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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 Garcia[1] and Pierre A. Roger[1]

Author for correspondence: Pierre A. Roger. Tel: +33 4 9182 8571. Fax: +33 4 9182 8570. e-mail: rogerpa@esil.univ-mrs.fr

[1] Laboratoire de Microbiologie IRD, Université de Provence – ESIL, Case 925, 163 Avenue de Luminy, 13288 Marseille cedex 9, France

[2] School of Biomolecular and Biomedical Sciences, Faculty of Science and Technology, Griffith University, Brisbane, Queensland 4111, Australia

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 H_2+CO_2 or formate for growth and produces CH_4. 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 CH_4 and CO_2.

Waterlogged ricefields, because of anoxic conditions developing after flooding, are major anthropogenic sources of CH_4 (Minami *et al.*, 1994), one of the major greenhouse gases (Lelieveld *et al.*, 1993). In ricefields, H_2 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*, *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.

DSM 11106^T) uses H_2+CO_2 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 H_2+CO_2. *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 H_2+CO_2 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 FPi^T was 31 mol%.

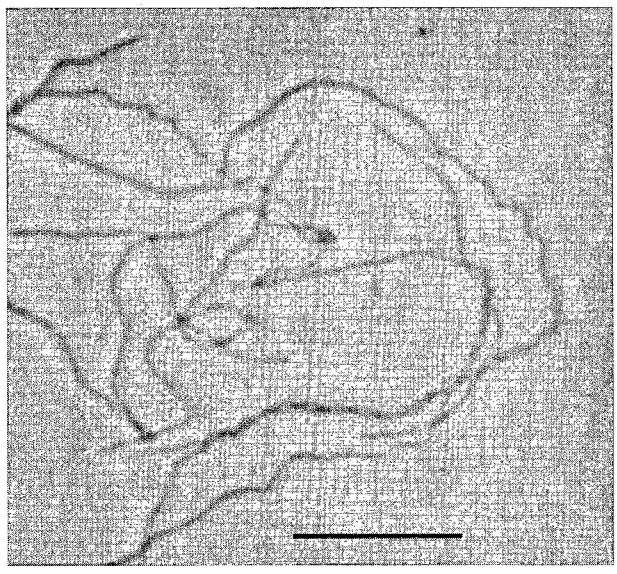

Fig. 1. Phase-contrast micrograph of strain FPi^T showing long chains of cells; bar, 10 μm.

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 FPi^T are *Methanobacterium palustre* and *Methanobacterium formicicum* since both form filaments and use H_2+CO_2 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.

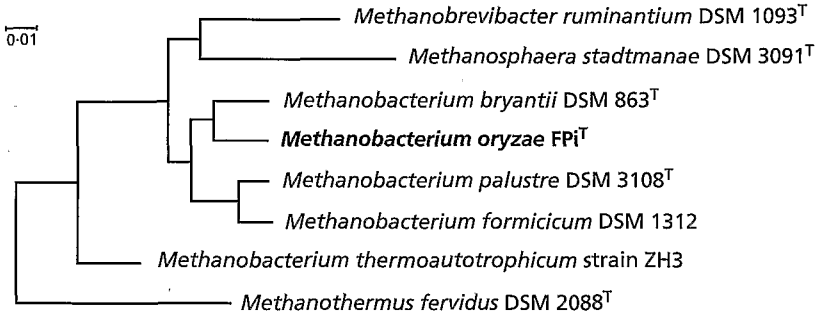

Fig. 2. Dendrogram showing the position of strain FPi^T amongst members of the order *Methanobacteriales*. Bar, 10 nucleotide changes per 100 nucleotides.

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 H_2+CO_2 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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References

Asakawa, S., Morii, H., Akagawa-Matsushita, M., Koga, Y. & Hayano, K. (1993). Characterization of *Methanobrevibacter arboriphilicus* SA isolated from a paddy field soil and DNA-DNA hybridization among *M. arboriphilicus* strains. *Int J Syst Bacteriol* **43**, 683–686.

Asakawa, S., Akagawa-Matsushita, M., Morii, H., Koga, Y. & Hayano, K. (1995). Characterization of *Methanosarcina mazei* TMA isolated from a paddy field soil. *Curr Microbiol* **31**, 34–38.

Boone, D. R., Whitman, W. B. & Rouvière, P. (1993). Diversity and taxonomy of methanogens. In *Methanogenesis. Ecology, Physiology, Biochemistry and Genetics*, pp. 35–80. Edited by J. G. Ferry. New York: Chapman & Hall.

Bryant, M. P. & Boone, D. (1987). Isolation and characterization of *Methanobacterium formicicum* MF. *Int J Syst Bacteriol* **37**, 171.

Bryant, M. P., Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1967). *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. *Arch Microbiol* **59**, 20–31.

Conrad, R., Bak, F., Seitz, H. J., Thebrath, B., Mayer, H. P. & Schütz, H. (1989). Hydrogen turnover by psychrophilic homoacetogenic and mesophilic methanogenic bacteria in anoxic paddy soil and lake sediment. *FEMS Microbiol Ecol* **62**, 285–294.

Dong, X. & Stams, A. J. M. (1995). Evidence for H_2 and formate formation during syntrophic butyrate and propionate degradation. *Anaerobe* **1**, 35–39.

Felsenstein, J. (1993). PHYLIP (Phylogenetic Inference Package) version 3.51c. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA, USA.

Fetzer, S., Bak, F. & Conrad, R. (1993). Sensitivity of methanogenic bacteria from paddy soil to oxygen and desiccation. *FEMS Microbiol Ecol* **12**, 107–115.

Garcia, J.-L. (1990). Taxonomy and ecology of methanogens. *FEMS Microbiol Rev* **87**, 297–308.

Grosskopf, R., Janssen, P. A. & Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl Environ Microbiol* **64**, 960–969.

Hungate, R. E. (1969). A roll tube method for cultivation of strict anaerobes. In *Methods in Microbiology*, vol. 3B, pp. 117–132. Edited by J. R. Norris & D. W. Ribbons. London: Academic Press.

Joulian, C., Ollivier, B., Neue, H.-U. & Roger, P. A. (1996). Microbiological aspects of methane emission by a ricefield soil from the Camargue (France): 1. methanogenesis and related microflora. *Eur J Soil Biol* **32**, 71–80.

Joulian, C., Ollivier, B., Patel, B. K. C. & Roger, P. A. (1998). Phenotypic and phylogenic characterization of dominant culturable methanogens isolated from ricefield soils. *FEMS Microbiol Ecol* **25**, 135–145.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

König, H. (1984). Isolation and characterization of *Methanobacterium uliginosum* sp. nov. from a marshy soil. *Can J Microbiol* **30**, 1477–1481.

Kudo, Y., Nakajima, T., Miyaki, T. & Oyaizu, H. (1997). Methanogen flora of paddy soils in Japan. *FEMS Microbiol Ecol* **22**, 39–48.

Lelieveld, J., Crutzen, P. J. & Brül, C. (1993). Climate effect of atmospheric methane. *Chemosphere* **26**, 739–768.

Macy, J. M., Snellen, J. E. & Hungate, R. E. (1972). Use of syringe methods for anaerobiosis. *Am J Clin Nutr* **25**, 1318–1323.

Mah, R. A. & Smith, M. R. (1981). The methanogenic bacteria. In *The Prokaryotes*, vol. 1, pp. 948–977. Edited by M. P. Starr, H. Stolp, H. G. Trüper, A. Balows & H. G. Schlegel. Berlin: Springer.

Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1996). The ribosomal database project (RDP). *Nucleic Acids Res* **24**, 82–85.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Minami, K., Mosier, A. & Sass, R. (1994). *CH_4 and NO_2 Global Emission and Controls from Ricefields and Other Agricultural and Industrial Sources.* NIAES Series 2. Tokyo: Yokendo.

Raimbault, M. (1981). Inhibition de la formation de méthane par l'acétylène chez *Methanosarcina barkeri. Cah ORSTOM Ser Biol* **43**, 45–51.

Rajagopal, B. S., Belay, N. & Daniels, L. (1988). Isolation and characterization of methanogenic bacteria from rice paddies. *FEMS Microbiol Ecol* **53**, 153–158.

Schink, B. (1992). Syntrophism among prokaryotes. In *The Prokaryotes*, 2nd edn, pp. 276–299. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Schütz, H., Seiler, W. & Conrad, R. (1989). Processes involved in formation and emission of methane in rice paddies. *Biogeochemistry* **7**, 33–53.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stams, A. J. M. (1994). Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie Leeuwenhoek* **66**, 271–294.

Takai, Y. (1970). The mechanism of methane fermentation in flooded paddy soil. *Soil Sci Plant Nutr* **16**, 238–244.

Zellner, G. & Winter, J. (1987). Secondary alcohols as hydrogen donors for CO_2-reduction by methanogens. *FEMS Microbiol Lett* **44**, 323–328.

Zellner, G., Bleicher, K., Braun, E., Kneifel, H., Tindall, B. J., Conway de Macario, E. & Winter, J. (1989). Characterization of a new mesophilic, secondary alcohol-utilizing methanogen, *Methanobacterium palustre* sp. nov. from a peat bog. *Arch Microbiol* **151**, 1–9.