Methanoculleus chikugoensis sp. nov., a novel methanogenic archaeon isolated from paddy field soil in Japan, and DNA–DNA hybridization among Methanoculleus species

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INTRODUCTION

Methane is one of the greenhouse gases, and paddy fields are reported to be an important source of atmospheric methane (Intergovernmental Panel on Climate Change, 1995). Methane is produced by methanogenic archaea, and microbiological studies of methanogens are important for understanding methane emissions from paddy fields. In order to understand the ecology of the methanogenic archaea in paddy field soil, the characterization and identification of methanogenic isolates from paddy field soil are necessary. Almost all methanogens isolated so far from paddy field soil belong to the families Methanobacteriaceae and Methanosarcinaceae (Rajagopal et al., 1988; Conrad et al., 1989; Asakawa et al., 1993, 1995; Chen et al., 1993; Fetzer et al., 1993; Min et al., 1997; Großkopf et al., 1998; Joulian et al., 1998, 2000; Takeda, 1998; Adachi, 1999; Kubota & Takeda, 1999). Kudo et al. (1997) demonstrated by analysis of 16S rDNA clones amplified using PCR from

The DDBJ accession number for the 16S rDNA sequence of strain MG62T is AB038795.
DNA extracted from soil that Methanogenium-like methanogens existed in Japanese paddy soils, and a strain of Methanoculleus marisnigri has been isolated recently from a rice field in France by Joulian et al. (1998). These results suggest that members of the family Methanomicrobiaceae also exist in paddy field soil.

In this work, we isolated and characterized a methanogenic archaeon from a Japanese paddy field soil after enrichment with 2-propanol/CO₂. We selected 2-propanol/CO₂ as a substrate and used a slightly saline medium (6 g NaCl l⁻¹) in order to enrich methanogens belonging to the family Methanomicrobiaceae, since many species within this family are known to utilize secondary alcohols and they usually prefer slightly saline conditions (0-1 M NaCl or more) (Widdel et al., 1988; Boone et al., 1993). Phenotypic, genotypic and phylogenetic data show that the strain should be assigned as a novel species within the genus Methanoculleus, for which we propose the name Methanoculleus chikugensis sp. nov. We also discuss genomic relationships among Methanoculleus species.

**METHODS**

**Source of strain MG62² and reference strains.** The sampling site was a paddy field plot (Gray Lowland Soil) with long-term application of rice straw compost at the Kyushu National Agricultural Experiment Station, Chikugo, Fukuoka, Japan (Asakawa et al., 1998). The sampling was performed on 29 August 1995 as described previously (Asakawa et al., 1993).

*Methanoculleus marisnigri* JR1⁻ (DSM 14988), *Methanoculleus thermophilus* CR-1⁻ (DSM 23733), *Methanoculleus palmei INSULZ⁻ (DSM 42733), *Methanoculleus oldenburgensis* CB1⁻ (DSM 62163), *Methanoculleus bourgensis* MS⁻ (DSM 30455) and *Methanoculleus oldenburgensis* R/C/E/R⁻ (DSM 27272) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

**Media and growth conditions.** The basal medium used for enrichment and isolation was a modification of LPBM (Ziebuhr, 1977) and contained (l⁻¹ distilled water): 0.75 g KH₂PO₄, 0.75 g KH₂PO₄, 1.0 g NH₄Cl, 0.36 g MgCl₂, 6H₂O, 6.0 g NaCl, 1.0 g sodium acetate, 2.0 g yeast extract (Difco), 2.0 g Polypepton (Nihon Pharmaceutical), 10 ml 2-propanol, 9 ml trace mineral solution (Mori et al., 1983), 10 ml vitamin solution (Balch et al., 1979), 0.5 ml 0.2% (w/v) resazurin, 0.5 g L-cysteine hydrochloride hydrate, 0.5 g Na₂S·9H₂O and 4.8 g NaHCO₃. For roll tubes, 1.7% (w/v) agar was added to the basal medium. The gas phase was N₂ (4:1, 203 kPa) when the substrates 2-propanol, sodium formate, 2-butanol and cyclopentanol were used. These substrates, cysteine and Na₂S were added from anoxic, sterile stock solutions. The media were prepared anoxically, basically according to Hungate technique (Hungate, 1969; Balch et al., 1979; Koga et al., 1987). Cultures were grown in 120 ml serum bottles containing 10 ml medium or in 18 mm (outside diameter) by 180 mm test tubes with 5 ml medium.

Cells for lipid and DNA analyses were grown in 11 serum bottles containing 100 ml medium at 27 °C for strain MG62², 37 °C for *Methanoculleus bourgensis*, *Methanoculleus marisnigri*, *Methanoculleus oldenburgensis* and 55 °C for *Methanoculleus thermophilus*.

The specific growth rate was calculated by monitoring the OD₅₆₀. Media with different pH values were prepared by varying the ratio of the H₂/CO₂ mixture and the concentration of NaHCO₃ or Na₂CO₃. The H₂/CO₂ mixture was repeatedly repurified to 203 kPa with the original ratio.

Methane production was determined by GC as described previously (Asakawa et al., 1995).

**Enrichment and isolation.** Enrichment was performed in 11 serum bottles sealed with butyl rubber stops and aluminium seals containing 100 ml medium with 2-propanol/CO₂ as growth substrate. A moist soil sample (about 20 g) was inoculated into the medium and the bottle was incubated with shaking (120 r.p.m.) at 30 °C. About 80% of the culture was replaced fortnightly by fresh medium. After seven successive transfers, roll tubes were prepared. Many colonies formed within a few weeks, but they did not produce methane after inoculating into liquid medium. Some of the colonies that formed after 1 month showed fluorescence when observed with an epifluorescence microscope. Therefore, these colonies were inoculated into liquid medium and methane production was examined. Isolation of colonies from roll tubes was repeated and well-isolated colonies were picked and inoculated into the liquid medium. To test for purity, a culture was inoculated into the basal medium without NaHCO₃ but containing 1% (w/v) glucose and VL medium as described previously (Asakawa et al., 1993). The gas phase was N₂. The preparations were incubated statically for 43 d at 30 °C. No growth was obtained and no contaminants were detected microscopically. The pure culture was designated strain MG62².

**Microscopy.** Phase-contrast and epifluorescence microscopy were carried out by using a Nikon Optiphot microscope and an Olympus VANOX microscope, respectively. Gram staining was carried out by the Hucker method. Cells for electron microscopy were collected from a late exponential phase culture and purified according to Sowers (1995). The preparations were used for cultivation of the isolate and reference strains was DSMZ (1993) supplemented with Polypepton (Nihon Pharmaceutical) instead of Trypticase. The liquid medium and methane production was examined.

**Lipid analysis.** Cells were harvested at late exponential phase and stored at −20 °C until use. Extraction of total lipids and analysis of lipid component parts were conducted as described previously (Koga et al., 1993, 1998). The nomenclature for archaeal ether lipids proposed by Nishihara et al. (1987) is used in this paper.

**G+C content of DNA and DNA–DNA hybridization.** Cells of methanogenic strains harvested at late exponential phase were used for DNA isolation. DNA was isolated and purified according to the method of Sowers (1995). The G+C content of the DNA was determined by HPLC (Tamaoka & Komagata, 1984) with the equipment described previously (Asakawa et al., 1995).

DNA–DNA hybridization was performed according to the microtitration plate method (Ezaki et al., 1989; Sawada et al., 1995). Aliquots of 100 μl denatured DNA solution (3 μg ml⁻¹) in PBS-Mg buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.1 M MgCl₂) were added to each well and the plate was kept at 30 °C overnight for immobilization of DNA. The solution was then dis-
RESULTS

Morphology

Cells of strain MG62T were irregularly coccolid and 1–2 μm in diameter (Fig. 1). The cells stained Gram-negative. Motility was not observed, but the cells were flagellated (Fig. 1b). SDS and hypotonic conditions lysed cells when the method described by Boone & Whitman (1988) was used. This indicates that strain MG62T has a proteinaceous cell wall.

Surface colonies on agar medium in roll tubes were 0·3–1·0 mm in diameter, round or ellipsoidal, entire, convex, smooth and yellow to ochre-yellow.

Physiology

Strain MG62T grew on 2-propanol (134 mM)/CO₂ and was also able to utilize H₂/CO₂, formate (40 mM), 2-butanol (109 mM)/CO₂, and cyclopentanol (110 mM)/CO₂ but not acetate (80 mM), methanol (250 mM), ethanol (110 mM) or trimethylamine (40 mM) as substrates for growth and methanogenesis. Growth on cyclopentanol/CO₂ was very slow. Yeast extract or Polypepton (Nihon Pharmaceutical), which is equivalent to Trypticase Peptone (BBL) and Casitone (Difco), and acetate were required for growth and methane production when growing on H₂/CO₂.

The generation time was 46 h (specific growth rate 0·015 h⁻¹) in 100 ml DSM 141 medium in a 1 l bottle with H₂/CO₂ (4:1, 203 kPa) at pH 7 and 27 °C. Fig. 2 shows the effect of temperature on growth and methane production. Strain MG62T grew at 15–40 °C. Growth was fastest at 25–30 °C. Growth occurred between pH 6·7 and 8·0 and was fastest at pH 6·7–7·2 (data not shown). Strain MG62T grew well in the presence of 0–0·3 M NaCl and optimum growth was observed at 0·1 M (data not shown).
Strain MG62 \textsuperscript{T} had values of less than 50\% with type strains of 67–104\% relatedness to each other.

**Lipids**

Strain MG62\textsuperscript{T} contained archaeol and caldarchaeol as core lipids, galactose as glycolipid sugar and aminopentanetetrol and glycerol as phospholipid polar head groups. Glucose was not detected. Aminopentanetetrol was methylated.

**G + C content and DNA–DNA hybridization**

The G + C content of DNA from strain MG62\textsuperscript{T} was 62.2 ± 1.1\% (mean ± standard deviation, n = 3).

Strain MG62\textsuperscript{T} exhibited DNA–DNA hybridization values of less than 50\% with type strains of *Methanoculleus* species (Table 1). *Methanoculleus olentangyi* RC/ER\textsuperscript{T}, *Methanoculleus bourgensis* MS2\textsuperscript{T} and *Methanoculleus oldenburgensis* CB1\textsuperscript{T} had values of 67–104\% relatedness to each other.

**16S rDNA sequence analysis**

The 16S rDNA sequence of strain MG62\textsuperscript{T} (1430 bp) was compared with those available in DNA databases. The highest 16S rDNA similarity, of 98\%, was obtained between strain MG62\textsuperscript{T} and *Methanoculleus marisnigri* strains JR1\textsuperscript{T} and CoCam (Joulian et al., 1998), followed by 97.4\% to *Methanoculleus palmoi* INSULUZ\textsuperscript{T}. Strain MG62\textsuperscript{T} showed similarities of 94.5–96.3\% to the other species of the genus *Methanoculleus* and at most 91.9\% to genera within the families *Methanomicrobiaceae*, *Methanoplanaceae* and *Methanocorpusculaceae*.

**DISCUSSION**

The morphological and physiological features of strain MG62\textsuperscript{T}, such as cell form, flagellation, proteinaceous cell wall, substrate utilization and growth requirements, are similar to those found in members of the genera *Methanogenium*, *Methanoculleus*, *Methanofollis* and *Methanocorpusculum* (Romesser et al., 1979; Zellner et al., 1987, 1989, 1999; Maestrojua et al., 1990). The lipid component composition of strain MG62\textsuperscript{T} exhibited the characteristic features, i.e. caldarchaeol as a core lipid, galactose as a glycolipid sugar, aminopentanetetrol and glycerol as phospholipid polar head groups, of the order *Methanomicrobiales* (Koga et al., 1998), which has been proposed to be separated from ‘*Methanosarcinales*’ by Boone et al. (1993). This suggests strongly that strain MG62\textsuperscript{T} belongs to the order *Methanomicrobiales*. The G + C content of DNA of the genera *Methanogenium* and *Methanocorpusculum* is 47–52 mol\%, while that of the genera *Methanoculleus* and *Methanofollis* ranges from 49 to 62 mol\% (Romesser et al., 1979; Rivard & Smith, 1982; Corder et al., 1983; Ferguson & Mah, 1983; Harris et al., 1984; Zabel et al., 1984, 1985; Olivier et al., 1985, 1986; Zellner et al., 1987, 1989, 1990, 1998, 1999; Widdel et al., 1988; Xun et al., 1989; Zhao et al., 1989; Maestrojua et al., 1990; Blotevogel et al., 1991). The DNA G + C content of strain MG62\textsuperscript{T} was 62 mol\%, which indicates that the strain belongs to the genus *Methanoculleus* or *Methanofollis*. 16S rDNA sequence analysis revealed that strain MG62\textsuperscript{T} should be assigned to the genus *Methanoculleus* and that it was most closely related to *Methanoculleus marisnigri*, although the similarity was no more than 98.3\%. The results of DNA–DNA hybridization

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**Table 1.** DNA–DNA hybridization between *Methanoculleus chikugoensis* MG62\textsuperscript{T} and type strains of *Methanoculleus* species

<table>
<thead>
<tr>
<th>Strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Methanoculleus chikugoensis</em> MG62\textsuperscript{T} (= JCM 10825\textsuperscript{T} = DSM 13459\textsuperscript{T})</td>
<td>100</td>
<td>39</td>
<td>16</td>
<td>40</td>
<td>30</td>
<td>21</td>
<td>42</td>
</tr>
<tr>
<td>2. <em>Methanoculleus marisnigri</em> JR1\textsuperscript{T} (= DSM 1498\textsuperscript{T})</td>
<td>47</td>
<td>100</td>
<td>12</td>
<td>26</td>
<td>34</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td>3. <em>Methanoculleus thermophilus</em> CR-1\textsuperscript{T} (= DSM 23733\textsuperscript{T})</td>
<td>16</td>
<td>22</td>
<td>100</td>
<td>21</td>
<td>35</td>
<td>20</td>
<td>31</td>
</tr>
<tr>
<td>4. <em>Methanoculleus palmoi</em> INSULUZ\textsuperscript{T} (= DSM 4273\textsuperscript{T})</td>
<td>18</td>
<td>15</td>
<td>7</td>
<td>100</td>
<td>18</td>
<td>19</td>
<td>30</td>
</tr>
<tr>
<td>5. <em>Methanoculleus oldenburgensis</em> CB1\textsuperscript{T} (= DSM 6216\textsuperscript{T})</td>
<td>25</td>
<td>16</td>
<td>6</td>
<td>25</td>
<td>100</td>
<td>77</td>
<td>104</td>
</tr>
<tr>
<td>6. <em>Methanoculleus bourgensis</em> MS2\textsuperscript{T} (= DSM 3045\textsuperscript{T})</td>
<td>14</td>
<td>20</td>
<td>7</td>
<td>15</td>
<td>67</td>
<td>100</td>
<td>84</td>
</tr>
<tr>
<td>7. <em>Methanoculleus olentangyi</em> RC/ER\textsuperscript{T} (= DSM 2772\textsuperscript{T})</td>
<td>19</td>
<td>23</td>
<td>15</td>
<td>19</td>
<td>76</td>
<td>80</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 2. Phenotypic characters of type strains of Methanoculleus species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>1–2</td>
<td>1–3</td>
<td>1.0–1.3</td>
<td>1.25–2.0</td>
<td>1.0</td>
<td>1–2</td>
<td>1.0–1.5</td>
</tr>
<tr>
<td>Flagellation</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>6.7–7.2</td>
<td>8.0</td>
<td>7.0</td>
<td>6.9–7.5</td>
<td>8.0</td>
<td>6.7</td>
<td>ND</td>
</tr>
<tr>
<td>NaCl (M)</td>
<td>0–1</td>
<td>0–1</td>
<td>0–25</td>
<td>0–25</td>
<td>ND</td>
<td>0–4–0.17</td>
<td>0–17</td>
</tr>
<tr>
<td>G+C content (mol %)†</td>
<td>62.2 (Lc)</td>
<td>61.2 (Bd)</td>
<td>59 (Bd)</td>
<td>59.5 (Lc), 59 (Tm)</td>
<td>48.6 (Tm)</td>
<td>59 (Bd)</td>
<td>54.4 (Bd)</td>
</tr>
</tbody>
</table>

* H, H2/CO2; f, formate; p, 2-propanol/CO2; b, 2-butanol/CO2.
† Determined by: Lc, HPLC; Bd, buoyant density; Tm, thermal denaturation.

experiments not only supported this assignment but also revealed that the genomic relatedness between strain MG62T and the type strains of each of the Methanoculleus species was too low to identify strain MG62T as belonging to one of the existing species of the genus Methanoculleus (Table 1). In addition, strain MG62T exhibited distinctive features, i.e. optimum temperature and lipid component parts. The optimum temperature (25–30 °C) for growth of strain MG62T is the lowest among the Methanoculleus species (Table 2). Methanoculleus marisnigrig JR1T was originally reported with a temperature optimum of 20–25 °C (Romesser et al., 1979); however, this has been emended to 40 °C by Maestrojua et al. (1990). Strain MG62T did not contain glucose as a glycolipid sugar, which has been detected in all strains of the order Methanomicrobiales studied so far (Koga et al., 1998). On the basis of the phenotypic, genotypic and phylogenetic characteristics described above, we propose the name Methanoculleus chikugoensis sp. nov. for strain MG62T.

DNA–DNA hybridization also revealed that Methanoculleus olentangyi RC/ER2T, Methanoculleus burgensis MS2T and Methanoculleus oldenburgensis CB1T were genomically closely related (Table 1). Xun et al. (1989) also reported a DNA hybridization value of 66% between Methanoculleus olentangyi RC/ER2T and Methanoculleus burgensis MS2T. The 16S rRNA sequence similarity between Methanoculleus olentangyi RC/ER2T and Methanoculleus burgensis MS2T was 97.7% (the 16S rRNA sequence of Methanoculleus oldenburgensis CB1T is not available), which also supports their close relationship. There seem to be few distinguishing phenotypic features of these three species (Table 2), with the single exception that the G+C content of Methanoculleus oldenburgensis CB1T is 48.6 mol %. These results indicate that not only Methanoculleus olentangyi and Methanoculleus burgensis, as reported already by Xun et al. (1989) and Boone et al. (1993), but also Methanoculleus oldenburgensis CB1T are subjective synonyms. Since Methanoculleus olentangyi is the senior synonym, Methanoculleus olentangyi must be the name of the united species. However, as Methanoculleus burgensis is the type species of the genus Methanoculleus, the genus would be lost. Therefore, we postpone the proposal of this synonymy until the genus Methanoculleus is conserved by an Opinion of the Judicial Commission. We will submit a Request for an Opinion on this matter later.

Description of Methanoculleus chikugoensis sp. nov.

Methanoculleus chikugoensis (chi.ku.go.en’nis. N. L. adj. chikugoensis of Chikugo, the city in Fukuoka, Japan, from where the strain was isolated).

Cells are irregularly coccoid, 1.0–2.0 µm in diameter, with a proteinaceous cell wall, and flagellated. Strictly anaerobic. Cells grow on H2/CO2, formate, 2-propanol/CO2, 2-butanol/CO2 and cyclopentanol/CO2 as substrates for methanogenesis. Acetate, ethanol, methanol and methylamines are not utilized. The optimum temperature and pH for growth are 25–30 °C and 6.7–7.2. Cells contain archaeol and caldarchaeol as core lipids, aminopentanetetrol and glycerol as phospholipid polar head groups and galactose as glycolipid sugar, but glucose is absent. The type strain is MG62T (= JCM 10825T = DSM 13459T), which was isolated from a paddy field soil in Chikugo, Fukuoka, Japan.
ACKNOWLEDGEMENTS

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REFERENCES


methanogenic strain (A2) of *Methanobrevibacter arborophilus*. 


