***Methanocalculus taiwanensis* sp. nov., isolated from an estuarine environment**

¹ Department of Botany,
National Chung Hsing
University, Taichung,
40227 Taiwan,
Republic of China

² Food Research &
Development Institute,
Hsinchu, Taiwan,
Republic of China

Mei-Chin Lai,¹ Sheng-Chung Chen,¹ Chin-Ming Shu,¹ Ming-Shing Chiou,²
Chia-Chi Wang,¹ Ming-Jen Chuang,¹ Tong-Yung Hong,¹ Chia-Chi Liu,¹
Li-Jane Lai¹ and Jack Jay Hua²

Author for correspondence: Mei-Chin Lai. Tel: +886 4 22840419 ext. 612. Fax: +886 4 22874740.
e-mail: mclai@dragon.nchu.edu.tw

Two novel hydrogenotrophic methanogens, designated strains P2F9704a^T and P2F9705, were isolated from an estuary in Eriln Shi, Taiwan. The cells of strain P2F9704a^T were non-motile, irregular cocci 0.9–1.4 µm in diameter. They stained Gram-negative. The cells catabolized formate and H₂+CO₂ to produce methane, but did not utilize acetate, methanol, trimethylamine, ethanol or secondary alcohols as methanogenic substrates. The optimal growth parameters for strain P2F9704a^T were pH 6.7, 37 °C and 0.5% NaCl. Acetate was required for cell growth even though it was not a substrate for methanogenesis. The trace element tungsten was not required but slightly stimulated the growth of strain P2F9704a^T. However, tungsten extended the growth ranges relating to temperature, pH and salt. The sequences of the 16S rRNA genes of strains P2F9704a^T and P2F9705 were nearly identical and possessed 99.1 and 98.5% similarity to the genes of *Methanocalculus pumilus* and *Methanocalculus halotolerans*, respectively. In addition, strain P2F9704a^T possessed 14 and 12% DNA relatedness with respect to *Methanocalculus pumilus* and *Methanocalculus halotolerans*, respectively. In addition, the optimal salt concentrations, the cellular protein profiles and the molecular masses of surface-layer protein subunits of strain P2F9704a^T were different from those of the other two known *Methanocalculus* species. On the basis of these observations, it is proposed that these two organisms should be placed in a new species, namely *Methanocalculus taiwanensis*. The type strain is P2F9704a^T (= OCM 671^T = CCRC 16182^T = DSM 14663^T).

Keywords: archaea, methanogen, *Methanocalculus*, estuarine, tungsten

INTRODUCTION

Strictly anaerobic methanogens are the only archaea that are truly ubiquitous. Methanogenic species have been isolated from virtually every habitat in which anaerobic biodegradation of organic compounds occurs, including freshwater and marine sediments, the digestive and intestinal tracts of animals, and anaerobic waste digesters (Boone *et al.*, 1993b; Ferry, 1997; Jones *et al.*, 1987; Wolfe, 1996). Moreover, isolates have also been obtained from extreme environ-

ments such as geothermal springs, deep-sea hydrothermal vents and hypersaline environments (Boone *et al.*, 1993a; Jones *et al.*, 1983; Huber *et al.*, 1982; Ollivier *et al.*, 1994; Stetter *et al.*, 1993).

The order *Methanomicrobiales* comprises three families (*Methanomicrobiaceae*, *Methanocorpusculaceae* and *Methanospirillaceae*) and nine genera (Boone *et al.*, 1993b; Rouvière *et al.*, 1992) of hydrogenotrophic methanogens (Garcia *et al.*, 2000). The family *Methanomicrobiaceae* contains seven genera, i.e. *Methanomicrobium*, *Methanolacinia*, *Methanogenium*, *Methanoculleus*, *Methanoplanus*, *Methanofollis* and *Methanocalculus*. The morphology of cells in the family *Methanomicrobiaceae* includes small rods, highly irregular cocci, and plane-shaped cells. The cell walls are proteinaceous. All strains can use H₂ + CO₂

Published online ahead of print on 7 June 2002 as DOI 10.1099/ijse.0.01730-0.

The GenBank accession numbers for the 16S rDNA sequences of strains P2F9704a^T and P2F9705 are AF172443 and AF411470, respectively.

and formate as substrates of methanogenesis. In addition, some can use secondary alcohols (Garcia *et al.*, 2000). Among them is *Methanocalculus*, a newly described genus that was first proposed by Ollivier *et al.* (1998); it encompasses the irregular cocci of *Methanocalculus halotolerans*, which was isolated from an offshore oil well. *Methanocalculus halotolerans* grows optimally at 5% NaCl but tolerates NaCl concentrations from 0 to 12%. This growth range is the widest reported, to date, for any hydrogenotrophic methanogen (Garcia *et al.*, 2000; Ollivier *et al.*, 1998). Recently, another *Methanocalculus* species (*Methanocalculus pumilus*) was isolated from a waste-disposal site in Japan. It grows optimally at 1% NaCl and tolerates only concentrations up to 7% NaCl (Mori *et al.*, 2000). Here, we report the isolation and characterization of two new *Methanocalculus* isolates, strains P2F9704a^T and P2F9705, from the estuarine environment of Eriln Shi, Taiwan.

METHODS

Source of organisms. *Methanocalculus pumilus* MHT-1^T (= DSM 12632^T = JCM 10627^T) was kindly provided by Dr Koji Mori of Gifu University, Japan. *Methanocalculus halotolerans* SEBR 4845^T (= OCM 470^T) was obtained from the Oregon Collection of Methanogens, USA.

Sampling site. The sampling site was the estuary environment at Eriln Shi, Taiwan. The water temperature in this subtropical environment was 30–34 °C during the summer, and the salinity at this estuary was around 1 ‰ (w/v). The sample was collected in a stainless steel sampling basket. From there, it was immediately transferred to a sterile Oak-Ridge bottle that had been equilibrated overnight inside a Coy anaerobic chamber with an N₂/CO₂ ratio of 4:1. The sample was then transported (within 3 h) to the laboratory under anoxic conditions.

Media and culture techniques. The modified anaerobic technique of Hungate was utilized (Balch *et al.*, 1979; Sowers & Noll, 1995). Sterilized media were prepared under an oxygen-free N₂/CO₂ (4:1) atmosphere. The MB medium used contained the following (per litre deionized water): MgCl₂·6H₂O, 1.0 g; KCl, 0.5 g; NaCl, 5 g; CaCl₂·2H₂O, 0.1 g; K₂HPO₄, 0.4 g; NH₄Cl, 1.0 g; cysteine·HCl, 0.25 g; NaHCO₃, 4.0 g; yeast extract, 2 g; tryptone, 2 g; and resazurin, 0.001 g. Vitamin (Wolin *et al.*, 1963) and trace-element (Ferguson & Mah, 1983) solutions without tungstate were each added to a final concentration of 1 ‰ (v/v). The pH of the MB medium was 7.0. An MB/W medium was made from MB medium prepared with a 1 ‰ (v/v) trace-element solution containing tungstate (Na₂WO₄, 0.3 mg l⁻¹). MM medium was MB medium without the addition of yeast extract and tryptone. All of the constituents except sulfide were dissolved in water, boiled and then cooled under an oxygen-free N₂/CO₂ (4:1) atmosphere. The medium was distributed into serum bottles (Wheaton Scientific) or Hungate tubes (Belleco Glass) under the same atmosphere. The anaerobic tubes were then sealed and autoclaved at 121 °C for 20 min. Sodium sulfide from a sterilized anoxic stock solution was added to a final concentration of 1 mM before inoculation. For solid roll-tube medium, agar was added at 20 g l⁻¹. To measure the effect of pH on growth, the ratio of N₂ to CO₂ in the gas phase and the concentration of

NaHCO₃ in the medium were modified to obtain pH values between 5.6 and 8.3.

Enrichment and isolation. The enrichment was begun immediately after the sample had been brought to the laboratory. In an anaerobic chamber, the estuarine water sample (5 ml) was added to 160 ml serum bottles that contained 45 ml MB medium with sodium formate (50 mM) as the catabolic substrate. Vancomycin (100 mg l⁻¹) was added to inhibit the growth of non-methanogenic organisms. The culture was incubated in the dark at room temperature for one month. A large amount of methane was detected in the cultures; 5 ml culture was then transferred anaerobically into a new bottle of sterile MB medium with formate. This procedure was repeated for four successive transfers. The culture was then diluted and transferred into molten MB agar, and the roll-tube technique was performed. Colonies grew on the inner wall of the glass tube after 2–3 weeks. In a Coy anaerobic chamber, colonies were picked with disposable, sterilized inoculation needles and transferred to anaerobic tubes containing 5 ml MB medium. Cultures from a single colony were further incubated at 30 °C for 1–2 weeks. Two methane-producing cultures, derived from two morphological distinct colonies, were further purified with repeated serial dilutions with vancomycin until it was free of contamination by non-methanogenic bacteria. The axenic nature of the culture was established on the basis of microscopic examination, the presence of a single colony type in roll tubes, and the absence of growth in anaerobically prepared Bacto thioglycollate medium.

Determination of catabolic substrates. The catabolic substrates tested under N₂/CO₂ (4:1) were sodium formate (100 mM), sodium acetate (50 mM), trimethylamine (40 mM), methanol (50 mM), ethanol (48 mM), 2-propanol (48 mM), iso-butanol (48 mM) and 2-butanol (48 mM). H₂ was tested for by pressurizing the culture tubes with H₂ (100%, 200 kPa). Utilization of the substrates was determined in MB/W media by monitoring methane production. Methane production was determined by GC with flame-ionization detection (Lai *et al.*, 1999).

Antibiotic susceptibility. The sensitivity of strain P2F9704a^T to ampicillin, penicillin, spectinomycin, kanamycin, tetracycline and chloramphenicol (each at a concentration of 100 µg ml⁻¹) was tested in MB/W medium with sodium formate (100 mM) at 37 °C. Growth was determined from methane production.

Whole-cell protein profile and surface-layer protein study. Whole-cell proteins were extracted from cell pellets by adding loading buffer containing 4% sodium dodecyl sulfate at a ratio of 1 ml buffer per OD unit. An OD unit was the amount of cells found in 1 ml culture with an absorbance of 1.0. Surface-layer proteins were isolated according to the protocol of König (1995). SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with Coomassie blue R-250.

Determination of growth rates. Specific growth rates were calculated from the methane production, which was analysed by linear regression of the logarithm of the total amount of methane that accumulated over time (Lai *et al.*, 2000). Inocula were grown under conditions similar to the experimental conditions.

Microscopy. An Olympus BH-2 microscope was used for phase-contrast microscopy. Preparations for negative staining and ultrathin sectioning were performed as described

previously (Lai & Shih, 2001). Electron micrographs were taken using a model JEM-1200EXII and 200cx (JEOL) equipment. For scanning electron micrographs, samples were prepared as described previously (Lai & Chen, 2001), and cells were sputter-coated with gold and observed with a Topcon model ABT-150S scanning electron microscope.

Phylogenetic analysis. DNAs from strains P2F9704^T and P2F9705 were isolated by the general procedure of Jarrell *et al.* (1992). Approximately 30 ng DNA was used as a template for PCR amplification of an approximately 1300 bp portion of the 16S rRNA gene. The PCR amplification primers used for strain P2F9704^T were 5'-GCTCAGTAACACGTG GATAACC-3' and 5'-GCAGATTCCCCTACGGCTACC-3'. The sequences were checked by the CHECK-PROBE program from the Ribosomal Database Project (Maidak *et al.*, 1996), and corresponded to positions 20–40 and 1444–1424 in the 16S rDNA (rDNA) nucleotide sequence of *Methanocalculus halotolerans* SEBR 4845^T (accession no. AF033672). For strain P2F9705, the PCR amplification primers used were 5'-CGACTAAGCCATGCGAGTC-3' and 5'-GTGACGGG-CGGTGTGTGCAAG-3', which corresponded to positions 34–52 and 1338–1320 in the 16S rDNA nucleotide sequence of *Methanocalculus halotolerans*. The following primers were also used for sequencing: 5'-GCTCAGTAACACGTGGA-TAACC-3', 5'-CGACTAAGCCATGCGAGTC-3', 5'-TAGACTTGGGACCGGGAGAGG-3', 5'-TGGGTCTCGC-T CGTTG-3', 5'-GTGACGGGCGGTGTGTGCAAG-3' and 5'-GCAGATTCCCCTACGGCT ACC-3' (*Methanocalculus halotolerans* SEBR 4845^T, sequence positions 20–42, 34–52, 572–592, 979–964, 1338–1320 and 1444–1424, respectively). The resulting sequences were assembled to produce an approximately 1369 bp contiguous rDNA sequence for strain P2F9704^T and 1260 bp sequence for strain P2F9705. The gene sequences of the archaea used were obtained from the GenBank database. The similarity matrix was obtained based on the analytic results of the Ribosomal Database Project (<http://rdp.cme.msu.edu.htm/>). Multiple sequence alignments were analysed by using the CLUSTAL W package at the Biology Workbench (<http://workbench.sdsc.edu/>). Distances were computed with the CLUSTAL TREE package at the same website, using the neighbour-joining model, and fed to the program DRAWGRAM of the program package PHYLIP version 3.5c (Felsenstein, 1995). A bootstrap confidence analysis was performed with the SEQBOOT program of the PHYLIP package by using 500 replicates.

DNA–DNA hybridization. Cells of *Methanocalculus* species were harvested at late exponential phase and used for DNA isolation. DNA was isolated and purified by a modification of the methods of Jarrell *et al.* (1992) and Johnson (1985). DNA–DNA hybridization experiments were performed by using the dot-blot technique (Sambrook & Russell, 2001) with a VersiTag fluorescein labelling system (NEN Life Science). Target DNA (500 ng) denatured by 0.8 M NaOH was blotted on to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech), and the labelled DNA was reassociated in a solution containing 50% formamide, 5 × Denhardt's solution (50 × Denhardt's contains 1% Ficoll, 1% polyvinylpyrrolidone and 1% bovine serum albumin) and 0.5% (w/v) SDS in 5 × standard saline citrate buffer (1 × standard saline citrate is 0.15 M NaCl plus 0.015 M sodium citrate). After incubation overnight at 42 °C, the blots were analysed with a Renaissance nucleic acid chemiluminescence reagent with an anti-fluorescein horseradish peroxidase conjugate detection system supplied by NEN Life Science. Hybridization signals were detected by autoradiography. Duplicate tests were performed for each assay,

and self-hybridization of the probe with homologous target DNA was set to 100%.

RESULTS

Enrichment and isolation

Water samples (5 ml) from the estuarine environment of Eriln Shi, Taiwan, were inoculated into eight bottles of MB medium (45 ml) containing methanol, trimethylamine, acetate or formate as methanogenic substrate. After one month incubation at room temperature, methanogenesis occurred in all enrichments. The formate enrichment was further enriched by four successive transfers, and then this culture was inoculated into roll-tube MB agar medium for further isolation. Under the fluorescence microscope, two fluorescent-positive colonies were picked and transferred to 5 ml MB medium with formate in a Coy anaerobic chamber. One was a large, yellowish, circular colony (strain P2F9704^T). The other colony possessed an irregular margin (strain P2F9705). Methane-producing cultures from the single colonies were further purified by serial dilution in medium with vancomycin until contamination by non-methanogens was not detectable.

Morphology, S-layer, and cell structure

Cells of strain P2F9704^T were non-motile, irregular cocci 0.9–1.4 µm in diameter. Strain P2F9705 also comprised irregular cocci, but the cells were motile and possessed flagella. When observed under the scanning electron microscope, the cell shape for strain P2F9704^T often resembled a soy-sauce dish (Fig. 1a); interestingly, angular edges were observed under negative staining (Fig. 1b). Refractive areas were observed under light microscopy of both strain P2F9704^T (Fig. 1c) and strain P2F9705.

Both strains stained as Gram-negative and lysed immediately upon the addition of SDS to 0.01% (w/v), indicating that a proteinaceous cell wall was present (Boone & Whitman, 1988). Ultrathin sections of strain P2F9704^T showed that the S-layer was closely associated with the cytoplasmic membrane (Fig. 1d). In this regard, cells of P2F9704^T resembled *Methanocalculus halotolerans* and differed from cells of *Methanocalculus pumilus* (Mori *et al.*, 2000; Ollivier *et al.*, 1998). The S-layer protein of strain P2F9704^T was composed by two different subunits, while the S-layers of *Methanocalculus halotolerans* and *Methanocalculus pumilus* were composed of only a single subunit. The molecular masses of the S-layer proteins of strain P2F9704^T, *Methanocalculus halotolerans* and *Methanocalculus pumilus* were as follows: 112 000 and 95 100; 91 800; and 94 300, respectively (see Table 1).

Sensitivity to antibiotics

Strains P2F9704^T and P2F9705 were both resistant to ampicillin, penicillin, kanamycin, tetracycline and spectinomycin and sensitive to chloramphenicol.

Fig. 1. (a) Scanning electron micrograph of strain P2F9704a^T (bar, 0.33 µm). (b) Negatively stained cell of strain P2F9704a^T (bar, 0.2 µm). (c) Phase-contrast micrograph of strain P2F9704a^T, showing irregular coccoid cells with refractive areas (bar, 2.5 µm). (d) Transmission electron micrograph of an ultrathin section of strain P2F9704a^T (bar, 0.1 µm).

Table 1. Characteristics of *Methanocalculus* species

Strains: 1, strain P2F9704a^T; 2, strain P2F9705; 3, *Methanocalculus halotolerans* SEBR 4845^T; 4, *Methanocalculus pumilus* MHT-1^T. All strains are irregular cocci, use H₂/CO₂ and sodium formate as substrates for methanogenesis, require acetate, and are lysed by addition of SDS.

Character/reference	1	2	3	4
Cell size (µm)	0.9–1.4	0.8–1.4	0.8–1.0	0.8–1.0
Flagella	—	+	+	—
Refractive area*	Yes	Yes	Yes	ND
Temp. optimum/range (°C)	37/25–42	37/28–37	38/25–45	35/24–45
pH optimum/range	6.7/5.6–8.3	7.1/ND	7.6/7.0–8.4	6.5–7.5/ND
NaCl optimum/range (%)	0.5/0–4	1.0/0–3	5.0/0–12	1.0/0–7
S-layer (<i>M_r</i>)	111 800; 95 100	ND	91 800	94 300
Habitat	Estuary	Estuary	Oilfield	Waste disposal site
Reference	This study	This study	Ollivier <i>et al.</i> (1998)	Mori <i>et al.</i> (2000)

ND, Not determined.

* Refractive area observed by phase microscopy.

Fig. 2. Influence of temperature (a), pH (b) and salinity (c) on the growth of strain P2F9704a^T in MB medium (○) and in MB/W medium (●) with formate as the catabolic substrate. Specific growth rates were calculated from methane production, and are the means of triplicate cultures.

Growth substrates, requirements and optimal growth conditions

Strain P2F9704a^T can use only formate and H₂ + CO₂ to produce methane. Under an N₂/CO₂ atmosphere, it could not produce methane from acetate, methanol, trimethylamine, ethanol, 2-propanol, iso-butanol or 2-butanol. Acetate (20 mM) was required for cell growth. The doubling times of strain P2F9704a^T in minimal medium (MM/W) and in MB/W (containing yeast extract and tryptone) with formate plus acetate were 8.2 and 7.1 h, respectively. Without the addition of acetate in the MM/W medium, cells could not be subcultured more than twice.

The optimal growth temperature for strain P2F9704a^T was 37 °C, but it did not grow at temperatures below

Fig. 3. Cell-protein profile of *Methanocalculus* species determined by SDS-PAGE. Lane 1, strain P2F9704a^T; lane 2, *Methanocalculus halotolerans*; lane 3, *Methanocalculus pumilus*. Lane M, protein marker. Arrows indicate polypeptide bands with significant differences between strain P2F9704a^T and the other *Methanocalculus* species.

28 °C or above 45 °C (Fig. 2a). Upon the addition of the trace element tungsten, the optimal growth temperature remained at 37 °C. However, the minimum growth temperature was extended to 25 °C (Fig. 2a). Cells grew over a wide range of pH, from 6.3 to 8.3; the optimal pH for growth was 6.7, growth at higher pH values being poor (Fig. 2b). The addition of tungsten increased the pH tolerance of strain P2F9704a^T, and cells grew well over a pH range of 5.6 to 8.3 (Fig. 2b). Strain P2F9704a^T tolerated NaCl concentrations of 0.1 and 3.0%. The optimal salt concentration for growth was 0.5% (Fig. 2c). A similar tungsten effect also occurred with regard to salt tolerance. With the addition of tungsten, the optimal salt concentration for growth remained at 0.5%, but cells also grew well within a 0–4% NaCl range (Fig. 2c). Growth was not observed at 6% NaCl. Although tungsten was not required for cell growth, it stimulated cell growth slightly. Under optimal growth conditions (0.5% NaCl, pH 6.7 and 37 °C), the doubling times of strain P2F9704a^T grown in MB and MB/W were 13.4 and 11.4 h, respectively.

Whole-cell protein analysis

Whole-cell protein from strain P2F9704a^T, *Methanocalculus halotolerans* and *Methanocalculus pumilus* was extracted and analysed. As indicated in Fig. 3, the

Fig. 4. Phylogenetic relationships of strains P2F9704a^T, P2F9705 and related species, based on the 16S rRNA gene sequences. The PHYLIP program was used to estimate phylogenetic distance from the distance-matrix data. Bootstrap values at nodes are the percentages of 500 replicates. Scale bar, base substitutions per 100 bases.

protein profile of P2F9704a^T was distinct from those of the other two *Methanocalculus* species.

16S rRNA gene analysis

The 16S rDNAs of strain P2F9704a^T (1369 nt) and P2F9705 (1260 nt) were sequenced and shown to possess 99.7% sequence similarity. The phylogenetic tree was constructed using a selection of different methanogen sequences obtained from the GenBank database. These two new isolates were shown to be close relatives of *Methanocalculus halotolerans* and *Methanocalculus pumilus* (Fig. 4). The similarities of the 16S rDNA sequences of strain P2F9704a^T to *Methanocalculus pumilus* and *Methanocalculus halotolerans* sequences were 99.1 and 98.5, respectively.

DNA–DNA hybridization

In DNA–DNA hybridization experiments, the DNA of strain P2F9704a^T exhibited 14% hybridization with the DNA of *Methanocalculus pumilus* and 12% hybridization with the DNA of *Methanocalculus halotolerans*.

DISCUSSION

Two mesophilic, hydrogenotrophic methanogens, strains P2F9704a^T and P2F9705, were isolated from water samples from an estuarine environment in Eriln Shi, Taiwan. Comparison of 16S rRNA sequences indicated that these two new strains were phylogenetically related to *Methanocalculus pumilus* and *Methanocalculus halotolerans* and distinct from the other known methanogens (less than 91% similarity). Characteristics that differentiate strains P2F9704a^T and P2F9705 from *Methanocalculus halotolerans* and *Methanocalculus pumilus* are listed in Table 1. They are all mesophilic, neutrophilic methanogens that use only

formate and H₂+CO₂ as catabolic substrates and require acetate for cell growth. The 16S rRNA sequence similarities of strain P2F9704a^T with respect to *Methanocalculus pumilus* and *Methanocalculus halotolerans* were 99.1 and 98.5%, respectively. Although, these differences in the 16S rRNA sequences do not strongly support the formation of a new species (Stackebrandt & Goebel, 1994), the formation of a new species is strongly indicated by the DNA–DNA hybridization analysis that revealed 14% relatedness with the DNA to *Methanocalculus pumilus* and 12% relatedness to that of *Methanocalculus halotolerans*. Additionally, the molecular mass of the S-layer protein (Table 1), the whole-cell protein profiles (Fig. 3) and the optimal salt range for growth of strain P2F9704a^T were different from those of *Methanocalculus halotolerans* and *Methanocalculus pumilus*. These phylogenetic, phenotypic and physiological distinctions indicate that strain P2F9704a^T may represent a new *Methanocalculus* species, which we have named *Methanocalculus taiwanensis* (as it is the first new methanogenic species isolated from Taiwan). In addition, strain P2F9705 (= OCM 672) is a member of the same (or a closely related) species.

The stimulatory effect of tungsten on the growth of methanogens was first reported in the late 1970s for *Methanococcus vannielii* (Jones & Stadtman, 1977). Cell growth was dramatically enhanced by the addition of tungsten when formate was the carbon and energy source, but not during growth on H₂ and CO₂. Within the order *Methanomicrobiales*, growth stimulated by tungsten has been described for *Methanofollis liminatans* (Zellner *et al.*, 1990, 1999) and *Methanofollis aquaemaris* (Lai & Chen, 2001). Also, growth that required tungsten has been reported for *Methanoculleus palmolei* (Zellner *et al.*, 1998), *Methanocorpusculum parvum* (Zellner *et al.*, 1987) and *Methanofollis tationis* (Zabel *et al.*, 1984). The effect of tungsten in *Methanocalculus* has not been observed before, and

our observation of strain P2F9704a^T revealed that tungsten was not required but does slightly stimulate cell growth. Moreover, the addition of tungsten increased cells' capacity to tolerate extremes of environmental pH, temperature and salt (Fig. 2). These results indicated that tungsten is not only important in the survival of cells but also in facing environmental challenges.

Most halotolerant and halophilic methanogenic species that have been described are methylotrophic (Boone *et al.*, 1993a; Zhilina, 1986), and it was suggested by Ollivier *et al.* (1994) that the use of methylotrophic substrates by methane-producing bacteria in halophilic environments predominates over H₂ and acetate utilization. *Methanocalculus halotolerans* grows optimally at 5% NaCl (w/v) and tolerates up to 12% NaCl, a range which is the widest reported, to date, for any hydrogenotrophic methanogen (Ollivier *et al.*, 1998). Therefore, Ollivier *et al.* (1998) described the new genus *Methanocalculus* on the basis of this salt tolerance. However, Mori *et al.* (2000) recently reported that *Methanocalculus pumilus*, a heavy-metal-tolerant methanogen isolated from a waste-disposal site, grew optimally at 1.0% NaCl and only tolerated a salt range of 0–7% NaCl. Here, we report two more *Methanocalculus* isolates, strains P2F9704a^T and P2F9705, from the estuarine environment of Eriln Shi, Taiwan. The optimal salt concentrations for the growth of strains P2F9704a^T and P2F9705 were 0.5% and 1.0%, respectively, and these two *Methanocalculus* strains tolerate NaCl concentrations only up to 4%. Moreover, we have also isolated and characterized two new *Methanocalculus* species from aquaculture fish-ponds (Lai & Lin, 2001). Their optimal concentration of NaCl for growth is 1.0%, but they can tolerate up to 12% NaCl. The occurrence of these new species indicated that *Methanocalculus* is widely distributed in nature and is highly diversified in terms of adaptation to salt.

Description of *Methanocalculus taiwanensis* sp. nov.

Methanocalculus taiwanensis (tai.wa.nen'sis. N.L. adj. *taiwanensis* of Taiwan, indicating the source of the type strain).

Irregular coccoid cells that stain Gram-negative, are non-motile, and are 0.9–1.4 µm in diameter. Lysed by 0.1 g SDS l⁻¹. Cells are resistant to ampicillin, penicillin, kanamycin, spectinomycin and tetracycline but sensitive to chloramphenicol. Catabolic substrates used include H₂/CO₂ and formate, but not acetate, methanol, trimethylamine, ethanol, 2-propanol, isobutanol or 2-butanol. Acetate is required for cell growth. Tungsten was not required but stimulated growth; addition of tungsten as a trace element greatly extends the ranges of temperature, pH and salt allowing growth. Optimal growth occurs in the presence of 0.5% NaCl (w/v) at pH 6.7 at 37 °C. Type strain is strain P2F9704a^T (= OCM 671^T = CCRC 16182^T = DSM 14663^T), which was isolated from the

water of an estuary in Eriln Shi near Wong-Kong, Taiwan. The reference strain is strain P2F9705.

ACKNOWLEDGEMENTS

The authors would like to thank Pei-Chi Chao and Yen-Shiun Lin from the Regional Instruments Centre at National ChungHsing University for operating the electron microscopes. We thank Dr Pi-Fang Linda Chang from the department of Plant Pathology of National ChungHsing University for help and supplies during the DNA–DNA hybridization experiments. This work was partially supported by the Council of Agriculture, The Executive Yuan of the Republic of China. M.-C.L. and S.-C.C. contributed equally to this paper.

REFERENCES

- Balch, W. E., Fox, G. E., Margum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Boone, D. R. & Whitman, W. B. (1988). Proposal of minimal standards for describing new taxa of methanogenic bacteria. *Int J Syst Bacteriol* **38**, 212–219.
- Boone, D. R., Mathrani, I. M., Liu, Y., Menaia, J. A. G. F., Mah, R. A. & Boone, J. E. (1993a). Isolation and characterization of *Methanohalophilus portucalensis* sp. nov. and DNA reassociation study of the genus *Methanohalophilus*. *Int J Syst Bacteriol* **43**, 430–437.
- Boone, D. R., Whitman, W. B. & Rouvière, P. (1993b). Diversity and taxonomy of methanogens. In *Methanogenesis*, pp. 35–80. Edited by J. G. Ferry. New York: Chapman & Hall.
- Felsenstein, J. (1995). PHYLIP (Phylogenetic Inference Package) version 3.57c. Seattle: University of Washington.
- Ferguson, T. J. & Mah, R. A. (1983). Isolation and characterization of an H₂-oxidizing thermophilic methanogen. *Appl Environ Microbiol* **45**, 265–274.
- Ferry, J. G. (1997). Methane: small molecules, big impact. *Science* **278**, 1413–1414.
- Huber, H., Thomm, M., König, H., Thies, G. & Stetter, K. O. (1982). *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. *Arch Microbiol* **132**, 47–50.
- Garcia, J.-L., Patel, B. K. C. & Ollivier, B. (2000). Taxonomic, phylogenetic, and ecological diversity of methanogenic archaea. *Anaerobe* **6**, 205–226.
- Jarrell, K. F. D., Hebert, F. A. M. & Kalmokoff, M. L. (1992). A general method of isolating high molecular weight DNA from methanogenic archaea (archaeobacteria). *Can J Microbiol* **38**, 65–68.
- Johnson, J. L. (1985). DNA reassociation and DNA hybridization of bacterial nucleic acids. *Methods Microbiol* **18**, 33–74.
- Jones, J. B. & Stadtman, T. C. (1977). *Methanococcus vannielii*: culture and effects of selenium and tungsten on growth. *J Bacteriol* **130**, 1404–1406.
- Jones, W. J., Leigh, J. A., Mayer, F., Woese, C. R. & Wolfe, R. S. (1983). *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from submarine hydrothermal vent. *Arch Microbiol* **136**, 254–261.
- Jones, W. J., Nagle, J. D., Jr & Whitman, W. B. (1987). Methanogens and the diversity of Archaeobacteria. *Microbiol Rev* **51**, 135–177.
- König, H. (1995). Isolation and analysis of cell walls from methanogenic archaea. In *Archaea: a Laboratory Manual*, vol. 2, *Methanogens*, pp. 315–328. Edited by K. R. Sowers & H. T. Schreier. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lai, M.-C. & Chen, S.-C. (2001). *Methanofollis aquaemaris* sp. nov., a methanogen isolated from an aquaculture fish pond. *Int J Syst Evol Microbiol* **51**, 1873–1880.

- Lai, M.-C. & Lin, C.-C. (2001). Isolation and characterization of a new methanogen from an aquaculture fishpond, I-56. In *Abstracts of the 101st General Meeting of the American Society for Microbiology*. Washington, DC: American Society for Microbiology.
- Lai, M.-C. & Shih, C.-J. (2001). Characterization of *Methanococcus voltae* strain P2F9701a, a new methanogen isolated from estuarine environment. *Curr Microbiol* **42**, 432–437.
- Lai, M.-C., Shu, C.-M., Chiou, M.-S., Hong, T.-Y., Chuang, M.-J. & Hua, J. J. (1999). Characterization of *Methanosarcina mazei* strain N2M9705 isolated from an aquaculture fishpond. *Curr Microbiol* **39**, 79–84.
- Lai, M.-C., Shu, C.-M., Chen, S.-C., Lai, L.-J., Chiou, M.-S. & Hua, J. J. (2000). *Methanosarcina mazei* strain O1M9704, methanogen with novel tubule isolated from estuarine environment. *Curr Microbiol* **41**, 15–20.
- 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.
- Mori, K., Yamamoto, H., Kamagata, Y., Hastu, M. & Takamizawa, K. (2000). *Methanocalculus pumilus* sp. nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site. *Int J Syst Evol Microbiol* **50**, 1723–1729.
- Ollivier, B., Caumette, P., Garcia, J.-L. & Mah, R. A. (1994). Anaerobic bacteria from hypersaline environments. *Microbiol Rev* **58**, 27–38.
- Ollivier, B., Fardeau, M.-L., Cayol, J.-L., Magot, M., Patel, B. K. C., Prensier, G. & Garcia, J.-L. (1998). *Methanocalculus halotolerans* gen. nov., sp. nov., isolated from an oil-producing well. *Int J Syst Bacteriol* **48**, 821–828.
- Rouvière, P., Mandelco, L., Winker, S. & Woese, C. R. (1992). A detailed phylogeny for the *Methanomicrobiales*. *Syst Appl Microbiol* **15**, 363–371.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sowers, K. R. & Noll, K. M. (1995). Techniques for anaerobic growth. In *Archaea: a Laboratory Manual*, vol. 2, *Methanogens*, pp. 15–48. Edited by K. R. Sowers & H. T. Schreier. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- 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.
- Stetter, K. O., Huber, R., Blochl, E., Kurr, M., Eden, R. D., Fielder, M., Cash, H. & Vance, L. (1993). Hyperthermophilic Archaea are thriving in deep North Sea and Alaskan reservoirs. *Nature* **365**, 743–745.
- Wolfe, R. S. (1996). 1776–1996: Alessandro Volta's combustible air. *ASM News* **62**, 529–534.
- Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1963). Formation of methane by bacterial extracts. *J Biol Chem* **238**, 2882–2886.
- Zabel, H. P., König, H. & Winter, J. (1984). Isolation and characterization of a new coccoid methanogen, *Methanogenium tationis* spec. nov. from a solfatara field on Mount Tatío. *Arch Microbiol* **137**, 308–315.
- Zellner, G., Alten, C., Stackebrandt, E., Conway de Macario, E. & Winter, J. (1987). Isolation and characterization of *Methanocorpusculum parvum* gen. nov., spec. nov., a new tungsten requiring, coccoid methanogen. *Arch Microbiol* **147**, 13–20.
- Zellner, G., Sleytr, U. B., Messner, P., Kneifel, H. & Winter, J. (1990). *Methanogenium liminatans* spec. nov., a new coccoid, mesophilic methanogen able to oxidize secondary alcohols. *Arch Microbiol* **153**, 287–293.
- Zellner, G., Messner, P., Winter, J. & Stackebrandt, E. (1998). *Methanoculleus palmolei* sp. nov., an irregularly coccoid methanogen from an anaerobic digester treating wastewater of a palm oil plant in North-Samatra, Indonesia. *Int J Syst Bacteriol* **48**, 1111–1117.
- Zellner, G., Boone, D. R., Keswani, J., Whitman, W. B., Woese, C. R., Hagelstein, A., Tindall, B. J. & Stackebrandt, E. (1999). Reclassification of *Methanogenium tationis* and *Methanogenium liminatans* as *Methanofollis tationis* gen. nov., comb. nov. and *Methanofollis liminatans* comb. nov. and description of a new strain of *Methanofollis liminatans*. *Int J Syst Bacteriol* **49**, 247–255.
- Zhilina, T. N. (1986). Methanogenic bacteria from hypersaline environments. *Syst Appl Microbiol* **7**, 216–222.