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

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

1 Department of Botany, National Chung Hsing University, Taichung, 40227 Taiwan, Republic of China
2 Food Research & Development Institute, Hsinchu, Taiwan, Republic of China

Two novel hydrogenotrophic methanogens, designated strains P2F9704aT and P2F9705, were isolated from an estuary in Eriln Shi, Taiwan. The cells of strain P2F9704aT 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 P2F9704aT 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 P2F9704aT. However, tungsten extended the growth ranges relating to temperature, pH and salt. The sequences of the 16S rRNA genes of strains P2F9704aT 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 P2F9704aT 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 P2F9704aT 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 P2F9704aT (= OCM 671T = CCRC 16182T = DSM 14663T).

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

INTRODUCTION

Strictly anaerobic methanogens are the only archaean 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 environments 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, Methanofovillus 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/ijs.0.01730-0.
The GenBank accession numbers for the 16S rDNA sequences of strains P2F9704aT and P2F9705 are AF172443 and AF411470, respectively.

Keywords: archaea, methanogen, Methanocalculus, estuarine, tungsten
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 P2F9704aT and P2F9705, from the estuarine environment of Eriln Shi, Taiwan.

METHODS

Source of organisms. Methanocalculus pumilus MHT-1T (= DSM 12632T = JCM 10627T) was kindly provided by Dr Koji Mori of Gifu University, Japan. Methanocalculus halotolerans SEBR 4845T (= OCM 470T) 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/O2 (4:1) atmosphere. The MB medium was made from Bacto thioglycollate medium, prepared Bacto NaHCO3 solution containing tungstate (Na2WO4, 0.3 mg 1−1). 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. SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with Coomasie blue R-250.

Determination of growth rates. Specific growth rates were determined in MB medium with 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 medium by monitoring 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. SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with Coomasie 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 P2F9704a\(^\text{a}\) 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 P2F9704a\(^\text{a}\) were 5'-GCTCAGTAAACACGTGGATAACC-3' and 5'-GCAGATTCCTACGGTCTACC-3'.

The sequences were checked by the CHECK-PROC program from the Ribosomal Database Project (http://rdp.cme.msu.edu/). Multiple sequence alignments were analysed by using the CLUSTAL W package at the Biology Workbench (http://workbench.sdsc.edu/). Distances were computed with the 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 fluorescent 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 P2F9704a\(^\text{a}\)). 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 P2F9704a\(^\text{a}\) 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 P2F9704a\(^\text{a}\) 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 P2F9704a\(^\text{a}\) (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 P2F9704a\(^\text{a}\) showed that the S-layer was closely associated with the cytoplasmic membrane (Fig. 1d). In this regard, cells of P2F9704a\(^\text{a}\) resembled *Methanocalculus halotolerans* and differed from cells of *Methanocalculus pumilus* (Mori et al., 2000; Ollivier et al., 1998). The S-layer protein of strain P2F9704a\(^\text{a}\) 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 P2F9704a\(^\text{a}\), *Methanocalculus halotolerans* and *Methanocalculus pumilus* were as follows: 112000 and 95100; 91800; and 94300, respectively (see Table 1).

**Sensitivity to antibiotics.**

Strains P2F9704a\(^\text{a}\) 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<sup>T</sup> (bar, 0.33 µm). (b) Negatively stained cell of strain P2F9704a<sup>T</sup> (bar, 0.2 µm). (c) Phase-contrast micrograph of strain P2F9704a<sup>T</sup>, showing irregular coccolid cells with refractive areas (bar, 2.5 µm). (d) Transmission electron micrograph of an ultrathin section of strain P2F9704a<sup>T</sup> (bar, 0.1 µm).

Table 1. Characteristics of *Methanocalculus* species

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

ND, Not determined.

* Refractive area observed by phase microscopy.
Methanocalculus taiwanensis sp. nov.

Fig. 2. Influence of temperature (a), pH (b) and salinity (c) on the growth of strain P2F9704aT 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 P2F9704aT 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 P2F9704aT 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 P2F9704aT was 37 °C, but it did not grow at temperatures below 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 P2F9704aT, and cells grew well over a pH range of 5·6 to 8·3 (Fig. 2b). Strain P2F9704aT 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 P2F9704aT grown in MB and MB/W were 13·4 and 11·4 h, respectively.

Whole-cell protein analysis

Whole-cell protein from strain P2F9704aT, Methanocalculus halotolerans and Methanocalculus pumilus was extracted and analysed. As indicated in Fig. 3, the
protein profile of P2F9704a\(^{\text{T}}\) was distinct from those of the other two *Methanocalculus* species.

### 16S rRNA gene analysis

The 16S rDNAs of strain P2F9704a\(^{\text{T}}\) (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\(^{\text{T}}\) 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\(^{\text{T}}\) 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\(^{\text{T}}\) and P2F9705, were isolated from water samples from an estuarine environment in Erlih 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\(^{\text{T}}\) and P2F9705 from *Methanocalculus halotolerans* and *Methanocalculus pumilus* are listed in Table 1. They are all mesophilic, neutrophilic methanogens that use only formate and H\(_2\) + CO\(_2\) as catabolic substrates and require acetate for cell growth. The 16S rRNA sequence similarities of strain P2F9704a\(^{\text{T}}\) 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\(^{\text{T}}\) were different from those of *Methanocalculus halotolerans* and *Methanocalculus pumilus*. These phylogenetic, phenotypic and physiological distinctions indicate that strain P2F9704a\(^{\text{T}}\) 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 H\(_2\) and CO\(_2\). Within the order *Methanomicrobiales*, growth stimulated by tungsten has been described for *Methanollis limina* (Zellner et al., 1990, 1999) and *Methanollis aquaeamars* (Lai & Chen, 2001). Also, growth that required tungsten has been reported for *Methanoculleus palmolei* (Zellner et al., 1998), *Methanococpus parvum* (Zellner et al., 1987) and *Methanollis tationis* (Zabel et al., 1984). The effect of tungsten in *Methanocalculus* has not been observed before, and
our observation of strain P2F9704aT 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 methylo trophic (Boone et al., 1993a; Zhilina, 1986), and it was suggested by Ollivier et al. (1994) that the use of methylo trophic substrates by methane-producing bacteria in halophilic environments predominate 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 P2F9704aT and P2F9705, from the estuarine environment of Eriln Shi, Taiwan. The optimal salt concentrations for the growth of strains P2F9704aT 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 fishponds (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 cocccid 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 P2F9704aT (= OCM 671T = CCRC 16182T = DSM 14663T), 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 Chung Hsing University for operating the electron microscopes. We thank Dr Pi-Fang Linda Chang from the department of Plant Pathology of National Chung Hsing 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**


M.-C. Lai and others


