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[†], 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[†] 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[†] 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[†] 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[†] 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[†] (=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.

Rice paddy fields are one of the major sources of the greenhouse gas methane, contributing about 10–25 % of global methane emission (Dentener *et al.*, 2001; Neue,

1993). Methane emission from rice paddy fields is attributed to methanogenic *Archaea* (methanogens), the key organisms responsible for the final step of the anoxic degradation of organic substances. The Rice Cluster I (RC-I) lineage, which hitherto has been comprised solely of 16S rRNA gene clone sequences, plays a key role in methane production in rice paddy fields (Lu & Conrad, 2005; Conrad *et al.*, 2006). Despite their numerical

Abbreviation: RC-I, Rice Cluster I.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *mcrA* gene sequences of strain SANAET[†] are AB196288 and AB300467, respectively.

significance and cosmopolitan dispersal, RC-I methanogens have so far escaped isolation.

Recently, we have successfully isolated a novel methanogenic archaeon, 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. Here we give a detailed description of strain SANAET, including morphological, physiological and genetic properties. We propose that strain SANAET 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 N₂/CO₂ (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 H₂ 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⁻¹ (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⁺ and 14 mM Cl⁻). Antibiotics were evaluated with cultures supplemented at final concentrations of 100 µg antibiotic ml⁻¹. 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, H₂ 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 cells, a 16S rRNA-targeted oligonucleotide probe specific for strain SANAET, SANAET136 (Sakai *et al.*, 2007), was used.

Cells of strain SANAET were non-motile, rod-shaped, 1.8–2.4 µm long and 0.3–0.6 µm wide, and occurred singly (Fig. 1). Coccoid cells were observed in a late-exponential phase culture with H₂ or formate (Fig. 2). Fluorescence *in situ* hybridization probe targeting of SANAET cells demonstrated that both cell types belong to strain SANAET (Fig. 2). Cells of strain SANAET autofluoresced in epifluorescence microscopy, indicating the presence of the methanogen-specific coenzyme F₄₂₀. The cells stained

Fig. 1. Transmission electron micrograph of strain SANAET. Bar, 1 µm.

Fig. 2. Photomicrographs of rod and coccoid cells of strain SANAE^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 μ m.

Gram-negative and resisted disruption by less than 2% (w/v) SDS. Colonies of strain SANAE^T in a deep agar medium were white to cream, reaching a diameter of 1–1.5 mm after 6 months incubation with H₂ as a substrate.

Strain SANAE^T required acetate as carbon source. Yeast extract supported growth; however, it was not required. H₂ 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^T grew between 25 and 40 °C with an optimum at 35–37 °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⁻¹ (0–17 mM). In medium containing 2–4 g NaCl l⁻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⁻¹ (86 mM). Under optimal conditions (pH 7.0, 37 °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^T was 56.6 mol%. Analysis of its 16S rRNA gene sequence affiliates strain SANAE^T with sequences in the RC-I lineage (previously published in Sakai *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^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^T was *Methanomethyl-ovorans 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^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^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^T is an H₂-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 H₂ (Lueders *et al.*, 2001; Sizova *et al.*, 2003; Erkel *et al.*, 2005, 2006; Lehmann-Richter *et al.*, 1999). Erkel *et al.* (2006) constructed a complete genome sequence of an RC-I methanogen from an enrichment culture growing on H₂ and revealed that the RC-I methanogen had a full set of genes involved in methanogenesis from H₂/CO₂. Abundant populations of RC-I *Archaea* were detected in an acidophilic methanogenic consortium supplemented with H₂ as sole energy source (Sizova *et al.*, 2003). The 16S rRNA gene sequences from those RC-I enrichments were determined and are available from the Genbank/EMBL/DBJ databases as AM114193 for the metagenome (the genome contained three *rrn* operons) and AF524853 for the acidophilic consortium. The sequence similarities between these RC-I enrichments and strain SANAE^T were in the range 92.2–94.2%, indicating that strain SANAE^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 *et al.*, 1998). In fact, as described above, the 16S rRNA gene sequence similarities between strain SANAE^T and members of the *Methanosarcinales* and *Methanomicrobiales* were also low. Additionally, the bootstrap values of both 16S rRNA 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^T, is certainly a monophyletic taxon at order level within the class 'Methanomicrobia' (Garrity *et al.*, 2003). Additionally, the physiological properties of strain SANAE^T distinguish it clearly from members of the order *Methanosarcinales*, which utilize acetate and simple methylated compounds in contrast to

Fig. 3. Phylogenetic tree of the class 'Methanomicrobia' based on comparative analyses of 16S rRNA gene sequences, showing the placement of strain SANAET. The tree was calculated based on a distance matrix analysis of 16S rRNA gene sequences (neighbour-joining tree). The 16S rRNA gene sequence of *Methanobacterium formicicum* (AY196659) was used as the outgroup. The sequences of *Methanotherix* ('*Methanosaeta*') *thermophila* and RC-I MRE50 were inferred from their complete genomic sequences, and the position of each sequence is indicated after their accession number in parentheses. Bootstrap support (>50% indicated only) was obtained from neighbour-joining (first value), maximum-parsimony (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 the number of changes per sequence position.

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 was obtained from neighbour-joining (first value), maximum-parsimony (second value) and maximum-likelihood (third value) methods 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); *Methanosaetaceae* (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.

Characteristic	SANAET	<i>Methanomicrobiales</i>				<i>Methanosarcinales</i>	
		<i>Methanomicrobiaceae</i>	<i>Methanospirillaceae</i>	<i>Methanocorpusculaceae</i>	Genus <i>Methanocalculus</i>	<i>Methanosaetaceae</i>	<i>Methanosarcinaceae</i>
Cell morphology	Rod, coccus*	Curved or irregular rod, irregular coccus, or irregular plate or disk	Curved rod	Coccus or irregular coccus	Irregular coccus	Rod	Coccus, irregular coccus, or flat polygons
Gram reaction	–	–	–	–	–	±	±
DNA G + C content (mol%)	56.6	38.7–62.2	45	48.5–52	50.8–55	49.0–55.7	36.3–55
Motility	–	±	+ (weakly)	±	–	–	±
Optimum growth temperature (°C)	35–37	20–55	30–37	30–37	35–38	34–55	20–50
Optimum pH	7	6.0–7.9	6.6–7.4	6.5–7.0	6.5–7.6	6.7–7.6	6.0–9.5
Substrate utilization							
H ₂ /CO ₂	+	+	+	+	+	–	±
Formate	+	+	+	+	+	–	–
Acetate	–	–	–	–	–	+	±
Secondary alcohols	–	±	–	±	–	–	–
Methanol or methylamines	–	–	–	–	–	–	+
Growth requirements							
Yeast extract	–	±	–	±	–	±	±
Acetate	+	±	–	±	+	ND	ND

*Cocci are observed in the late-exponential culture.

strain SANAET^T. However, the phenotypic distinction between strain SANAET^T 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^T 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(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 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.

Methanocella paludicola (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).

Cells occur singly and almost all of the cells are rod-shaped; however, coccoid 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 H₂ and formate. Acetate is required for growth and yeast extract supported growth. Growth occurs between 25 and 40 °C with the optimum at 35–37 °C. The pH range is 6.5–7.8; optimum growth occurs at pH 7.0. Growth was observed in medium containing less than 1 g NaCl l⁻¹ (17 mM). The DNA G + C content is 56.6 mol%.

The type strain, isolated from a rice paddy soil at Nagaoka, Niigata, Japan, is SANAET^T (=JCM 13418^T=NBRC 101707^T=DSM 17711^T).

Description of *Methanocellaceae* fam. nov.

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*).

The description is the same as for the genus *Methanocella*.

The type genus is *Methanocella* gen. nov.

Description of *Methanocellales* ord. nov.

Methanocellales (Me.tha.no.cel.la'les. N.L. fem. n. *Methanocella* type genus of the order; *-ales* ending to donate an

order; N.L. fem. pl. n. *Methanocellales* the order of the genus *Methanocella*).

The order *Methanocellales* is defined on the basis of a phylogenetic tree constructed by comparative 16S rRNA gene sequence analysis from one isolated strain and a wide variety of environmental clones. The organism uses H₂ or formate for growth and methane production.

The type genus is *Methanocella* gen. nov.

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References

- Boone, D. R., Whitman, W. B. & Rouvière, P. (1993). Diversity and taxonomy of methanogens. In *Methanogenesis*, pp. 35–80. Edited by J. G. Ferry. New York: Chapman & Hall.
- Boone, D. R., Whitman, W. B. & Koga, Y. (2001a). Order II. *Methanomicrobiales*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 246–267. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.
- Boone, D. R., Whitman, W. B. & Koga, Y. (2001b). Order III. *Methanosarcinales*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 248–293. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.
- Chong, S. C., Liu, Y., Cummins, M., Valentine, D. L. & Boone, D. R. (2002). *Methanogenium marinum* sp. nov., a H₂-using methanogen from Skan Bay, Alaska, and kinetics of H₂ utilization. *Antonie van Leeuwenhoek* **81**, 263–270.
- Conrad, R., Erkel, C. & Liesack, W. (2006). Rice Cluster I methanogens, an important group of *Archaea* producing greenhouse gas in soil. *Curr Opin Biotechnol* **17**, 262–267.
- DeLong, E. F. (1992). *Archaea* in coastal marine environments. *Proc Natl Acad Sci U S A* **89**, 5685–5689.
- Dentener, F., Derwent, R., Dlugokencky, E., Holland, E., Isaksen, I., Katima, J., Kirchhoff, V., Matson, P., Midgley, P. & Wang, M. (2001). Climate change 2001, the scientific basis. *Third Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge: Cambridge University Press.
- Dianou, D., Miyaki, T., Asakawa, S., Morii, H., Nagaoka, K., Oyaizu, H. & Matsumoto, S. (2001). *Methanoculleus chikugoensis* sp. nov., a novel methanogenic archaeon isolated from paddy field soil in Japan, and DNA–DNA hybridization among *Methanoculleus* species. *Int J Syst Evol Microbiol* **51**, 1663–1669.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. H. Phillips. Washington, DC: American Society for Microbiology.
- Erkel, C., Kemnitz, D., Kube, M., Ricke, P., Chin, K.-J., Dedysh, S., Reinhardt, R., Conrad, R. & Liesack, W. (2005). Retrieval of first genome data for rice cluster I methanogens by a combination

- of cultivation and molecular techniques. *FEMS Microbiol Ecol* **53**, 187–204.
- Erkel, C., Kube, M., Reinhardt, R. & Liesack, W. (2006). Genome of Rice Cluster I *Archaea* – the key methane producers in the rice rhizosphere. *Science* **313**, 370–372.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Garrity, G. M., Bell, J. A. & Lilburn, T. G. (2003). Taxonomic outline of the Procaryotes. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, Release 4.0. New York: Springer. doi:10.1007/bergeysoutline200310
- Großkopf, R., Stubner, S. & Liesack, W. (1998). Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. *Appl Environ Microbiol* **64**, 4983–4989.
- Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W. & Saunders, J. R. (1996). Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl Environ Microbiol* **62**, 668–675.
- Imachi, H., Sekiguchi, Y., Kamagata, Y., Ohashi, A. & Harada, H. (2000). Cultivation and in situ detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. *Appl Environ Microbiol* **66**, 3608–3615.
- Imachi, H., Sekiguchi, Y., Kamagata, Y., Hanada, S., Ohashi, A. & Harada, H. (2002). *Pelotomaculum thermopropionicum* gen. nov., sp. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. *Int J Syst Evol Microbiol* **52**, 1729–1735.
- Imachi, H., Sekiguchi, Y., Kamagata, Y., Loy, A., Qiu, Y.-L., Hugenholtz, P., Kimura, N., Wagner, M., Ohashi, A. & Harada, H. (2006). Non-sulfate-reducing, syntrophic bacteria affiliated with *Desulfotomaculum* cluster I are widely distributed in methanogenic environments. *Appl Environ Microbiol* **72**, 2080–2091.
- Jiang, B., Parshina, S. N., van Doesburg, W., Lomans, B. P. & Stams, A. J. M. (2005). *Methanomethylovorans thermophila* sp. nov., a thermophilic, methylotrophic methanogen from an anaerobic reactor fed with methanol. *Int J Syst Evol Microbiol* **55**, 2465–2470.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kamagata, Y. & Mikami, E. (1991). Isolation and characterization a novel thermophilic *Methanosaeta* strain. *Int J Syst Bacteriol* **41**, 191–196.
- Lai, M.-C. & Chen, S.-C. (2001). *Methanofollis aquaemaris* sp. nov., a methanogen isolated from an aquaculture fish pond. *Int J Syst Evol Microbiol* **51**, 1873–1880.
- Lai, M.-C., Chen, S.-C., Shu, C.-M., Chiou, M.-S., Wang, C.-C., Chuang, M.-J., Hong, T.-Y., Liu, C.-C., Lai, L.-J. & Hua, J.-J. (2002). *Methanocalculus taiwanensis* sp. nov., isolated from an estuarine environment. *Int J Syst Evol Microbiol* **52**, 1799–1806.
- Lai, M.-C., Lin, C.-C., Yu, P.-H., Huang, Y.-F. & Chen, S.-C. (2004). *Methanocalculus chunghsingensis* sp. nov., isolated from an estuary and a marine fishpond in Taiwan. *Int J Syst Evol Microbiol* **54**, 183–189.
- Lehmann-Richter, S., Großkopf, R., Liesack, W., Frenzel, P. & Conrad, R. (1999). Methanogenic archaea and CO₂-dependent methanogenesis on washed rice roots. *Environ Microbiol* **1**, 159–166.
- Lomans, B. P., Maas, R., Luderer, R., Op den Camp, H. J., Pol, A., van der Drift, C. & Vogels, G. D. (1999). Isolation and characterization of *Methanomethylovorans hollandica* gen. nov., sp. nov., isolated from freshwater sediment, a methylotrophic methanogen able to grow on dimethyl sulfide and methanethiol. *Appl Environ Microbiol* **65**, 3641–3650.
- Lu, Y. & Conrad, R. (2005). In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* **309**, 1088–1090.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.
- Lueders, T., Chin, K. J., Conrad, R. & Friedrich, M. (2001). Molecular analyses of methyl-coenzyme M reductase alpha-subunit (*mcrA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environ Microbiol* **3**, 194–204.
- Lyimo, T. J., Pol, A., Op den Camp, H. J. M., Harhangi, H. R. & Vogels, G. D. (2000). *Methanosarcina semesiae* sp. nov., a dimethylsulfide-utilizing methanogen from mangrove sediment. *Int J Syst Evol Microbiol* **50**, 171–178.
- Ma, K., Liu, X. & Dong, X. (2006). *Methanosaeta harundinacea* sp. nov., a novel acetate-scavenging methanogen isolated from a UASB reactor. *Int J Syst Evol Microbiol* **56**, 127–131.
- Mikucki, J. A., Liu, Y., Delwiche, M., Colwell, F. S. & Boone, D. R. (2003). Isolation of a methanogen from deep marine sediments that contain methane hydrates, and description of *Methanoculleus submarinus* sp. nov. *Appl Environ Microbiol* **69**, 3311–3316.
- Mori, K., Yamamoto, H., Kamagata, Y., Hatsu, M. & Takamizawa, K. (2000). *Methanocalculus pumilus* sp. nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site. *Int J Syst Evol Microbiol* **50**, 1723–1729.
- Neue, H.-U. (1993). Methane emission from rice fields. *Bioscience* **43**, 466–474.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sakai, S., Imachi, H., Sekiguchi, Y., Ohashi, A., Harada, H. & Kamagata, Y. (2007). Isolation of key methanogens for global methane emission from rice paddy fields: a novel isolate affiliated with the clone cluster Rice Cluster I. *Appl Environ Microbiol* **73**, 4326–4331.
- Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H. & Nakamura, K. (1998). Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. *Microbiology* **144**, 2655–2665.
- Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A. & Harada, H. (2000). *Syntrophothermus lipocalidus* gen. nov., sp. nov., a novel thermophilic, syntrophic, fatty-acid-oxidizing anaerobe which utilizes isobutyrate. *Int J Syst Evol Microbiol* **50**, 771–779.
- Sekiguchi, Y., Yamada, T., Hanada, S., Ohashi, A., Harada, H. & Kamagata, Y. (2003). *Anaerolinea thermophila* gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain *Bacteria* at the subphylum level. *Int J Syst Evol Microbiol* **53**, 1843–1851.
- Sekiguchi, Y., Imachi, H., Susilorukmi, A., Muramatsu, M., Ohashi, A., Harada, H., Hanada, S. & Kamagata, Y. (2006). *Tepidanaerobacter syntrophicus* gen. nov., sp. nov., an anaerobic, moderately thermophilic, syntrophic alcohol- and lactate-degrading bacterium isolated from thermophilic digested sludges. *Int J Syst Evol Microbiol* **56**, 1621–1629.
- Simankova, M. V., Parshina, S. N., Tourova, T. P., Kolganova, T. V., Zehnder, A. J. & Nozhevnikova, A. N. (2001). *Methanosarcina lacustris* sp. nov., a new psychrotolerant methanogenic archaeon from anoxic lake sediments. *Syst Appl Microbiol* **24**, 362–367.
- Singh, N., Kendall, M. M., Liu, Y. & Boone, D. R. (2005). Isolation and characterization of methylotrophic methanogens from anoxic marine sediments in Skan Bay, Alaska: description of *Methanococcoides alaskense* sp. nov., and emended description of *Methanosarcina baltica*. *Int J Syst Evol Microbiol* **55**, 2531–2538.

Sizova, M. V., Panikov, N. S., Tourova, T. P. & Flanagan, P. W. (2003). Isolation and characterization of oligotrophic acido-tolerant methanogenic consortia from a *Sphagnum* peat bog. *FEMS Microbiol Ecol* **45**, 301–315.

Sprenger, W. W., van Belzen, M. C., Rosenberg, J., Hackstein, J. H. P. & Keltjens, J. T. (2000). *Methanomicrococcus blatticola* gen. nov., sp. nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. *Int J Syst Evol Microbiol* **50**, 1989–1999.

von Klein, D., Arab, H., Völker, H. & Thomm, M. (2002). *Methanosarcina baltica*, sp. nov., a novel methanogen isolated from the Gotland Deep of the Baltic Sea. *Extremophiles* **6**, 103–110.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Wu, S.-Y., Chen, S.-C. & Lai, M.-C. (2005). *Methanofollis formosanus* sp. nov., isolated from a fish pond. *Int J Syst Evol Microbiol* **55**, 837–842.