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Phenotypic and phylogenetic characterization of dominant culturable methanogens isolated from ricefield soils

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Abstract

Populations of the four major trophic groups of methanogens were enumerated by most probable numbers (MPN) on selective media in a sample of 13 soils representative of major types of rice soils. Dominant strains were isolated and their phenotypic and phylogenetic characteristics were studied. MPN counts ranged from 10^2 to 10^6 g^{-1} d.w. on H_2, from <10 to 10^4 g^{-1} d.w. on acetate, from <10 to 10^5 on methanol, and from 50 to 10^6 on formate. In most soils, counts of hydrogenotrophs were higher than counts of acetotrophs, partly because acetotrophs were aggregated sarcinae difficult to separate into individual cells. Methylotrophs other than acetotrophic sarcinae were not recorded. In most soils, rods enumerated on formate were 5–400 times less abundant than those enumerated on H_2, indicating that hydrogenotrophic-non-formatotrophic rods are probably dominant in ricefields. Dominant strains isolated comprised: 15 hydrogenotrophic-non-formatotrophic rods affiliated to *Methanobacterium bryantii*; three hydrogenotrophic-formatotrophic rods affiliated to *Mb. formicicum*; one hydrogenotrophic-formatotrophic rod not affiliated to a sequenced species; two sarcinae affiliated to *Methanosarcina barkeri* and *Methanosarcina mazei;* and one irregular coccus affiliated to *Methanoculleus marisnigri* – a species so far isolated from marine sediments only. Results from classical counts of methanogens and strains isolated from ricefields suggest the dominance of *Methanobacterium* spp. (mostly responsible for CH_4 production from H_2/CO_2) and *Methanosarcina* spp. (mostly responsible for CH_4 production from acetate) among culturable organisms. Both genera are probably ubiquitous. In particular, *Mb. bryantii* was isolated from 12 of the 13 soils.

Keywords: Methane; Methanogen; MPN count; Ricefield; Soil; Phylogeny; *Methanobacterium*

1. Introduction

Methane (CH_4) has a high potential for absorbing infrared radiations and is therefore one of the major gases involved in the greenhouse effect [1,2]. Up to 70–80% of atmospheric CH_4 is biogenic [3]. Waterlogged ricefields, because of anoxic conditions developing after flooding, are one of the major anthropogenic sources of CH_4 [4], however, the estimation of their contribution to the global CH_4 budget remains relatively imprecise [5].

Methane-producing bacteria are strict anaerobes

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Table 1
Primers used for amplification and sequencing of 16S rRNA gene of methanogens

Primer	*E. coli* position	Sequence 5′-3′
Forward		
MS1[a]	406	CCA CTC TTA ACG GGG TG
meth-ArFd3[b]	7	G(A)TC CGT TTG ATC CTG GCG G
FARCH-9[b]	9	CTG GTT GAT CCT GCC AG
F2	785	CAG GAT TAG ATA CCC TGG TAG
F3	907	AAA CTC AAA GGA ATT GAC GG
F4	1391	TGT ACA CAC CGC CCG T
Reverse		
MS2[a]	1342	ACA CGC GAT TAC TAC GC
meth-ArchR1	337	GCT GCG CCC CGT AGG GCC
R2	519	GTA TTA CCG CGG CTG CTG
R4	907	CCG TCA ATT CAT TTG AGT TT
R6	1494	TAC GGT TAC CTT GTT ACG AC
Rd1[b]	1542	AAG GAG GTG ATC CAG CC

[a]Primers used for partial 16S rRNA gene amplification (about 940 bp).
[b]Primers used for complete 16S rRNA gene amplification and sequencing.

belonging to the *Archaea* domain. They are commonly isolated from natural anoxic environments, including freshwater and marine sediments, wet and waterlogged soils, the rumen, and the gut of insects [6–8]. They play an important role in these environments by performing the last step of anaerobic decomposition of organic matter, which is mineralized into CH_4 and CO_2. Radiotracer experiments showed that H_2 and acetate are the main energy sources used by methanogens in ricefields [9–11]. Those substrates result from fermentative metabolism or syntrophic associations degrading reduced compounds such as butyrate and propionate [12–14].

Despite numerous studies providing indirect evidence of the occurrence of methanogens in ricefields, few reports are available on their density [10,15–17], and species present in ricefields [11,17–20]. The only fully characterized species isolated from a ricefield are *Methanobrevibacter arboriphilicus* strain SA [21] and *Methanosarcina mazei* strain TMA [22]. Recently, a phylogenetic study has reported the presence of *Methanosarcina*, *Methanogenium*, *Methanosaeta*, and *Methanobacterium* in Japanese ricefield soils [23].

This paper reports on the study of the four major trophic groups of methanogens in ricefield soils. Populations were enumerated in a sample of 13 soils representative of major rice soils. Dominant strains were isolated and their phenotypic and phylogenetic characteristics were studied.

2. Materials and methods

2.1. Soils

Soils were collected from a range of ricefields in three countries (France, the Philippines, and USA), providing a representative sample with a broad range of physico-chemical properties: pH ranged from 4.5 to 7.8; organic C content ranged from 0.88 to 3.72%; organic N content ranged from 0.10 to 0.38%; available P ranged from 2 to 22 ppm. Soils were collected at the end of the crop cycle as composite samples. They were air-dried at ambient temperature as large clods and stored at room temperature. Before use, they were crushed and passed through a 5 mm sieve.

2.2. Reference bacterial strains

Methanobacterium bryantii (DSM 863), *Methanobacterium formicicum* (DSM 1535), *Methanobacterium wolfei* (DSM 2970), and *Methanobacterium thermoautotrophicum* (DSM 1053) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh (DSMZ), Braunschweig, Germany.

2.3. Cultivation and media

Hungate anaerobic techniques [24,25] were used

throughout this work. Basal medium contained (per liter): 1 g of NH_4Cl; 0.3 g of KH_2PO_4; 0.3 g of K_2HPO_4; 0.2 g of $MgCl_2{\cdot}6H_2O$; 0.1 g of $CaCl_2{\cdot}2H_2O$; 0.1 g of KCl; 0.5 g of CH_3COONa anhydrous; 0.6 g of NaCl; 0.5 g of cysteine-HCl; 10 ml of trace element solution [26]; and 0.001 g of rezasurin. The pH of the medium was adjusted to 7 using a 10 M KOH solution. The medium was then boiled under a stream of O_2-free N_2 and cooled at room temperature. It was distributed anaerobically into 60-ml serum bottles (20 ml/bottle) and into 20-ml Hungate tubes (5 ml/tube). Hungate tubes and serum bottles were flushed with N_2/CO_2 (80/20 v/v) and sterilized for 45 min at 110°C. After sterilization, 0.01 ml of 2% $Na_2S.9H_2O$ and 0.05 ml of 10% $NaHCO_3$ (sterile, anaerobic solutions) were injected per ml of basal medium into the culture vessels.

Four selective media were prepared by adding to the sterilized basal medium, one of the four following substrates: formate (40 mM), methanol (40 mM), acetate (20 mM), which were added from sterile and anaerobic stock solution, or H_2/CO_2 (80/20 v/v, 2 bar), which was injected in the gas phase. For enumerations, these media were supplemented with 1 g l^{-1} of yeast extract (Difco Laboratories, Detroit, MI) and 1 g l^{-1} of bio-Trypticase (BioMérieux, Craponne, France). For preparing solid media, 1.6% agar (Difco) was used. For growth requirement studies, vitamin (1% of Pfennig-Widdel [27] solution), various concentrations of yeast extract, and bio-Trypticase were added.

2.4. Counts of methanogens

The population density of the major trophic groups of methanogens were estimated by the most probable number (MPN) method (3 tubes per dilution). Successive 10-fold serial soil suspension dilutions were inoculated in the four selective media described above. Trophic groups of methanogens are detected, at least, on one of these media. Counts were duplicated by using two composite soil samples for each soil type. Methanogen growth was assayed by measuring CH_4 produced after 60 days of incubation at 37°C. Inoculated tubes containing medium supplemented with 1 g l^{-1} of yeast extract and 1 g l^{-1} of bio-Trypticase, where no substrate was added, served as control. A tube was considered positive when CH_4 produced was as least 5% higher than in the control. Populations were expressed as MPN per g dry soil. Because MPN counts have a low accuracy, counts (average of two replicates) were considered different if their ratio was higher than four [28].

Table 2
Counts of major trophic groups of methanogens

Soil	Origin of soil	MPN of methanogens (g^{-1} soil d.w.) on			
		H_2/CO_2	Formate	Methanol	Acetate
Camargue	France	9.6×10^3	1.8×10^3	2.5×10^3	5.0×10^3
California 3	USA	1.0×10^2	1.3×10^2	2.8×10^2	1.8×10^2
Amurao	The Philippines	5.7×10^2	1.1×10^3	2.6×10^2	2.3×10^2
Binalonan	The Philippines	4.8×10^3	2.0×10^2	1.2×10^2	2.4×10^2
Bugallon	The Philippines	3.8×10^2	6.7×10^1	2.0×10^1	2.7×10^1
Lal-lo	The Philippines	2.1×10^2	5.3×10^1	$<10^1$	4.7×10^1
Luisiana	The Philippines	1.9×10^3	9.0×10^3	5.0×10^2	1.2×10^2
Maahas	The Philippines	7.0×10^4	1.0×10^3	2.9×10^2	1.3×10^2
Maahas+salt[a]	The Philippines	1.3×10^5	7.0×10^3	1.8×10^2	5.0×10^2
Maahas+GM[b]	The Philippines	5.0×10^4	1.5×10^3	1.8×10^2	5.0×10^2
Maligaya	The Philippines	1.8×10^4	4.3×10^2	2.2×10^2	1.8×10^2
Pila	The Philippines	3.6×10^4	9.0×10^1	4.0×10^3	2.9×10^3
San Dionisio	The Philippines	1.7×10^2	6.0×10^1	2.0×10^2	$<10^1$
Tiaong	The Philippines	2.3×10^6	1.6×10^6	1.9×10^5	2.2×10^4
Urdaneta	The Philippines	7.3×10^2	6.0×10^1	6.5×10^1	1.3×10^2

[a]0.66 kg NaCl m^{-2} applied before transplanting.
[b]Green manure (20 t ha^{-1} fresh weight) incorporated before transplanting.

Table 3
Origin, morphology, and energy sources of isolated strains

Soil	Strain	Form	Energy sources used for				
			Isolation	Growth			
				H_2/CO_2	Formate	Methanol	Acetate	Alcohols
Camargue	RiH2	Rod	H_2/CO_2	+	−	−	−	2-P
	FCam	Rod	Formate	+	+	−	−	2-P
	CoCam	Coccoid	Formate	+	+	−	−	−
	Sar	Sarcina	Methanol	+	−	+	+	−
California 3	H2Cal3	Rod	H_2/CO_2	+	−	−	−	2-P
Amurao	TAH2	Rod	H_2/CO_2	+	−	−	−	2-P
Binalonan	PBH2	Rod	H_2/CO_2	+	−	−	−	2-P
Bugallon	PDH2	Rod	H_2/CO_2	+	−	−	−	2-P
Lal-lo	CBH2	Rod	H_2/CO_2	+	−	−	−	2-P
Luisiana	LuiH2	Rod	H_2/CO_2	+	−	−	−	2-P
Maahas	MaH2	Rod	H_2/CO_2	+	−	−	−	2-P
	H2Sol2	Rod	H_2/CO_2	+	−	−	−	2-P
	FSol1	Rod	Formate	+	+	−	−	−
Maahas+salt[a]	SelH2	Rod	H_2/CO_2	+	−	−	−	2-P
Maahas+GM[b]	GMH2	Rod	H_2/CO_2	+	−	−	−	2-P
Maligaya	MaliH2	Rod	H_2/CO_2	+	−	−	−	2-P
Pila	PiH2	Rod	H_2/CO_2	+	−	−	−	2-P
	FPi	Rod	Formate	+	+	−	−	−
	SarPi	Sarcina	Methanol	+	−	+	+	B
San Dionisio	IAH2	Rod	H_2/CO_2	+	−	−	−	2-P
Tiaong	TiaH2	Rod	H_2/CO_2	+	+	−	−	−
Urdaneta	PAH2	Rod	H_2/CO_2	+	−	−	−	2-P

[a,b]As in Table 2.
2-P: 2-propanol; B: butanol.

2.5. Enrichment and isolation procedure

Pure cultures of methanogens were obtained from the last positive tubes, by three repeated applications of the agar shake dilution method [24]. For final purification, single colonies were picked up, using an anaerobic glove box with a N_2/H_2 atmosphere (95/5 v/v). Colonies were diluted in liquid media and the last positive dilution was checked for purity by microscopic examination and by verifying the absence of growth in a medium containing 1 g l^{-1} bio-Trypticase and 1 g l^{-1} yeast extract, to which was added either (1) 20 mM glucose (to detect fermentative bacteria) or (2) 20 mM sulfate and 20 mM lactate (to detect sulfate-reducing bacteria).

2.6. Morphology and growth characteristics

Growth characteristics of the isolates were determined in duplicate Hungate tubes. Media were enriched with 1 g l^{-1} of yeast extract and, when necessary, 1 g l^{-1} of bio-Trypticase. The temperature range for growth was tested from 15°C to 50°C in water baths. Optimum NaCl concentration was tested at 37°C using concentrations ranging from 0 to 60 g l^{-1}. The pH range for growth was determined by adding various concentration of 10% $NaHCO_3$, 8% Na_2CO_3, or 0.5 M HCl solutions to the culture medium, to obtain pHs ranging from 4 to 9. The following substrates were tested for growth: 2 bar H_2/CO_2, 40 mM formate, 40 mM methanol, 20 mM acetate, 10 mM 1-propanol, 10 mM 2-propanol, 10 mM 1-butanol, and 10 mM 2-butanol.

2.7. Analytical techniques

Bacterial cultures were observed with a Nikon Optiphot microscope equipped with a Nikon FX35 camera and a Zeiss, standard 20, microscope equipped with epifluorescence. Bacterial growth was quantified (1) with a Shimadzu UV 160 A spectrophotometer by measuring turbidity increase at

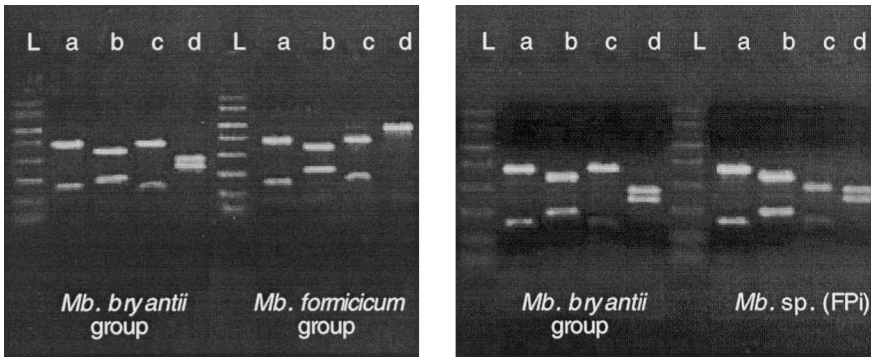

Fig. 1. Restriction profiles of the isolated rods. *Mb. bryantii* group included strains RiH2, H2Cal3, TAH2, PBH2, PDH2, CBH2, LuiH2, MaH2, H2Sol2, SelH2, GMH2, MaliH2, PAH2, IAH2 and PiH2; *Mb. formicicum* group included strains FCam, FSol1 and TiaH2. L, ladder; a, *Bam*HI; b, *Cfo*I; c, *Sau*3A; d, *Taq*I.

580 nm directly in Hungate tubes, or (2) through CH_4 production for aggregated forms such as members of the genus *Methanosarcina* or methanogenic cultures with low density. Methane was measured with a Girdel series 30 flame ionization detector gas chromatograph (column SP 1000 with chromosorb WAW and 1% H_3PO_4) operated at 150°C with N_2 as carrier gas.

2.8. Phylogenetic analysis

2.8.1. Restriction profile

Part of the 16S rRNA gene (about 940 bp) of the isolated *Methanobacterium* strains was amplified. The sequences of forward (MS1), and reverse (MS2) primers used were determined by comparing the 16S rRNA sequences of *Methanobacterium* species available in the GenBank database. Primer sequences are described in Table 1. Primers were synthesized by Bioprobe® systems (Montreuil, France). PCR was performed directly from 1 μl of culture. The PCR product was digested with four restriction enzymes, *Bam*HI, *Cfo*I, *Sau*3A and *Taq*I (Boehringer Mannheim, Germany).

2.8.2. 16S rRNA gene sequencing

Semi-purified DNA was extracted for amplification of the 16S rRNA gene using the following protocol. Pure cultures (50 ml) were centrifuged for 20 min at $10\,000\times g$ and pellets were resuspended in 50 μl of lysis buffer (50 mM Tris-HCl, pH 7.2; 50 mM EDTA; 3% sodium dodecyl sulfate). Suspensions were microwaved, with lids open, at 600 W (15 s on, 5 s off; repeated four times), and were then placed at 80°C for 2 h. This treatment was repeated three times. Then, 350 μl of lysis buffer was added and the suspensions were placed at 80°C. After overnight incubation, suspensions were mixed vigorously with 400 μl of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged for 15 min at $10\,000\times g$. The top aqueous phase was removed carefully to avoid collecting cell material from the interface. Then, 10 μl of cold isopropanol and 5 μl of 5 M sodium acetate (pH 5.8) were added to the aqueous phase. The suspensions were centrifuged, the nucleic acid pellets were washed with 500 μl of 70% cold ethanol and dried in a desiccator. Pellets were resuspended in 50 μl of TE buffer (10 mM Tris-HCl; 1 mM EDTA) and stored at −20°C until use.

The sequences of primers used for amplification and sequencing are described in Table 1. The 16S rRNA gene was amplified as described by Love et al. [29] and Redburn and Patel [30] using Farch9 and Rd1 as PCR primers. In addition, a new forward primer, meth-ArFd3, specific to *Methanobacterium* strains, was used for amplification. The purified PCR product was sequenced directly with an ABI automated DNA sequencer, using a Prism dideoxy terminator cycle sequencing kit, as recommended by the manufacturer (Applied Biosystems, Ltd., Foster City, CA). Primers used were described by Redburn and Patel [30]. In addition, a new reverse primer specific to *Methanobacterium* strains, meth-ArchR1, was used to sequence the beginning of the gene. New primers were synthesized by CMCB (University of the Southern Cross, Lismore, Australia).

Table 4
Evolutionary similarity matrix for methanogens from ricefields and various methanogens

	% Similarity																								
	Strain Sar	Strain SarPi	Strain RiH2	Strain FCam	Strain FPi	Strain CoCam	*Methanobacterium bryantii*	*Methanobacterium formicicum*	*Methanosarcina barkeri*	*Methanosarcina acetivorans*	*Methanosarcina mazei*	*Methanoculleus marisnigri*	*Methanococcoides methylutens*	*Methanococcus vannielii*	*Methanocorpusculum parvum*	*Methanogenium cariaci*	*Methanoplanus limicola*	*Methanolobus tindarius*	*Methanomicrobium mobile*	*Methanosaeta concilii*	*Methanospirillum hungatei*	*Methanosphaera stadtmaniae*	*Methanobrevibacter ruminantium*
Strain Sar																									
Strain SarPi	97.7																								
Strain RiH2	73.6	74.1																							
Strain FCam	72.9	72.9	95.4																						
Strain FPi	72.5	73.9	96.4	94.9																					
Strain CoCam	79.5	79.5	75.5	75.1	75.3																				
Mb. bryantii	72.7	73.4	98.9	94.9	96.6	75.2																			
Mb. formicicum	73.4	73.3	94.8	97.7	94.9	76.0	94.9																		
Ms. barkeri	98.7	97.3	72.6	71.9	72.2	78.4	72.5	72.3																	
Ms. acetivorans	97.9	97.5	71.5	71.7	72.1	78.6	71.0	72.2	97.7																
Ms. mazei	97.4	99.9	72.9	71.6	73.1	78.4	72.5	72.0	97.3	97.6															
Mc. marisnigri	79.4	79.2	75.6	75.4	75.4	98.4	75.4	76.0	78.9	79.2	78.8														
M. methylutens	91.4	91.4	73.4	73.8	74.0	79.6	72.9	74.5	91.2	91.5	91.0	80.0													
M. vannielii	71.1	71.3	78.2	77.2	78.4	70.1	77.7	77.4	70.3	69.0	69.9	69.7	71.7												
M. parvum	72.8	73.3	68.5	69.6	69.5	84.6	68.1	69.9	73.3	73.1	73.3	85.9	73.8	64.8											
M. cariaci	77.5	77.3	75.6	75.0	75.0	92.4	74.7	75.2	77.0	77.4	76.8	92.6	79.1	71.5	84.2										
M. limicola	77.5	78.3	75.0	75.2	74.7	91.9	74.6	77.3	77.4	77.7	91.9	75.7	78.9	70.6	84.8	95.0									
M. tindarius	90.3	90.3	72.1	72.3	73.2	79.0	71.3	72.4	90.9	91.0	90.2	79.9	92.8	68.9	74.2	78.8	78.5								
M. mobile	77.0	76.9	74.2	75.1	74.3	90.1	73.9	75.5	76.7	76.9	76.2	90.3	76.9	70.4	83.5	92.6	93.5	77.6							
M. concilii	83.6	84.6	75.6	74.7	75.7	80.0	75.4	74.9	83.8	83.4	84.3	80.5	84.0	68.4	72.3	79.0	78.5	83.5	77.3						
M. hungatei	77.3	77.7	73.2	74.2	74.2	87.3	72.9	74.9	77.2	76.5	76.8	87.2	78.1	68.8	83.2	88.0	87.9	78.3	85.8	78.7					
M. stadtmaniae	70.8	71.6	89.0	89.0	89.3	72.4	89.5	89.8	71.0	70.1	71.2	72.8	71.2	74.7	68.4	71.2	72.2	70.2	70.9	72.7	70.9				
M. ruminantium	72.1	73.5	90.9	89.8	90.4	75.3	91.5	90.4	72.5	71.8	72.7	75.0	73.7	76.1	69.0	74.9	74.0	71.9	73.1	74.6	72.7	89.5			
M. bourgense	78.5	78.5	73.9	73.4	73.8	97.5	73.6	74.5	78.3	78.8	78.5	97.3	78.4	68.5	85.2	91.6	91.3	78.9	90.8	79.8	86.5	71.6	73.2		

The 16S rRNA sequences of the isolated strains and 16S rRNA sequences of various members of the *Archaea* domain, obtained from the RNA Database Project and from the GenBank database, were manually aligned using the sequence editor ae2 [31]. Positions at which the sequences and/or alignment were uncertain were omitted from the analysis, and pairwise evolutionary distances for unambiguous nucleotides were computed by the method of Jukes and Cantor [32]. Dendrograms were constructed from these distances by the neighbor-joining method. All programs are available as part of the PHYLIP package [33].

3. Results

3.1. Estimation of methanogenic populations

Population of methanogens were quantified in the 13 ricefield soils using four substrates (Table 2). MPN counts ranged from 10^2 to 2×10^6 g^{-1} d.w. for hydrogenotrophs, from 50 to 2×10^6 g^{-1} d.w. for formatotrophs, from <10 to 2×10^5 g^{-1} d.w. for methylotrophs, and from <10 to 2×10^4 g^{-1} d.w. for acetotrophs.

Hydrogenotrophs were dominant in eight of the 13 soils, being 5–400 times more abundant than formatotrophs, 9–650 times more abundant than methylotrophs, and 5–538 times more abundant than acetotrophs. In Tiaong soil, both hydrogenotrophs and formatotrophs were dominant; their populations were similar and exhibited the highest density among the soils tested. In California 3 and Amurao soils, the four methanogenic trophic groups exhibited approximately the same densities within each soil.

When methanol or acetate was used as energy source, *Methanosarcina*-like forms were dominant and population densities were of the same order on both substrates in most of the soils. When H_2 or formate was used as energy source, rods were dominant. They showed the typical fluorescence of methanogens under UV light and phenotypic characteristics similar to members of the *Methanobacterium* genus. Population densities were usually higher when enumerated on H_2/CO_2.

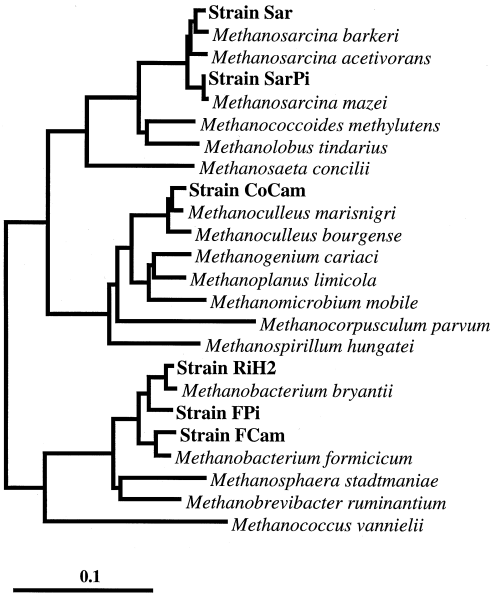

Fig. 2. Dendrogram showing the position of the methanogenic strains isolated from ricefields among representative methanogens of various origins.

3.2. Isolation

The dominant morphotypes isolated on H_2 or formate were rods, which were observed as single cells or chains, depending on the growth phase. In Camargue and Pila soils, irregular coccoid cells were also present in tubes containing formate as energy source, but rods still dominated. Enrichments on acetate or methanol led to the development of sarcina-like morphotypes. Their isolation was further performed on methanol. In addition, in some enrichments we observed the presence of spirilla showing a fluorescence typical of methanogens under UV microscopic examination. Attempts to isolate these spirilla were unsuccessful.

Twenty-two methanogenic strains were isolated from the 13 soils studied (Table 3). We found 19 rods, phenotypically related to the genus *Methanobacterium*, among which 15 were hydrogenotrophs unable to utilize formate, and four used both H_2 and formate as energy sources. *Methanosarcina*-like strains were present in all soils; two strains were isolated. An irregular methanogenic coccus was observed in tubes containing formate in Camargue and Pila soils; one strain was isolated.

3.3. Phylogenetic and phenotypic characterization

As isolated *Methanobacterium* strains were too numerous to be all studied for their phenotypic characteristics and sequenced, we first characterized them from their RFLP profiles using four restriction enzymes (*Bam*HI, *Cfo*I, *Sau*3A, and *Taq*I) and about 940 bp of the 16S rRNA gene. We compared their profiles with those of the four *Methanobacterium* species with known 16S rRNA gene sequences (*Mb. bryantii*, *Mb. formicicum*, *Mb. wolfei*, and *Mb. thermoautotrophicum*). These four species exhibited four different profiles. The 19 isolated rods exhibited three profiles (Fig. 1), suggesting that three species of *Methanobacterium* were present. The 15 hydrogenotrophic-non-formatotrophic rods had a profile similar to that of *Mb. bryantii*, and three of the four formatotrophic-hydrogenotrophic rods had a profile similar to that of *Mb. formicicum*. A profile different from the four reference profiles was obtained for one formatotrophic-hydrogenotrophic rod (strain FPi). Then, we sequenced the complete 16S rRNA gene of one strain of each group (strain RiH2 for *Mb. bryantii* group, strain FCam for *Mb. formicicum* group, and strain FPi for the unknown *Methanobacterium* sp. group), using 10 primers (Table 1). The analysis of the sequences confirmed the determination of the three species of *Methanobacterium* isolated. Finally, we used the other isolated strains of *Methanobacterium* to sequence the most variable region of their 16S rRNA gene, located at the beginning of the gene, using primers meth-ArFd3 and meth-ArchR1 (Table 1). Results confirmed the groups established with the restriction profiles.

These studies showed that the 15 hydrogenotrophic-non-formatotrophic rods were related to the species *Mb. bryantii*. Three of the four formatotrophic rods were related to *Mb. formicicum*. The formatotrophic rod, strain FPi, was significantly distant from currently sequenced *Methanobacterium* species.

To determine the phylogeny of all isolated methanogens, we sequenced the complete or almost complete 16S rRNA gene, using 10 primers (Table 1), of the six different species isolated: the three rods, as

Table 5
Major characteristics of the isolated strains and related species[a]

Microorganism	Culture conditions								ARNr 16S phylogeny	
	Optimum (and range)			Substrate for growth					Most closely related species	Similarity (%)
	Temperature (°C)	Salinity (g/l)	pH	H_2/CO_2	Formate	Methanol	Acetate	Alcohols		
Methanobacterium										
bryantii[b]	37–39	n.d.	6.9–7.2	+	−	−	−	+		
formicicum[b]	37–45	n.d.	6.6–7.8	+	+	−	−	±		
RiH2 (Camargue)[c]	35 (25–42)	0–25	6.8 (6.0–8.5)	−	+	−	−	2-P	*Mb. bryantii*	99.2
FCam (Camargue)[c]	40 (25–42)	5 (0–30)	7–7.2 (6.0–8.5)	+	+	−	−	2-P	*Mb. formicicum*	97.9
FPi (Pila)[c]	40 (20–42)	5 (0–30)	7 (6.0–8.0)	+	+	−	−	−	*Mb. bryantii*	96.5
Methanosarcina										
barkeri[b]	30–40	n.d.	neutral	+	−	+	+	−		
mazei[b]	30–40	n.d.	7–7.2	+	−	+	+	n.d.		
Sar (Camargue)[c]	35–37 (25–42)	0 (0–10)	(5.5–7.5)	+	−	+	+	−	*Ms. barkeri*	99.0
SarPi (Pila)[c]	40 (20–42)	(0–60)[d]	7.5 (6.0–8.5)	+	−	+	+	B	*Ms. mazei*	99.8
Methanoculleus										
marisnigri[b]	20–25	60	6.8–7.3	+	+	−	−	+		
CoCam (Camargue)[c]	37 (20–42)	(0–60)[d]	7 (6.8–8.2)	+	+	−	−	−	*Mc. marisnigri*	98.4

[a]Characteristics of related species according to Garcia [8].
[b]Reference strains.
[c]Soil of origin.
[d]Concentrations >60 g l^{-1} of NaCl not tested.
2-P: 2-propanol; B: 1-butanol.
n.d.: not determined.

described above (strains RiH2, FCam and FPi), the two sarcinae, and the irregular coccus. Sequences were aligned with the sequences of representative methanogens, and phylogenetic analysis was performed from the Jukes-Cantor similarity matrix [32] (Table 4). A dendrogram was obtained from this matrix by the FITCH method [33] (Fig. 2). Results indicated that five of the six isolated species were related phylogenetically but also phenotypically to known species (Table 5). RiH2 (sequence accession number AF028688) belongs to *Methanobacterium bryantii* (M59124) (average similarity of 99.2%), FCam (AF028689) to *Methanobacterium formicicum* (M36508) (average similarity of 97.9%). SarPi (AF028691) belongs to *Methanosarcina mazei* (U20151) (average similarity of 99.8%), and Sar (AF028692) to *Methanosarcina barkeri* (M59144) (average similarity of 99%). The irregular coccus, strain CoCam (AF028693), which uses H_2 and formate, belongs to *Methanoculleus marisnigri* (M59134) (average similarity of 98.4%). Strain FPi (AF028690) showed marked phylogenetic differences from its closest relatives, *Methanobacterium bryantii* (average similarity of 96.5%) and *Methanobacterium formicicum* (average similarity of 94.7%). In contrast to its phylogenetically closest relative, *Mb. bryantii*, it used formate as energy source (Table 5).

4. Discussion

We enumerated methanogens in dry soil samples originating from 13 ricefields representing a broad range of physico-chemical properties and geographical origins. Since acetate and H_2/CO_2 were demonstrated to be the major methanogenic substrates in wetland ricefields [9–11], enumerations were performed on selective media containing each of these energy sources. In addition, we also used two additional selective media containing (1) methanol, to record strict (non-acetoclastic) methylotrophs and (2) formate, since hydrogenotrophic methanogens do not necessarily use formate [8]. Formate might be important in the anaerobic degradation of organic matter, because, like H_2, it is involved in the interspecies transfer during oxidation of reduced compounds [14,34].

MPN counts of methanogens on H_2 ranged from 10^2 to 10^6 g^{-1} d.w.; counts on acetate ranged from <10 to 10^4 g^{-1} d.w. Populations were in a range similar to those reported in 29 Senegalese ricefields by Garcia et al. [15] (10^2–10^7 g^{-1} d.w.). In an Italian ricefield, Schütz et al. [10] and Mayer and Conrad [16] counted 10^4–10^5 acetotrophs g^{-1} d.w. and 10^5–10^6 hydrogenotrophs g^{-1} d.w.

In most soils, counts of hydrogenotrophs were higher than counts of acetotrophs. However, the lower counts of acetotrophs mostly resulted from a methodological bias, hydrogenotrophs being mostly rods, whereas acetotrophs were mostly sarcinae. Sarcinae are known to develop as dense aggregates, difficult to separate into individual cells, thus their populations are underestimated by MPN counts, which mostly record the number of aggregates [20].

In most soils, counts on methanol did not show significant differences with counts on acetate. Sarcinae dominated on both substrates; non-acetoclastic-methylotrophic methanogens, such as members of the genera *Methanococcoides* or *Methanolobus*, were not evidenced. These results suggest that methanogenesis from methanol released from the fermentation of pectin [35] originating from the photosynthetic aquatic biomass (aquatic macrophytes, micro- and macro-algae, and cyanobacteria) is probably mainly due to the activity of *Methanosarcina*.

In most soils, rods enumerated on formate were 5–400 times less abundant than those enumerated on H_2, indicating that hydrogenotrophic-non-formatotrophic rods were dominant in the ricefield soils tested, since all formatotrophs also use H_2/CO_2. In all soils, dominant hydrogenotrophic methanogens belonged to the genus *Methanobacterium*.

Twenty-two strains were isolated from the highest dilution tubes. They included: two sarcinae isolated on methanol, one irregular coccus isolated on formate, 16 hydrogenotrophic rods isolated on H_2/CO_2, and three formatotrophic rods isolated on formate. Among the hydrogenotrophic rods, one was found to be also a formatotroph (Table 3).

On the basis of phenotypic studies, all the isolated rods were affiliated to the genus *Methanobacterium*. Fifteen strains were related to *Mb. bryantii* and three to *Mb. formicicum*. One strain (FPi) presented sufficient phenotypic and phylogenetic differences from its closest relative, *Mb. bryantii* (average similarity

96.5%), to consider it a new species (work in progress).

Surprisingly, an irregular methanogenic coccus, phylogenetically related to *Methanoculleus marisnigri* (only isolated so far from marine sediments [36]) was isolated from the Camargue soil. This soil is of estuarine origin, but cannot be considered saline (exchangeable Na: 0.9 mEq/100 g). Members of the genus *Methanoculleus* are halotolerant microorganisms [37] and *M. marisnigri* in particular might be adapted to a wide range of NaCl concentrations. This probably explains its occurrence in marine but also in non-saline environments such as ricefields.

The isolated sarcinae were related to the species *Methanosarcina barkeri* and *Methanosarcina mazei*, which have already been isolated from a broad range of environments, including sediments and digesters. The latter species was also recently isolated from a Japanese ricefield [22].

Our results seem to indicate that, in ricefields, *Methanobacterium* spp. are mostly responsible for CH_4 production from H_2/CO_2 and *Methanosarcina* spp. for CH_4 production from acetate. Both genera are probably ubiquitous. In particular, *Mb. bryantii* was isolated from 12 of the 13 soils. However, beside members of these two genera, we have isolated a member of the genus *Methanoculleus*.

Although results from classical isolation of methanogens from ricefields suggest the ubiquity and dominance of *Methanobacterium* spp. among culturable organisms, Kudo et al. [23], using PCR amplification of archaeobacterial 16S ribosomal DNA from extracted soil DNA in nine Japanese ricefield soils, reported the presence of *Methanobacterium* in only one soil, where it was not dominant. On the other hand, similar to our results, this Japanese study also reported the presence of members of the genera *Methanosarcina* – including *Msr. mazei* and *Msr. barkeri* – and *Methanogenium* or *Methanoculleus*. From their results, Kudo et al. [23] concluded to the dominance of *Methanosarcina* in five soil samples, to that of *Methanogenium* in two soils and *Methanosaeta* in two soils.

The somewhat conflicting results regarding *Methanobacterium* occurrence may indicate that this genus is absent in most Japanese ricefield soils, whereas it is widely represented in soils of the Philippines, USA and France. However, these results might also be due to bias inherent in both methods. On the one hand the DNA extraction and 16S rDNA clone analysis methods used by Kudo et al. [23] possibly do not fully reflect the diversity of the methanogenic flora. On the other hand the MPN method we used is possibly selective for *Methanobacterium* and might lead to the erroneous conclusion of its dominance when it is only present.

At this moment it is difficult to say which technique yield the most reliable results in terms of presence and relative abundance of genera of methanogens.

All together, these results indicate that methanogen biodiversity in ricefields is not yet elucidated. It clearly demonstrates the need for simultaneous classical microbiological studies and in situ phylogenetic analysis to assess microbial populations in soils.

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