**Methanobacterium oryzae** sp. nov., a novel methanogenic rod isolated from a Philippines ricefield

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A rod (0.3–0.4 μm × 3–10 μm) to filamentous (up to 40 μm) non-motile methanogenic bacterium, designated strain FPiT (T = type strain), was isolated from ricefield soil in the Philippines. The strain uses H2+CO2 or formate for growth and produces CH4. Optimum growth temperature is 40 °C; no growth is observed at 15 °C or 45 °C. Optimum pH for growth is 7; no growth is observed at pH 5.5 or 9.0. Strain FPiT is halotolerant and grows at NaCl concentrations of 0–25 g l⁻¹. The G+C content of its DNA is 31 mol%. Based on 16S rRNA gene sequence analysis, the isolate was identified as a new species of the genus *Methanobacterium: Methanobacterium oryzae* sp. nov. The type strain is FPiT (T = DSM 11106T).

**Keywords:** Archaea, methanogens, *Methanobacterium oryzae*, phylogeny, ricefield soil

Methane-producing bacteria are strict anaerobes belonging to the domain *Archaea*. They are commonly isolated from natural anoxic environments, including freshwater and marine sediments, wet and waterlogged soils, the rumen and the gut of insects (Boone et al., 1993; Garcia, 1990; Mah & Smith, 1981). They play an important role in these environments by performing the last step of anaerobic decomposition of organic matter, which is mineralized to CH4 and CO2.

Waterlogged ricefields, because of anoxic conditions developing after flooding, are major anthropogenic sources of CH4 (Minami et al., 1994), one of the major greenhouse gases (Leleuvel et al., 1993). In ricefields, H2 and acetate are the main energy sources used by methanogens (Conrad et al., 1989; Schütz et al., 1989; Takai, 1970). These substrates are produced as a result of fermentative metabolism or the activity of syntrophic associations degrading reduced compounds such as butyrate and propionate (Dong & Stams, 1995; Schink, 1992; Stams, 1994).

Twenty-six genera of methane-producing bacteria have currently been described (Boone et al., 1993), but only strains of *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, *Methanoseta* and *Methanosarcina* have so far been isolated and cultivated from ricefield soils (Asakawa et al., 1993, 1995; Fetzer et al., 1993; Grosskopf et al., 1998; Joulain et al., 1998; Raimbault, 1981; Rajagopal et al., 1988). Joulain et al. (1996) reported on the presence of *Methanospirillum* in a French ricefield. By using a phylogenetic approach based on DNA extracted from soil, Kudo et al. (1997) provided evidence for the presence of *Methanosarcina*, *Methanogenium*, *Methanoseta* and *Methanobacterium* in Japanese ricefields. More recently, Grosskopf et al. (1998) provided evidence for the presence of *Methanosarcina*, *Methanoseta* and *Methanobacterium* in Italian rice soils on the basis of both molecular and cultivation studies. Currently, only a limited number of strains isolated from ricefields have been identified at the species level. They include *Methanobrevibacter arborphilicus* (Asakawa et al., 1993) and *Methanosarcina mazei* (Asakawa et al., 1995) isolated from Japanese ricefields. Based on phylogenetic and phenotypic characteristics, the isolation of five species of methanogens, *Methanobacterium bryantii*, *Methanobacterium formicicum*, *Methanosarcina barkeri*, *Methanosarcina mazei* and *Methanoculleus marisnigri* from 13 ricefield soils has been reported (Joulain et al., 1998). Characterization of a new species of a rod-shaped methanogen, designated strain FPiT is reported here. Strain FPiT (=...
Strain FPiT was isolated from a ricefield soil in the Philippines (Pila area, Luzon Province). Enrichments were performed in a medium containing formate as carbon and energy source (Joulian et al., 1998). Pure cultures were obtained by using Hungate anaerobic techniques and repeated application of the agar shake dilution method (Hungate, 1969; Macy et al., 1972). Methods for growing strains and determining temperature, pH and salinity ranges for growth, and substrate utilization by strain FPiT were described by Joulian et al. (1998). Methanobacterium bryantii DSM 863T was grown on H2 + CO2. Methanobacterium palustre DSM 3108T, Methanobacterium formicicum DSM 1535T and strain FPiT were grown on formate. The procedures used for DNA extraction, purification, 16S rRNA gene amplification, RFLP analysis and sequencing were also described by Joulian et al. (1998).

The 16S rRNA gene sequences obtained for strain FPiT and Methanobacterium palustre (1445 and 1400 nt, respectively) were manually aligned by using the sequence editor ae2 (Maidak et al., 1996) with the sequences of representative methanogens extracted from the GenBank and RDP databases (version 6.0). Positions of sequences and/or alignment ambiguity were omitted from the analysis, and pairwise evolutionary distances of 1335 nt were computed by the evolutionary distances of 1335 nt were computed by the method of Jukes & Cantor (1969). A dendrogram was obtained from the distance matrix by the neighbour-joining method (Felsenstein, 1993). All programs used form part of the PHYLIP package (Felsenstein, 1993). The DNA G+C content was determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) by using HPLC as described by Mesbah et al. (1989).

Several circular colonies 1–2 mm in diameter developed in solid media after 2–3 weeks incubation at 37 °C. Microscopic examination revealed the presence of non-motile rods (0.3–0.4 µm × 3–10 µm) occurring singly, in chains, and as aggregates in old cultures. Filamentous cells (up to 40 µm) were also frequently observed (Fig. 1). Strain FPiT showed the typical fluorescence of methanogens under UV light (420 nm) and phenotypic characteristics similar to that of the genus Methanobacterium. Strain FPiT grew at temperatures of 20–42 °C (but not at 15 °C or 45 °C), with an optimum around 40 °C. Growth occurred at pH 6.0–8.5 (but not at pH 5.5 or 9.0), with an optimum around 7.0. The isolate was halotolerant and grew at NaCl concentrations of 0–25 g l−1 with an optimum between 0 and 5 g l−1. Strain FPiT grew only on H2 + CO2 or formate as the sole carbon and energy source. It grew on both substrates in the absence of yeast extract, but growth was faster when 1 g l−1 yeast extract was added to the medium. Growth and methane production on secondary alcohols isobutanol and 2-propanol were negative. The mean DNA G+C composition of strain FPiT was 31 mol %.

Fig. 1. Phase-contrast micrograph of strain FPiT showing long chains of cells; bar, 10 µm.

The genus Methanobacterium currently comprises physiologically diverse species of which four are validated mesophilic and neutrophilic species, namely Methanobacterium bryantii, Methanobacterium uliginosum, Methanobacterium formicicum and Methanobacterium palustre. The most closely phenotypic relatives of strain FPiT are Methanobacterium palustre and Methanobacterium formicicum since both form filaments and use H2 + CO2 and formate as carbon and energy sources (Table 1). However, phylogenetic analysis indicated that strain FPiT was more related to Methanobacterium bryantii (similarity of 96.5%) than to Methanobacterium formicicum (similarity of 95.0%) or Methanobacterium palustre (similarity of 95.1%) (Fig. 2). It has been proposed that members of the same genus whose 16S rRNA sequence similarity is less than 97% should be regarded as separate species (Stackebrandt & Goebel, 1994). Based on this criterion alone, strain FPiT should be given species status. Interestingly, a recent study on the phylogenetic diversity of methanogens in ricefield soils (Grosskopf et al., 1998) reported the presence of a Methanobacterium species closely related to strain FPiT, indicating that this organism may be common in ricefields.

The 16S rRNA sequence of Methanobacterium uliginosum is not available for analysis. However, restriction endonuclease digestion (using four restriction enzymes, BamHI, CfoI, Sau3A and TaqI) of the partially amplified 16S rRNA revealed a matching RFLP profile for Methanobacterium uliginosum and Methanobacterium bryantii that substantially differed from that of strain FPiT (data not shown). From this,
Table 1. Major characteristics of strain FPI<sup>T</sup> and mesophilic and neutrophilic species of the genus *Methanobacterium*

<table>
<thead>
<tr>
<th>Species</th>
<th>1</th>
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<tr>
<td>Culture collection number</td>
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<td>DSM 1535&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSM 3108&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSM 863&lt;sup&gt;T&lt;/sup&gt;</td>
<td>DSM 2956&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>Source</td>
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<td>Domestic sludge</td>
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<td>Temperature range (°C)</td>
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<td>20–45</td>
<td>ND</td>
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<td>Optimum temperature (°C)</td>
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<td>37–39</td>
<td>40</td>
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<tr>
<td>pH range</td>
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<td>6–9–7.2</td>
<td>ND</td>
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<td>NaCl concn range (g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
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<td>&lt;30</td>
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<td>ND</td>
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<tr>
<td>Optimum NaCl concn (g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>G + C content (mol %)*</td>
<td>31 (Lc)</td>
<td>41–42 (Bd)</td>
<td>34 (T&lt;sub&gt;m&lt;/sub&gt;)</td>
<td>33–38 (Bd)</td>
<td>29 (T&lt;sub&gt;m&lt;/sub&gt;)</td>
</tr>
<tr>
<td>Substrates used†</td>
<td>H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;, formate</td>
<td>H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;, formate, iP, iB</td>
<td>H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;, iB, iP, iB</td>
<td>H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;, iB, iP, iB</td>
<td>H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;, iB, iP, iB</td>
</tr>
</tbody>
</table>

* Determined by: Lc, HPLC analysis; Bd, buoyant density method; or T<sub>m</sub>, melting point analysis.
† iP, 2-propanol; iB, isobutanol.

**Fig. 2.** Dendrogram showing the position of strain FPI<sup>T</sup> amongst members of the order *Methanobacteriales*. Bar, 10 nucleotide changes per 100 nucleotides.

it can be inferred that strain FPI<sup>T</sup> is distinct from *Methanobacterium uliginosum*, especially since strain FPI<sup>T</sup> and *Methanobacterium bryantii* are phylogenetically distinct, as reported above. This inference is also supported by the phenotypic differences observed between strain FPI<sup>T</sup> (grows on formate) and *Methanobacterium uliginosum* (does not grow on formate) (Table 1).

Our results are in agreement with previous phenotypic and DNA reassociation studies showing that *Methanobacterium palustre* is a new species of the genus *Methanobacterium* (Zellner et al., 1989). Our studies clearly establish that strain FPI<sup>T</sup> should be regarded as a new species of the genus *Methanobacterium*, *Methanobacterium oryzae* sp. nov.

**Description of Methanobacterium oryzae** sp. nov.

*Methanobacterium oryzae* (o.ry’zae. M.L. fem. n. oryza generic name of rice; M.L. gen. n. oryzae of rice).

Round colonies, 1–2 mm in diameter develop after 2–3 weeks of incubation. Cells are 0.3–0.4 µm in width and 3–10 µm in length, non-motile and rod-shaped, occurring singly or in chains (up to 40 µm in length), depending on their growth phase. Methanogen (domain *Archaea*). Optimum temperature for growth is 40 °C with no growth occurring at 15 °C and 45 °C. Optimum pH for growth is 7.0 with no growth occurring at pH 5.5 and 9.0. Cells are halotolerant and growth occurs in medium containing 0–25 g l<sup>−1</sup> NaCl. Growth substrates include H<sub>2</sub> + CO<sub>2</sub> and formate. No growth on 2-propanol or isobutanol. Yeast extract is not required for growth but its presence stimulates growth. The mean DNA G + C content is 31 mol % (as determined by HPLC). Isolated from a ricefield soil. The type strain is FPI<sup>T</sup> (= DSM 11106<sup>T</sup>).

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References


