12 The Family *Methanocellaceae*

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Abstract
Methanocellaceae is a family within the order *Methanocellales* Sakai et al. (Int J Syst Evol Microbiol 58:929–936, 2008). This order and family contains a single genus *Methanocella*. Hitherto three species within the genus *Methanocella* have been reported Sakai et al. (Int J Syst Evol Microbiol 58:929–936, 2008; Int J Syst Evol Microbiol 60:2918-2923, 2010), Lü and Lu (PLoS ONE 7: e35279, 2012a); all the reported species were isolated from rice field soil. Cells are nonmotile, irregular rods and anaerobic; energy metabolism is by reduction of CO_2 to CH_4 with H_2 as an electron donor; some species can also use formate as an electron donor.

Taxonomy, Historical and Current

Short Description of the Family

Methanocellaceae (Me.tha.no.cel.la′ce.ae N.L. fem. n. *Methanocella* type genus of the family; -*aceae* the ending to donate a family; N.L. fem. pl. n. *Methanocellaceae* the family of the genus *Methanocella*).

Phylogenetically, the family *Methanocellaceae* is placed in the order *Methanocellales* within the phylum Euryarchaeota (Sakai et al. 2008). The family contains a single genus *Methanocella*, which consists of three species: *Methanocella paludicola*, *Methanocella arvoryzae*, and *Methanocella conradii* (Sakai et al. 2008, 2010; Lü and Lu 2012a). Cells occur singly and almost all of the cells are rod-shaped. In some species, coccoid cells were observed in late-exponential culture. The cells are nonmotile. Methane is produced from H_2/CO_2 or formate. Acetate is required as a carbon source for growth.

Phylogenetic Structure of the Family and Its Genus

The family *Methanocellaceae* is a single family within the order *Methanocellales*. According to phylogenetic analysis based on the 16S rRNA gene, the order *Methanocellales* deeply branches between other orders of methanogens, *Methanosarcinales* and *Methanomicrobiales* (➋ *Fig. 12.1*). Phylogenetic analysis of the *mcrA* gene (encoding the subunit of methyl-coenzyme M reductase, a key enzyme in the methane production pathway) also indicates that the order *Methanocellales* is distinct from the orders *Methanosarcinales* and *Methanomicrobiales* (Sakai et al. 2008).

Molecular Analyses

Genome Comparison

Genome sequences are available for all three species of the family *Methanocellaceae* (Erkel et al. 2006; Sakai et al. 2011; Lü and Lu 2012b).

The genome of *M. paludicola* SANAE^T is a single circular chromosome of 2,957,635 bp length with a GC content of 54.9 %. Two clusters of rRNA operons and two more distantly located 5S rRNA genes are present in the genome. A total of 48 tRNA genes containing putative introns are scatted over the genome. 3,004 predicted protein-coding sequences (CDSs) were identified. 1,467 genes (48.8 % of the protein-coding genes) were assigned with a putative function while the remaining ones (1,537 genes; 51.2 % of the protein-coding genes) were annotated as hypothetical proteins.

The genome of *M. arvoryzae* MRE50^T is a circular chromosome with 3,179,916 bp length and a GC content of 54.6 %. There are 3,085 coding genes, three rRNA operons, and 54 tRNAs in the chromosome. Originally, the genome sequence was reported in 2006 as an environmental genotype by

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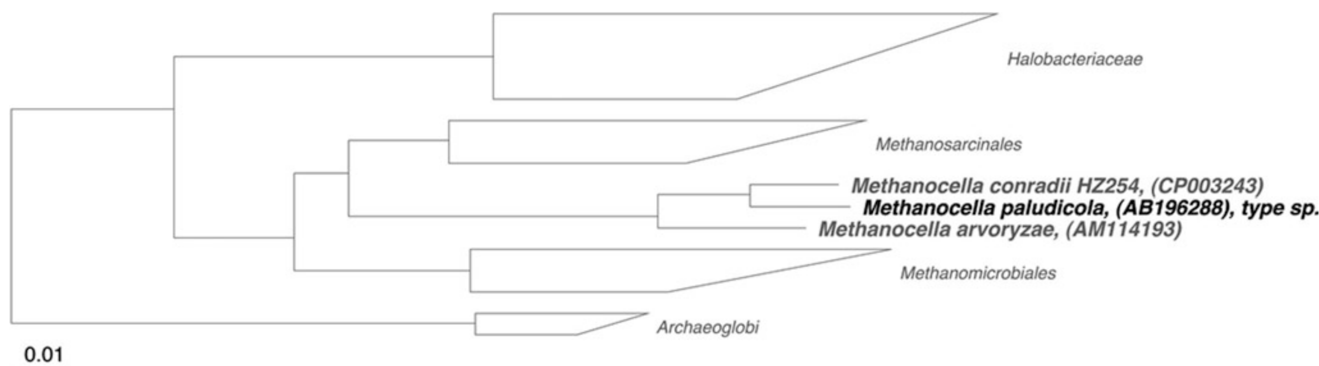

Fig. 12.1
Neighbor-joining phylogenetic tree showing the placement of the family *Methanocellaceae* based on 16S rRNA gene sequences. The tree was constructed by using a subset of sequences representative of closely related genera to stabilize tree topology. In addition, a 40 % conservational filter for the whole archaeal domain was used to remove hypervariable positions. The type species of the family is indicated in bold. The bar represents the number of changes per sequence position

metagenomic analysis of a methanogenic consortium (Erkel et al. 2006). Later, *M. arvoryzae* MRE50^T was isolated from the methanogenic consortium (Sakai et al. 2010), and the sequences of the 16S rRNA and *mcrA* genes obtained from strain MRE50^T perfectly matched those of the complete genome sequence. So, the genome sequence based on metagenomic analysis was considered to be the true genome sequence of *M. arvoryzae* MRE50^T.

M. conradii HZ254^T has so far the smallest circular chromosome of 2,378,438 bp length among the members of the family *Methanocellales*. The GC content of 52.7 % is the lowest among the genomes of the family *Methanocella*. The genome encodes two rRNA operons and a full complement of tRNA genes. 2,512 candidate genes were identified, of which 71.9 % could be assigned *a* possible function.

All three genomes contain sufficient genes to encode a full methanogenesis pathway using H_2/CO_2. Similarly to other obligate hydrogenotrophic methanogens, formate dehydrogenase complexes and a formate transporter, the key enzyme for the growth on formate as an alternative methanogenic substrate, were also found. No homologous genes for alcohol dehydrogenase, which is involved in methanogenesis from primary or secondary alcohols, were found. Although an incomplete pathway of potential methanogenesis from methanol was found in the genome of *M. arvoryzae* MRE50^T, none of the corresponding genes for utilizing methanol and other C1 compound were found in the *M. paludicola* SANAE^T and *M. conradii* HZ254^T genome. The ANI (average nucleotide identity) values among the three strains were between 69.4 % and 74.8 % (Lü and Lu 2012b).

Phenotypic Analyses

The main features of members of the family *Methanocellaceae* are listed in ➋ *Table 12.1*.

Methanocella **(Sakai et al. 2008)**

Me.tha.no.cel′la. N.Gr. n. *methane* (from N.Gr. n. *meth*(*yl*) and chemical suffix *-ane*) methane; L. fem. n. *cella* a room, and in biology a cell; N. L. fem. n. *Methanocella* a methane-producing cell.

Cells are nonmotile rods and occur singly. The cells autofluoresce under epifluorescence microscopy when excited with light near 420 nm in wavelength, which indicates the presence of the methanogen-specific coenzyme F_{420}. Methane is produced from H_2/CO_2 or formate. Acetate is required for growth. Some species also require yeast extract for its growth. The type species is *Methanocella paludicola*.

List of Species of the Genus *Methanocella*

Methanocella paludicola **(Sakai et al. 2008)**

(pa.lu.di′co.la. L. n. *palus -udis* swamp, muddy environment; L. suff. *-cola* derived from L. n. *incola* inhabitant, dweller; N.L. masc. n. *paludicola* an inhabitant of muddy environments).

Type strain is SANAE^T (=JCM 13418 = NBRC 101707 = DSM 17711), which was isolated from a Japanese rice field soil. Cells occur singly and almost all of the cells are rod-shaped (1.8–2.4 mm long by 0.3–0.6 mm wide); however, coccoid cells were occasionally observed in late-exponential culture. Cells are nonmotile; flagella-like structures were not observed. Cells stain Gram-negative and are resistant to lysis by 2 % (w/v) SDS. Freeze-etched preparations indicated that cells of strain SANAE^T are surrounded by an S-layer with hexagonal symmetry. Colonies in a deep agar medium are white to creamy, reaching a diameter of 1–1.5 mm after 6 months incubation with H_2/CO_2 as a substrate. Methane is produced from H_2/CO_2 and formate. Acetate and yeast extract are required for growth. L-glutamate can be used in

Table 12.1
Morphological and physiological characteristics of the species within the family *Methanocellaceae*

Characteristics	*Methanocella paludicola* SANAE^T	*Methanocella arvoryzae* MRE50^T	*Methanocella conradii* HZ254^T
Cell morphology	Rod, coccoid[a]	Rod, coccoid[a]	Rod
Cell width (μm)	0.3–0.6	0.4–0.7	0.2–0.3
Cell length (μm)	1.8–2.4	1.3–2.8	1.4–2.8
G+C content (%)[b]	54.9	54.6	52.7
Flagellum-like structure	–	+	+
Motility	–	–	–
Temperature range (optimum) (°C)	25–40 (35–37)	37–55 (45)	37–60 (55)
pH range (optimum)	6.5–7.8 (7)	6.0–7.8 (7)	6.4–7.2 (6.8)
NaCl range (optimum) (g L^{-1})	0–1 (0)	0–20 (0–2)	0–5 (0–1)
Substrate utilization			
H_2/CO_2	+	+	+
Formate	+	+	–
Acetate	–	–	–
Methanol or methylamines	–	–	–
Secondary alcohols	–	–	–
Growth requirements			
Acetate	+	+	+
Yeast extract	+	–	–

Abbreviations: – negative, + positive, *ND* not determined
[a]Coccoid cells are observed in the late-logarithmic culture
[b]The data were taken from genome information

substitution for yeast extract. Growth occurs between 25 and 40 °C with optimum at 35–37 °C. The pH range is 6.5–7.8; optimum growth occurs at pH 7.0. Growth is observed in media containing less than 1 g NaCl L^{-1}. The strain tolerates 100 μg mL^{-1} of ampicillin, penicillin G, vancomycin, kanamycin, bacitracin, and streptomycin, but not rifampicin, tetracycline, and chloramphenicol.

***Methanocella arvoryzae* (Sakai et al. 2010)**

(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).

Type strain is MRE50^T (= NBRC 105507 = DSM 22066), was isolated from a thermophilic methanogenic consortium, which was originally obtained from an Italian rice field soil. Cells occur singly and most of the cells are rods (1.3–2.8 mm long and 0.4–0.7 mm wide). Coccoid cells are also observed, especially in late-exponential phase cultures. Negative staining indicated that cells of strain MRE50^T might possess flagella; in good agreement with this finding, genes putatively encoding archaeal flagella were detected in the genome (Erkel et al. 2006). However, motility has not been observed. Freeze-etched preparations indicated that cells of strain MRE50^T were surrounded by an S-layer with hexagonal symmetry. Colonies in a deep agar medium are white to creamy, reaching a diameter of 0.1–1 mm after 2–3 weeks incubation with H_2/CO_2 as substrate. Methane is produced from H_2/CO_2 and formate. Acetate is required for growth and yeast extract enhances growth. Growth occurs at 37–55 °C with optimum at 45 °C. The pH range is from 6 to 7.8, optimum at pH 7.0. Growth is observed in media containing less than 20 g NaCl L^{-1}. The strain is resistant to 100 μg mL^{-1} of ampicillin, vancomycin, kanamycin, rifampicin, tetracycline, and streptomycin, but not to chloramphenicol.

***Methanocella conradii* (Lü and Lu 2012a)**

(con.rad′i.i. N.L. gen. masc. n. *conradii*, named after Ralf Conrad, who has pioneered the ecological studies on *Methanocella* methanogens in environmental samples).

The type strain is HZ254^T (= CGMCC 1.5162 = JCM 17849 = DSM 24694), isolated from a Chinese rice field soil. Cells are rods (1.4–2.8 μm long and 0.2–0.3 μm wide) and occur singly. A flagellum was observed by negative staining of the cells being consistent with the detection of a *fla* gene cluster in the genome (Lü and Lu 2012b). However, motility has not been observed. Cells lyse in 0.5 % but not in <0.1 % SDS. Colonies are nearly lens-shaped in roll-tube medium. Methane is produced from H_2/CO_2, but not from formate.

Acetate is required for growth and yeast extract can stimulate growth. Growth occurs at 37–60 °C (optimum 55 °C), at pH 6.4–7.2 (optimum 6.8) and with less than 5 g L^{-1} of NaCl (optimum 0–1 g L^{-1}). The strain tolerates 200 μg mL^{-1} of ampicillin, penicillin G, and kanamycin, but not apramycin, neomycin, rifampicin, and chloramphenicol.

Isolation, Enrichment, and Maintenance Procedures

Enrichments and isolation must be carried out under anaerobic conditions. A selective enrichment culture procedure has not yet been developed. However, cultivation under low hydrogen concentration, called co-culture method (Sakai et al. 2007, 2009), which is achieved by using an anaerobic, syntrophic, heterotrophic, and hydrogen-producing bacterium as partner organism, might be effective for enrichment of *Methanocella* methanogens. Indeed, *M. paludicola* $SANAE^T$ was isolated from such a culture using propionate as heterotrophic substrate (Sakai et al. 2007). Likewise cultivation of *Methanocella* members was reported in other syntrophic cultures (Sakai et al. 2009). In addition, incubation under moderately high temperature (e.g., 45–50 °C) with high concentration of hydrogen may also result in enrichment cultures with a high population density of *Methanocella* species (Fey et al. 2001; Lü and Lu 2012a). Therefore, high cultivation temperatures may also benefit cultivation of *Methanocella* members. Taken together, syntrophic culture conditions and moderately high incubation temperatures might be a good strategy to cultivate thermophilic species. For example, *Methanocella* species were predominant in syntrophic acetate-degrading enrichment cultures at 50 °C (Liu and Conrad 2010; Rui et al. 2011).

Once enrichment cultures are obtained, purification can be accomplished by repeated serial dilution with both liquid and solid media supplemented with H_2/CO_2 (ca. 150 kPa) or formate (40 mM). Since *Methanocella* species have limited growth factor requirement (➲ *Table 12.1*), isolation media should better be supplemented with acetate and/or yeast extract. The addition of antibiotics may be beneficial to inhibit growth of contaminating bacteria, and allow purification of the methanogen from the enrichment culture.

M. paludicola $SANAE^T$ was isolated from a rice paddy soil at Nagaoka, Niigata, Japan. Enrichment of *M. paludicola* $SANAE^T$ was achieved by application of the co-culture approach with *Syntrophobacter fumaroxidans* (Harmsen et al. 1998) as the hydrogen-producing syntrophic partner (Sakai et al. 2009). A primary enrichment culture was made from the rice paddy soil with propionate (20 mM) as the sole energy source and the addition of pregrown cells of a *S. fumaroxidans* culture. The cultivation was carried out at 37 °C under an atmosphere of N_2/CO_2 (80/20, v/v) without shaking. Once cells multiplied and propionate degradation and methane production were observed, the culture was successively transferred to fresh medium every 50–80 days. The co-culture enrichment with *S. fumaroxidans* and *Methanocella* members was established after five transfers. *M. paludicola* $SANAE^T$ was isolated from the co-culture enrichment into pure culture by applying the serial dilution method with liquid media under H_2/CO_2 (ca. 150 kPa) atmosphere.

M. arvoryzae $MRE50^T$ was isolated from a methanogenic consortium named MRE50, which was originally established in the year 2000, using Italian rice field soil as inoculum (Lueders et al. 2001). Isolation of *M. arvoryzae* $MRE50^T$ was performed at 45 °C using serial dilution method with liquid media under H_2/CO_2 and addition of streptomycin and vancomycin (each 100 μg mL^{-1}) to prevent growth of bacteria. Once contaminated bacteria were eliminated, the culture was transferred to fresh liquid medium supplemented with H_2/CO_2 and 1 mM acetate. Thereafter, the deep agar method under H_2/CO_2 growth condition was applied. Colony formation was observed only after 2–3 weeks of incubation. Colonies of strain $MRE50^T$ were white to creamy in color and had a diameter of 0.1–1 mm. The deep agar isolation procedure was repeated twice.

M. conradii $HZ254^T$ was isolated from a rice field soil located in Hangzhou, China. Enrichment of strain $HZ254^T$ was performed at 50 °C under ca. 150 kPa H_2/CO_2 (80/20, v/v) except that the preincubation was performed under an atmosphere of N_2. After at most 13 successive transfers over 338 days, the archaeal populations consisted exclusively of *Methanocella* species. Isolation was carried out using the roll-tube technique with a medium containing 1.5 % agar supplemented with 0.05 % yeast extract and tryptone and 1 mM acetate under a H_2/CO_2 atmosphere. Nearly lens-shaped colonies were formed after 5 months of incubation. The colonies were picked with Pasteur pipettes and further purified by serial dilution in liquid medium supplemented with 200 μg mL^{-1} kanamycin.

Cultures can be stored at room temperature for short-term preservation, at 4 °C for longer storage. It was shown that a culture was able to recover after 502 days storage at 4 °C (Lü and Lu 2012a). Cultures can also be preserved at −80 °C in liquid medium plus 15 % (v/v) glycerol.

Ecology

The main habitat of *Methanocella* members appears to be soil, especially rice field soil, since all species of the genus *Methanocella* have so far been isolated from rice field soil. In addition, 16S rRNA gene-based analyses indicate that *Methanocella*-related sequences are the major archaeal component in rice fields regardless of geographical location and season (Ramakrishnan et al. 2001; Krüger et al. 2005). In rice fields, *Methanocella* species colonize especially rice roots (Großkopf et al. 1998) and play a key role in CH_4 production from plant-derived carbon (Lu and Conrad 2005). *Methanocella* members are probably one of the most important methanogenic groups responsible for CH_4 emission from rice fields.

The question arises, why *Methanocella* members are dominant particularly in rice fields, in spite of the presence of other hydrogenotrophic methanogens, which seem to perform the same ecosystem function? The ecological specialty of *Methanocella* members was demonstrated by using the stable-isotope-probing technique. In situ pulse labeling of rice plants with $^{13}CO_2$ demonstrated that mainly *Methanocellaceae* living in the rhizosphere were assimilating the plant-derived carbon while producing methane (Lu and Conrad 2005). Labeling the archaeal communities on excised rice roots with $^{13}CO_2$ showed that ^{13}C was preferentially incorporated into *Methanocella* members when the incubation was done under a N_2 atmosphere in which low concentrations of H_2 were generated by fermentative bacteria living from rice root materials (Lu et al. 2005). However, *Methanobacteriales* or *Methanosarcinales* incorporated the ^{13}C when the rice roots were incubated under high hydrogen concentrations (Lu et al. 2005). Stable-isotope-probing analysis with ^{13}C-labeled propionate of anoxic rice field soil (Lueders et al. 2004) showed that ^{13}C was incorporated not only into rRNA of syntrophic propionate-oxidizing bacteria but also into methanogenic archaea, including *Methanocella* members. In methanogenic environments, the oxidation of propionate requires the syntrophic cooperation of propionate-oxidizing bacteria with hydrogenotrophic methanogens due to thermodynamic constraints. Therefore, propionate degradation can only proceed if hydrogen concentrations are kept low. Collectively, these results indicate that *Methanocella* members may have an ecological niche characterized by low H_2 concentrations, whereas other methanogens are active when H_2 concentrations are high. This interpretation is consistent with the successful cultivation and isolation of *Methanocella* members using the co-culture method (Sakai et al. 2007, 2009). Although affinity data for hydrogen of *Methanocella* members have not yet been reported, we postulate that they must have a comparatively high affinity for hydrogen. This may explain why *Methanocella* members are dominant species in rice fields, where hydrogen concentration is low (Conrad et al. 1986, 1989).

An additional characteristic of the *Methanocella* methanogens may be their moderately thermophilic mode of activity. Although rice field soils have a relatively low temperature regime (about 15–30 °C, Schütz et al. 1990), the presence of thermophilic methanogens was demonstrated in several studies. In fact, the methanogenic community in rice field soil produces CH_4 over a wide temperature range up to about 50 °C (Yao and Conrad 2000; Fey et al. 2001) and moderately thermophilic methanogens, mostly *Methanocellaceae* are ubiquitously present (Wu et al. 2006). Temperature increase was found to affect not only the rate of CH_4 production but also the composition of the methanogenic community and its function. Conrad et al. (2009) reported that when rice filed soil was incubated at moderate temperatures (<40 °C), CH_4 is always produced by a combination of aceticlastic and hydrogenotrophic methanogenesis involving *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteriales*, and *Methanocellales*, while at moderately high temperatures (>40 °C), CH_4 is formed exclusively by hydrogenotrophic methanogenesis with *Methanocellales* prevailing. Stable-isotope-probing analysis with ^{13}C-labeled acetate at 50 °C demonstrated that the bacterial community consisted mainly of members of the *Thermacetogenium* genus, i.e., thermophilic syntrophic acetate oxidizers, while the archaeal community consisted mainly of *Methanocella* members (Liu and Conrad 2010). Hence, *Methanocella* members are probably involved in syntrophic acetate oxidation at thermal conditions. The moderately thermophilic characteristics of *Methanocella* members helped to isolate two of the existing pure cultures of *Methanocella* species (Sakai et al. 2010; Lü and Lu 2012a). However, all these results were obtained from artificially incubated rice field soils. The selective advantage of moderate thermophily in real environments is presently unknown.

Finally, *Methanocellaceae* seem to have a rather high resistance to aeration. Initially, this was hypothesized because of the occurrence in the genome of many different genes involved in detoxification of oxygen species, including catalase and hyperoxide dismutase (Erkel et al. 2006; Lü and Lu 2012b). Such resistance is beneficial for methanogens inhabiting rice fields because of regular drainage and fallow under non-flooded and oxic conditions. Indeed, it was found that drainage and aeration does not impede methanogenic activity in rice field soil and that populations of methanogens are maintained at the same titer as under flooded and anoxic conditions (Krüger et al. 2005; Watanabe et al. 2009; Yuan et al. 2011; Ma et al. 2012). An intriguing observation was the detection of *Methanocellaceae* in dry upland soils, even in biological soil crusts of desert, which are most of the time under oxic conditions. In such soils, hydrogenotrophic *Methanocellaceae* together with presumably aceticlastic *Methanosarcinaceae* are the only methanogenic archaea. They can rapidly be activated to produce CH_4 and proliferate when the soil is wetted (Angel et al. 2011, 2012a, b). These observations indicate that *Methanocellaceae* are characterized by being able to tolerate oxic conditions and desiccation.

While all existing *Methanocella* species were isolated from rice fields and many of the studies involving *Methanocellaceae* were done in rice fields, *Methanocellaceae* have also been detected in a variety of other anaerobic environments by using 16S rRNA gene clone sequencing. These environments include acidic peatlands, acidic bog, rich minerotrophic fen, oil-contaminated groundwater, tropical rainforest, gold mine, lake sediment, river bank soil, and bromeliad tanks (Dojka et al. 1998; Zepp Falz et al. 1999; Jurgens et al. 2000; Donovan et al. 2004; Kaku et al. 2005; Cadillo-Quiroz et al. 2006, 2008, 2010; Martinson et al. 2010; Goffredi et al. 2011). *Methanocella* species apparently contribute to methane production not only in rice fields but also other anaerobic environments.

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