

## NOTE

***Methanobacterium oryzae* sp. nov., a novel methanogenic rod isolated from a Philippines ricefield**Catherine Joulian,<sup>1</sup> Bharat K. C. Patel,<sup>2</sup> Bernard Ollivier,<sup>1</sup> Jean-Louis Garcia<sup>1</sup> and Pierre A. Roger<sup>1</sup>

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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<sup>T</sup> (T = type strain), was isolated from ricefield soil in the Philippines. The strain uses H<sub>2</sub>+CO<sub>2</sub> or formate for growth and produces CH<sub>4</sub>. 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<sup>T</sup> is halotolerant and grows at NaCl concentrations of 0–25 g l<sup>-1</sup>. 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<sup>T</sup> (= DSM 11106<sup>T</sup>).**

**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 CH<sub>4</sub> and CO<sub>2</sub>.

Waterlogged ricefields, because of anoxic conditions developing after flooding, are major anthropogenic sources of CH<sub>4</sub> (Minami *et al.*, 1994), one of the major greenhouse gases (Lelieveld *et al.*, 1993). In ricefields, H<sub>2</sub> 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<sup>T</sup> is reported here. Strain FPi<sup>T</sup> (=

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

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DSM 11106<sup>T</sup>) uses H<sub>2</sub>+CO<sub>2</sub> 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<sup>T</sup> 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 (Jouliau *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<sup>T</sup> were described by Jouliau *et al.* (1998). *Methanobacterium bryantii* DSM 863<sup>T</sup> was grown on H<sub>2</sub>+CO<sub>2</sub>. *Methanobacterium palustre* DSM 3108<sup>T</sup>, *Methanobacterium formicicum* DSM 1535<sup>T</sup> and strain FPi<sup>T</sup> were grown on formate. The procedures used for DNA extraction, purification, 16S rRNA gene amplification, RFLP analysis and sequencing were also described by Jouliau *et al.* (1998). The 16S rRNA gene sequences obtained for strain FPi<sup>T</sup> 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<sup>T</sup> showed the typical fluorescence of methanogens under UV light (420 nm) and phenotypic characteristics similar to that of the genus *Methanobacterium*. Strain FPi<sup>T</sup> 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<sup>-1</sup> with an optimum between 0 and 5 g l<sup>-1</sup>. Strain FPi<sup>T</sup> grew only on H<sub>2</sub>+CO<sub>2</sub> 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<sup>-1</sup> yeast extract was added to the medium. Growth and methane production on secondary alcohols isobutanol

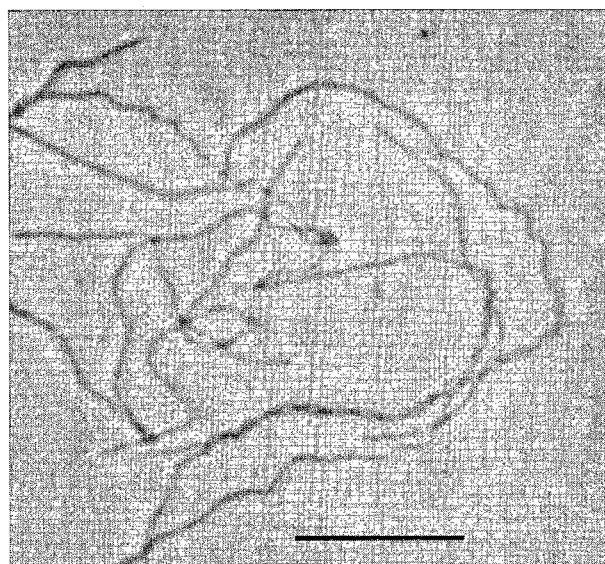


Fig. 1. Phase-contrast micrograph of strain FPi<sup>T</sup> showing long chains of cells; bar, 10 µm.

and 2-propanol were negative. The mean DNA G+C composition of strain FPi<sup>T</sup> 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<sup>T</sup> are *Methanobacterium palustre* and *Methanobacterium formicicum* since both form filaments and use H<sub>2</sub>+CO<sub>2</sub> and formate as carbon and energy sources (Table 1). However, phylogenetic analysis indicated that strain FPi<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup>, 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<sup>T</sup> (data not shown). From this,

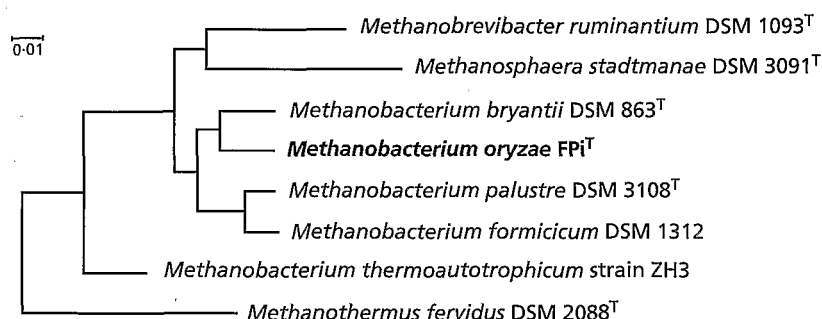
**Table 1.** Major characteristics of strain FPI<sup>T</sup> and mesophilic and neutrophilic species of the genus *Methanobacterium*

Strains: 1, FPI<sup>T</sup>; 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 <sup>T</sup>	DSM 1535 <sup>T</sup> (= OCM 55 <sup>T</sup> )	DSM 3108 <sup>T</sup> (= OCM 238 <sup>T</sup> )	DSM 863 <sup>T</sup> (= OCM 110 <sup>T</sup> )	DSM 2956 <sup>T</sup> (= OCM 176 <sup>T</sup> )
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 <sup>-1</sup> )	0–25	ND	<30	ND	ND
Optimum NaCl concn (g l <sup>-1</sup> )	5	ND	ND	ND	ND
G+C content (mol%)*	31 (Lc)	41–42 (Bd)	34 (T <sub>m</sub> )	33–38 (Bd)	29 (T <sub>m</sub> )
Substrates used†	H <sub>2</sub> + CO <sub>2</sub> , formate	H <sub>2</sub> + CO <sub>2</sub> , formate, iP, iB	H <sub>2</sub> + CO <sub>2</sub> , formate, iP, iB	H <sub>2</sub> + CO <sub>2</sub> , iP, iB	H <sub>2</sub> + CO <sub>2</sub>

\* 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>).

#### Acknowledgements

The authors acknowledge the kind contribution of the International Rice Research Institute, Los Baños, Philippines, in providing the soil sample from which the

strain was isolated. Funding from the Australian Research Council to B.K.C.P. is gratefully acknowledged.

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