly (GenBank/EMBL/DBJ 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 MRE50^T by FISH, we used probe MRE50_426 (5'-GACGATGGGA-CAGCCACC-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 MRE50^T 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 MRE50^T (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 archaeal cells, and that the archaeal 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 archaeon 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 F₄₂₀ 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 *Gracilibacter thermotolerans* JW/YJL-S1^T (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 MRE50^T 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/YJL-S1^T (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* SANAE^T. 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 F₄₂₀ 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 H_2/CO_2 and 1 mM acetate. Thereafter, we used deep agar and roll-tube methods under H_2/CO_2 growth conditions. Colony formation was observed only via the deep agar method after 2–3 weeks of incubation. Colonies of strain MRE50^T were white to cream and had a diameter of 0.1–1 mm. The deep agar isolation procedure was repeated twice to obtain strain MRE50^T.

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 H_2/CO_2 (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 MRE50^T. All cells in the culture were detected by this probe. The combined results of our purity tests thus indicated that the MRE50^T culture was axenic.

Cells of strain MRE50^T 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 F_{420} , which is typical of many methanogens. Coccoid 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 MRE50^T (Fig. 1b, c). Negative staining indicated that cells of strain MRE50^T 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_

Fig. 1. Photomicrographs of cells of strain MRE50^T. (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 MRE50^T are surrounded by an S-layer with hexagonal symmetry (Fig. 1e).

Strain MRE50^T 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), trimethylamine (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 MRE50^T 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 MRE50^T, 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 MRE50^T is affiliated with the order *Methanocellales* (Fig. 2). Strain MRE50^T 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 MRE50^T is a member of the order *Methanocellales*. Differential characteristics between strain MRE50^T 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 MRE50^T, but these antibiotics completely inhibit growth of *Methanocella paludicola* SANAET; (iii) *Methanocella paludicola* SANAET cannot grow above 40 °C, whereas strain MRE50^T shows growth up to 55 °C; and (iv) strain MRE50^T is able to grow

Fig. 2. Neighbour-joining phylogenetic trees showing the placement of strain MRE50^T based on 16S rRNA gene (a) and deduced McrA amino acid (b) sequences. The McrA tree is based on 144 deduced amino acid positions and distance corrections were based on the percentage acceptance mutations. The sequences of *Methanobacterium formicicum* MF^T (GenBank accession no. AY196659) and *Methanothermobacter thermautotrophicus* ΔH^T (NC_000916; *E. coli* positions 1040339–1042000) were used as outgroups for rooting the 16S rRNA and McrA trees, respectively (not shown). For sequences taken from complete genome sequences, their position on the genome is indicated in brackets. Bootstrap support was obtained from the neighbour-joining/maximum-parsimony/maximum-likelihood methods based on 1000 replicates; –, <50 %. Bars, 0.1 changes per sequence position.

Table 1. Differential characteristics between strain MRE50^T and *Methanocella paludicola* SANAET

Strains: 1, MRE50^T; 2, *Methanocella paludicola* SANAET (data from Sakai *et al.*, 2008). Both strains shown optimum growth at pH 7.

Characteristic	1	2
Cell width (µm)	0.4–0.7	0.3–0.6
Cell length (µm)	1.3–2.8	1.8–2.4
DNA G + C content (mol%)*	56.7 (54.6)	56.6
Flagellum-like structures	+	–
Temperature for growth (°C)		
Range	37–55	25–40
Optimum	45	35–37
pH range for growth	6.0–7.8	6.5–7.8
NaCl concentration for growth (g l ⁻¹)		
Range	0–20	0–1
Optimum	0–2	0
Antibiotic tolerance†		
Rifampicin	+	–
Tetracycline	+	–

*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 MRE50^T and *Methanocella paludicola* SANAET show divergence of 7.5 %. This level of divergence indicates that strain MRE50^T 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 MRE50^T could represent the type species of a new genus within the order *Methanocellales*. However, the aforementioned minor phenotypic differences between strain MRE50^T 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 thermophila* within the genus *Methanomethylovorans* (Ollivier *et al.*, 1986; Rivard & Smith, 1982; Lomans *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 MRE50^T 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. *oryza* rice; N.L. gen. n. *arvoryzae* of a rice paddy field).

Cells occur singly and most are rod-shaped. Coccoid cells are also observed, especially in late-exponential 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, MRE50^T (=NBRC 105507^T =DSM 22066^T), was isolated from a thermophilic methanogenic consortium, which was originally established from a rice field soil in Italy.

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