

NOTE

***Methanobacterium oryzae* sp. nov., a novel methanogenic rod isolated from a Philippines ricefield**Catherine Joulian,¹ Bharat K. C. Patel,² Bernard Ollivier,¹ Jean-Louis Garcia¹ and Pierre A. Roger¹

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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 FPi^T (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 FPi^T 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 FPi^T (= DSM 11106^T).

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 (Lelieveld *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*, *Methanobrevi-*

bacter, *Methanoculleus*, *Methanosaeta* 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; Joulian *et al.*, 1998; Raimbault, 1981; Rajagopal *et al.*, 1988). Joulian *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*, *Methanosaeta* and *Methanobacterium* in Japanese ricefields. More recently, Grosskopf *et al.* (1998) provided evidence for the presence of *Methanosarcina*, *Methanosaeta* 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 arboriphilicus* (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 (Joulian *et al.*, 1998). Characterization of a new species of a rod-shaped methanogen, designated strain FPi^T is reported here. Strain FPi^T (=

The GenBank accession numbers for the 16S rDNA sequences of strain FPi^T and *Methanobacterium palustre* are AF028690 and AF093061, respectively.

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DSM 11106^T) uses H₂+CO₂ or formate, is phylogenetically distinct from all other members of the genus *Methanobacterium*, and is proposed as a new species, *Methanobacterium oryzae* sp. nov.

Strain FPi^T 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 FPi^T were described by Joulian *et al.* (1998). *Methanobacterium bryantii* DSM 863^T was grown on H₂+CO₂. *Methanobacterium palustre* DSM 3108^T, *Methanobacterium formicicum* DSM 1535^T and strain FPi^T 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 FPi^T 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 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 FPi^T showed the typical fluorescence of methanogens under UV light (420 nm) and phenotypic characteristics similar to that of the genus *Methanobacterium*. Strain FPi^T 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 FPi^T 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

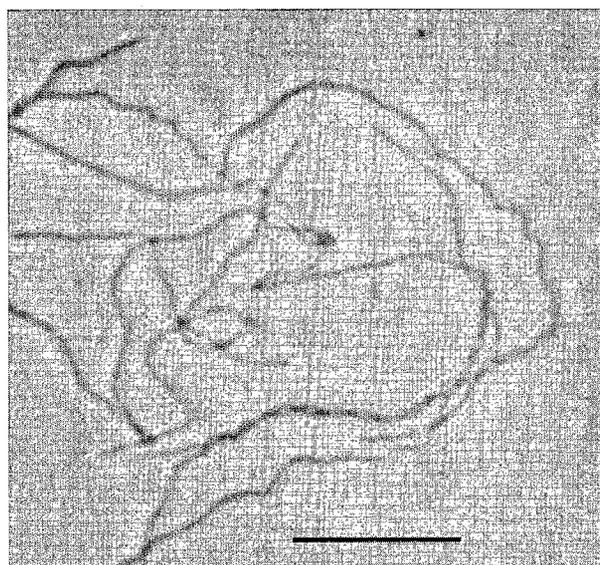


Fig. 1. Phase-contrast micrograph of strain FPi^T showing long chains of cells; bar, 10 µm.

and 2-propanol were negative. The mean DNA G+C composition of strain FPi^T was 31 mol %.

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 FPi^T 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 FPi^T 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 FPi^T 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 FPi^T, 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, *Bam*HI, *Cfo*I, *Sau*3A and *Taq*I) of the partially amplified 16S rRNA revealed a matching RFLP profile for *Methanobacterium uliginosum* and *Methanobacterium bryantii* that substantially differed from that of strain FPi^T (data not shown). From this,

Table 1. Major characteristics of strain FPI^T and mesophilic and neutrophilic species of the genus *Methanobacterium*

Strains: 1, FPI^T; 2, *Methanobacterium formicicum*; 3, *Methanobacterium palustre*; 4, *Methanobacterium bryantii*; 5, *Methanobacterium uliginosum*. Data from Bryant *et al.* (1967), Bryant & Boone (1987), König (1984), Zellner & Winter (1987) and Zellner *et al.* (1989).

| Species | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|--|
| Culture collection number | DSM 11106 ^T | DSM 1535 ^T (= OCM 55 ^T) | DSM 3108 ^T (= OCM 238 ^T) | DSM 863 ^T (= OCM 110 ^T) | DSM 2956 ^T (= OCM 176 ^T) |
| Source | Ricefield | Domestic sludge | Peat bog | Sewage sludge | Marshy soil |
| Temperature range (°C) | 20–42 | ND | 20–45 | ND | 15–45 |
| Optimum temperature (°C) | 40 | 37–45 | 33–37 | 37–39 | 40 |
| pH range | 6.0–8.5 | ND | ND | ND | 6.0–8.5 |
| Optimum pH | 7.0 | 6.6–7.8 | 7.0 | 6.9–7.2 | ND |
| NaCl concn range (g l ⁻¹) | 0–25 | ND | <30 | ND | ND |
| Optimum NaCl concn (g l ⁻¹) | 5 | ND | ND | ND | ND |
| G+C content (mol%)* | 31 (Lc) | 41–42 (Bd) | 34 (T _m) | 33–38 (Bd) | 29 (T _m) |
| Substrates used† | H ₂ + CO ₂ , formate | H ₂ + CO ₂ , formate, iP, iB | H ₂ + CO ₂ , formate, iP, iB | H ₂ + CO ₂ , iP, iB | H ₂ + CO ₂ |

* Determined by: Lc, HPLC analysis; Bd, buoyant density method; or T_m, melting point analysis.

† iP, 2-propanol; iB, isobutanol.

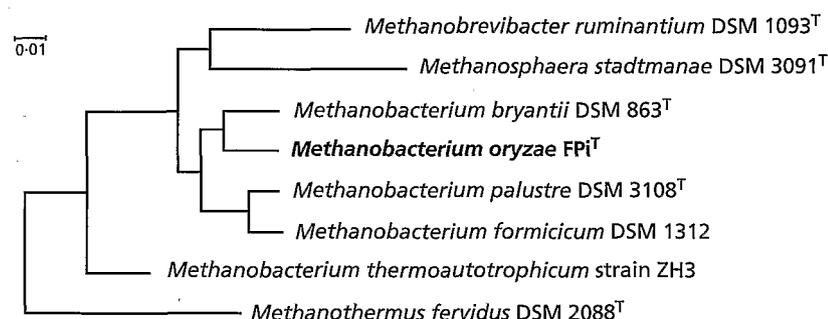


Fig. 2. Dendrogram showing the position of strain FPI^T amongst members of the order *Methanobacteriales*. Bar, 10 nucleotide changes per 100 nucleotides.

it can be inferred that strain FPI^T is distinct from *Methanobacterium uliginosum*, especially since strain FPI^T and *Methanobacterium bryantii* are phylogenetically distinct, as reported above. This inference is also supported by the phenotypic differences observed between strain FPI^T (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^T 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⁻¹ NaCl. Growth substrates include H₂ + CO₂ 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^T (= DSM 11106^T).

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