Methanospirillum lacunae sp. nov., a methane-producing archaeon isolated from a puddly soil, and emended descriptions of the genus *Methanospirillum* and *Methanospirillum hungatei*

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A mesophilic, hydrogenotrophic methanogen, designated strain Ki8-1^T, was isolated from soil. Cells were strictly anaerobic, Gram-stain-negative, non-sporulating, motile by means of a single flagellum or tufted flagella, and curved or wavy rod-shaped (11–25 µm long). The temperature and pH for optimum growth were 30 °C and 7.5. The strain grew best in basal medium without the addition of NaCl. Methane was produced from H₂ and formate. Acetate or yeast extract was required for growth. The G+C content of the genomic DNA of strain Ki8-1^T was 45.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain Ki8-1^T was a member of the genus *Methanospirillum* and showed 95.1 % sequence similarity to *Methanospirillum hungatei* NBRC 100397^T. On the basis of its phenotypic characteristics and phylogenetic position, strain Ki8-1^T is considered to represent a novel species of the genus *Methanospirillum*, for which the name *Methanospirillum lacunae* sp. nov. is proposed. The type strain is Ki8-1^T (NBRC 104920^T = JCM 16384^T = DSM 22751^T). Emended descriptions of the genus *Methanospirillum* and of *Methanospirillum hungatei* are also provided.

The production of methane has been regarded as a good source of renewable energy from biomass and its waste. Therefore, methane-producing archaea (methanogens), which are responsible for the final step of the anaerobic degradation of organic substances, have been investigated by both culture-dependent and culture-independent approaches (Bräuer *et al.*, 2006; Joye *et al.*, 2009; Narihiro *et al.*, 2009; Zengler *et al.*, 1999).

The methanogens are phylogenetically accommodated in the phylum *Euryarchaeota* (Garrity & Holt, 2001), and, at the time of writing, approximately 120 named species in 32 genera have been recognized according to the List of Prokaryotic names with Standing in Nomenclature (<http://www.bacterio.cict.fr/>). These methanogens have frequently been isolated from anoxic environments such as paddy fields, peatlands, wetlands, the intestinal tract of animals, anaerobic digestors, freshwater or marine sediments and hot springs (Jones *et al.*, 1987; Liu & Whitman, 2008).

Recently, we have successfully isolated a novel methanogen, designated strain Ki8-1^T, from anoxic soil. In this paper, we

describe the isolation and characterization of this strain and show that it represents a novel species of the genus *Methanospirillum*. In addition, we also provide emended descriptions of the genus *Methanospirillum* and of *Methanospirillum hungatei* according to our study and the report of Patel *et al.* (1976).

Soil was collected from a puddle at Kisarazu, Chiba, Japan. Spring water accumulates at this site year round, and therefore anaerobic conditions are maintained in the soil. The soil sample was kept in a sealed nylon bag with an O₂-absorbing and CO₂-generating agent (Anaero-Pack; Mitsubishi Gas Chemical) until inoculation to fresh medium.

For enrichment, 1 g of soil was used for inoculation into 20 ml of basal medium in a vial sealed with a tight-fitting butyl rubber stopper. The basal medium comprised (per litre): 0.54 g NH₄Cl, 0.14 g KH₂PO₄, 0.20 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, 2.5 g NaHCO₃ and 1.0 ml trace elements solution (Touzel & Albagnac, 1983) containing 3.0 mg Na₂WO₄·H₂O but lacking NaCl. Prior to inoculation, the pH of the medium was adjusted to 6.8, dissolved air was removed by flushing with H₂/CO₂ (4:1, v/v; approximately 150 kPa), and 10 ml vitamin solution l⁻¹ (Wolin *et al.*, 1963) and 10 ml of a sterile stock solution of

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *mcrA* gene sequences of strain Ki8-1^T are AB517986 and AB517988, respectively.

Na₂S (50.0 g l⁻¹) were added. The enrichment culture was cultivated at 25 °C for 3 weeks and transferred several times to fresh HAB medium, which added 0.82 g sodium acetate l⁻¹ and 1 mg ampicillin l⁻¹ to the above basal medium. After microbial growth was observed in the medium, the culture was spread on slants of HAB medium solidified with 1.5 % (w/v) agar, and microbial colonies appearing within a few months were picked and transferred to fresh medium. The purification procedure was repeated several times to establish an axenic culture with one strain, which was designated Ki8-1^T. After purification, the isolate was maintained in HAB medium without antibiotic. *M. hungatei* NBRC 100397^T, used as a reference, was also cultivated in HAB medium.

Cells of strain Ki8-1^T were curved or wavy rods with blunt ends, and were generally 0.5–0.6 µm wide and 10.9–25.1 µm long (Fig. 1a). Some cells were 8.2 × 0.5–26.4 × 0.7 µm in size. Weak motility was observed under phase-contrast microscopy. A single polar flagellum or tufted flagella were observed by electron microscopy (Fig. 1b, c). Cells of strain Ki8-1^T autofluoresced under epifluorescence microscopy, indicating the presence of the methanogen-specific coenzyme F₄₂₀. Cells stained Gram-negative by conventional Gram staining.

Strain Ki8-1^T was strictly anaerobic, i.e. was able to grow in HAB medium under an H₂/CO₂ (4:1, v/v) atmosphere, but could not grow under microaerobic or aerobic conditions. Strain Ki8-1^T required acetate or yeast extract for growth. Catalase and oxidase reactions were negative. Strain Ki8-1^T grew at 15–37 °C, with optimum growth at 30 °C. No growth was observed at 10 or 40 °C. The pH range for growth was 6.0–9.5, with optimum growth at pH 7.5. No growth was observed at pH 5.5 or 10.0. Growth of *M. hungatei* NBRC 100397^T was also investigated in the present study for comparison. *M. hungatei* NBRC 100397^T grew at 20–50 °C, with optimum growth at 37–45 °C. No

growth was observed at 15 or 55 °C. The pH range for growth was 6.5–10.0, with optimum growth at pH 7.0–9.0. No growth was observed at pH 6.0 or 10.5. Growth of strain Ki8-1^T and *M. hungatei* NBRC 100397^T was observed below 1 % (w/v) NaCl, and optimum growth for the two strains was observed in basal medium without the addition of NaCl. No growth was observed at 2 % (w/v) NaCl. Strain Ki8-1^T and *M. hungatei* NBRC 100397^T used H₂/CO₂ (4:1, v/v) and formate (10 mM) for growth and methane production. No growth occurred on acetate, pyruvate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol or trimethylamine (all at 10 mM). Strain Ki8-1^T and *M. hungatei* NBRC 100397^T were susceptible to chloramphenicol and tetracycline (both at 100 µg ml⁻¹), but were resistant to ampicillin, erythromycin, gentamicin, kanamycin, rifampicin, streptomycin and vancomycin (all at 100 µg ml⁻¹). Strain Ki8-1^T was weakly susceptible to bacitracin (100 µg ml⁻¹), whereas *M. hungatei* NBRC 100397^T was resistant to bacitracin. The generation time of strain Ki8-1^T in HAB medium at 30 °C and pH 7.5 was 32.3 h, based on an increase in turbidity, and that of *M. hungatei* NBRC 100397^T was 20.7 h at 40 °C and pH 7.5. The genomic DNA G+C content of strain Ki8-1^T was 45.3 mol%, as determined by the HPLC method described by Tamaoka & Komagata (1984). The morphological, biochemical and physiological properties that differentiate strain Ki8-1^T from *M. hungatei* are summarized in Table 1.

The 16S rRNA genes of strain Ki8-1^T and *M. hungatei* NBRC 100397^T were amplified by PCR with primers A10F (5'-TCYGGTTGATCCYGCCRG-3') and A1400R (5'-ACGGGCGGTGTGTGCAAG-3'), and almost-complete 16S rRNA gene sequences for the two strains (1260 and 1259 bases, respectively) were determined. After alignment using the ARB software (Ludwig *et al.*, 2004), phylogenetic

Fig. 1. Transmission electron micrographs of cells of strain Ki8-1^T. Bars: (a) 2 µm; (b, c) 0.5 µm.

Table 1. Morphological, biochemical and physiological properties that differentiate strain Ki8-1^T from *M. hungatei* NBRC 100397^T

Data from this study. Both strains are weakly motile, Gram-stain-negative, curved rods with polar or tufted flagella.

Characteristic	Ki8-1 ^T	<i>M. hungatei</i>
Cell width (µm)	0.5–0.6	0.4–0.5
Cell length (µm)	11–25 (often 8–26)	7.4–20 (often 15 to >100)
Temperature for growth (°C)		
Optimum	30	45
Range	15–37	20–50
pH for growth		
Optimum	7.5	7.0–9.0
Range	6.0–9.5	6.5–10.0
DNA G+C content (mol%)	45.3 (HPLC)	45–49.5 (<i>T_m</i>)*
Source	Soil	Sewage sludge

*Data from Ferry *et al.* (1974) and Patel *et al.* (1976).

trees were reconstructed by the neighbour-joining method with the program CLUSTAL_X (Saitou & Nei, 1987; Thompson *et al.*, 1997) and the maximum-likelihood method with the MORPHY software package (Adachi & Hasegawa, 1995). The topologies of the trees generated by these two methods were almost identical, and strain Ki8-1^T was located near *M. hungatei* NBRC 100397^T, which represents a single family, genus and species in the order *Methanomicrobiales* (Fig. 2). The 16S rRNA gene sequence of strain Ki8-1^T showed 95.1% similarity to that of *M. hungatei* NBRC 100397^T. Phylogenetically, strain Ki8-1^T formed a distinct subline branching with *M. hungatei* NBRC 100397^T at the periphery of the 16S rRNA gene sequence lineage that embraced related genera.

The sequence of the *mcrA* gene, encoding the alpha-subunit of methyl-coenzyme M reductase, was also determined for phylogenetic comparison between strain Ki8-1^T and *M. hungatei*. This gene was partially amplified from strain Ki8-1^T by PCR with primers MR1 and ME2 (Hales *et al.*, 1996; Simankova *et al.*, 2003). The amplified *mcrA* genes were cloned into pT7Blue T-vector (Takara Bio), and the sequence (1109 bases; GenBank/EMBL/DDBJ accession number for strain Ki8-1^T, AB517988) was determined by using M13 primers. The *McrA* amino acid sequence deduced from the *mcrA* gene sequence of strain Ki8-1^T showed 91.1% similarity to that of *M. hungatei* JF-1^T (GenBank/EMBL/DDBJ accession number CP000254).

Cells of strain Ki8-1^T were curved or wavy rods, strictly anaerobic and Gram-stain-negative, possessed a single polar flagellum or tufted flagella, used H₂/CO₂ or formate and required acetate. These properties are similar to those described for *M. hungatei* (Boone *et al.*, 2001; Ferry *et al.*, 1974), although *M. hungatei* also forms wavy filaments several hundred micrometres long. However, the 16S rRNA gene sequence of strain Ki8-1^T showed only 95% similarity to that of *M. hungatei* NBRC 100397^T, and the branching of strain Ki8-1^T and *M. hungatei* NBRC 100397^T was supported by bootstrap probability scores of 100% in the

two phylogenetic analysis methods employed. In addition, the temperature for growth of strain Ki8-1^T was different from that of *M. hungatei*. The optimum temperature and range for growth of strain Ki8-1^T were 30 and 15–37 °C, respectively, lower than those of *M. hungatei*.

On the basis of its phylogenetic position, morphology, and biochemical and physiological properties, strain Ki8-1^T is a member of the genus *Methanospirillum* separate from *M. hungatei*. We therefore suggest that strain Ki8-1^T represents a novel species of the genus *Methanospirillum*, for which the name *Methanospirillum lacunae* sp. nov. is proposed.

The optimum temperature and pH for growth of *M. hungatei* were originally given as 37 °C and pH 7.0 (Boone *et al.*, 2001; Ferry *et al.*, 1974), whereas values of 37–45 °C and pH 7.0–9.0 were found in the present study. Differences in these properties had already been suggested by Patel *et al.* (1976), and our study supported this conclusion. Therefore, emended descriptions of the genus *Methanospirillum* and of *M. hungatei* are also proposed here based on data given by Patel *et al.* (1976) and presented here.

Emended description of the genus *Methanospirillum* Ferry *et al.* 1974

Strictly anaerobic, mesophilic, neutrophilic or mildly alkaliphilic. Cells are Gram-stain-negative, non-sporulating and motile by polar, tufted flagella. Cells form curved or wavy rods with blunt ends. Produce methane from H₂ or formate. The G+C content of the genomic DNA is 45–49.5 mol%. Represent a distinct phylogenetic lineage in the family *Methanospirillaceae* based on 16S rRNA gene sequence analysis. The type species is *Methanospirillum hungatei*.

Emended description of *Methanospirillum hungatei* Ferry *et al.* 1974

The following properties are additional to those given for the genus. Cells are 0.4–0.5 × 7.4–10 µm in size, and often

Fig. 2. Phylogenetic tree showing the position of strain Ki8-1^T among related species based on 16S rRNA gene sequences. The tree was based on an alignment of 1255 bp of 16S rRNA gene sequences and was reconstructed by using the neighbour-joining method. Numbers at nodes are bootstrap percentages derived from 1000 replications (neighbour-joining method/maximum-likelihood method). Bar, 0.02 substitutions per nucleotide position.

form wavy filaments from 15 µm to several hundred micrometres long. Growth occurs at 20–50 °C, with optimum growth at 37–45 °C. The pH range for growth is 6.5–10.0, with optimum growth at pH 7.0–9.0. Growth occurs below 1 % NaCl (w/v), with optimum growth in basal medium without the addition of NaCl. Methane is produced from H₂ and formate, but not from acetate, pyruvate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol or trimethylamine. Acetate or yeast extract is required for growth. The G+C content of the genomic DNA of the type strain is 45–49.5 mol%.

The type strain, JF-1^T (=NBRC 100397^T), was isolated from sewage sludge.

Description of *Methanospirillum lacunae* sp. nov.

Methanospirillum lacunae (la.cu'nae. L. fem. n. *lacuna* pond; L. fem. gen. n. *lacunae* of a pond).

The following properties are additional to those given for the genus. Cells are 0.5–0.6 × 11–25 µm in size, and often from 8 to 26 µm in length. Growth occurs at 15–37 °C, with optimum growth at 30 °C. The pH range for growth is 6.0–9.5, with optimum growth at pH 7.5. Growth occurs below 1 % NaCl (w/v), with optimum growth in basal medium without the addition of NaCl. Methane is produced from H₂ and formate, but not from acetate, pyruvate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol or trimethylamine. Acetate or yeast extract is required for growth. The G+C content of the genomic DNA of the type strain is 45.3 mol%.

The type strain, Ki8-1^T (=NBRC 104920^T =JCM 16384^T =DSM 22751^T), was isolated from puddly soil in Kisarazu, Chiba, Japan.

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