Methanocalculus chunghsingensis sp. nov., isolated from an estuary and a marine fishpond in Taiwan

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Three novel halotolerant, hydrogenotrophic methanogens, designated strains K1F9705b^T, K1F9705c and O1F9704a, were isolated from an estuary in Eriln Shi, Taiwan, and from a nearby marine water aquaculture fishpond. These isolates were irregular cocci that stained Gram-negative. Strains K1F9705b^T and K1F9705c were non-motile, but strain O1F9704a was weakly motile with flagella. They were able to use formate and H₂/CO₂ to form methane, but they could not catabolize acetate, methanol, trimethylamine or secondary alcohols. Acetate was required for cell growth. Tungsten greatly stimulated the growth of strains K1F9705b^T and K1F9705c, but did not affect the growth of strain O1F9704a. Optimal pH and temperature for growth of these three isolates were respectively 7.2 and 37 °C. Optimal NaCl concentration for growth was 0.5 % for strain O1F9704a and 1.0 % for strains K1F9705c and K1F9705b^T. Moreover, all strains grew well at up to 8–12 % NaCl. Analysis of the 16S rRNA gene revealed that these isolates are members of the genus *Methanocalculus*, but are distinct from *Methanocalculus taiwanensis*, *Methanocalculus pumilus* and *Methanocalculus halotolerans*, with sequence similarities of 98.4, 98.3 and 98.2 %, respectively. In addition, strain K1F9705b^T possessed 85, 80, 37, 29 and 10 % DNA–DNA relatedness to strain K1F9705c, strain O1F9704a, *M. pumilus*, *M. halotolerans* and *M. taiwanensis*, respectively. Analysis of protein profiles and the *M_r* of surface (S)-layer glycoprotein subunits showed that these three new isolates are closely related to, but distinct from, known *Methanocalculus* species. A novel species, *Methanocalculus chunghsingensis* sp. nov., is proposed for strains K1F9705b^T, K1F9705c and O1F9704a. The type strain is K1F9705b^T (= OCM 772^T = DSM 14646^T).

Ollivier *et al.* (1998) first proposed the genus *Methanocalculus*, with *Methanocalculus halotolerans*, a hydrogenotrophic, halotolerant methanogen that was isolated from an oil-producing well, as the type species. Recently, *Methanocalculus pumilus*, a heavy-metal-tolerant methanogen that was isolated from a waste-disposal site (Mori *et al.*, 2000), and *Methanocalculus taiwanensis* strains P2F9704a^T and P2F9705, isolates from an estuary in Eriln Shi, near Wang-gong, Taiwan (Lai *et al.*, 2002), were reported. All these new *Methanocalculus* isolates are hydrogenotrophic methanogens with an irregularly coccoid shape. They all require acetate for growth and grow optimally at mesophilic

temperatures and neutral pH. The following *Methanocalculus* species grow in a relatively restricted NaCl range: *M. taiwanensis* and *M. pumilus* grow optimally at 1 % NaCl and can only tolerate up to 4 and 7 % NaCl, respectively (Mori *et al.*, 2000; Lai *et al.*, 2002). In contrast, *M. halotolerans* grows optimally at 5 % NaCl and can tolerate salt concentrations of 0–12 %. Such a wide salt-tolerance range is the widest reported to date for any hydrogenotrophic methanogen (Ollivier *et al.*, 1998; Garcia *et al.*, 2000). In this study, we report the characterization of three novel methanogenic strains, K1F9705b^T, K1F9705c and O1F9702c, that were isolated from an estuary and a marine fishpond in Taiwan. Phylogenetic evidence, DNA–DNA hybridization and phenotypic characteristics showed that these three strains constitute a novel species of the genus *Methanocalculus*, for which we propose the name *Methanocalculus chunghsingensis* sp. nov.

Sampling sites were the estuary environment at Eriln Shi, Wong-gong, Taiwan, and a nearby marine water aquaculture fishpond. Water temperature in this subtropical

Published online ahead of print on 18 July 2003 as DOI 10.1099/ij.s.0.02761-0.

Abbreviations: S-layer, surface layer; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains K1F9705b^T, K1F9705c and O1F9704a determined in this study are AF347025, AF321115 and AY234332, respectively.

environment was 30–34 °C during summer and salinity at this estuary was around 1 ‰ (w/v). The water source of the aquaculture fishpond was mixed sea water and ground-water, which was pumped into a man-made pond for the mixed cultivation of *Chanos chanos* (white mullet) and *Meretrix lusoria* (Lai & Chen, 2001). Strains K1F9705b^T and K1F9705c were isolated from sediment samples of the aquaculture fishpond, whereas strain O1F9704a was isolated from a sediment sample from the estuary. Samples were collected in a stainless-steel sampling basket. They were then transferred immediately 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 bottles were filled to the top, sealed and returned to the laboratory for processing within 3 h.

The modified anaerobic technique of Hungate (Balch *et al.*, 1979; Sowers & Noll, 1995) was utilized in this study. Sterilized media were prepared under an oxygen-free N₂/CO₂ (4:1) atmosphere. MB medium contained the following [(1 deionized water)⁻¹]: 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, 1 mg. Vitamin solution (Wolin *et al.*, 1963) and trace-element solution without tungstate (Ferguson & Mah, 1983) were each added to a final concentration of 1 ‰ (v/v). The pH of MB medium was 7.0. MB/W medium, made from MB medium, was prepared with 1 ‰ (v/v) trace-element solution that contained tungstate (Na₂WO₄, 0.3 mg l⁻¹). Minimal medium (MM) was MB medium without yeast extract or tryptone. All constituents except for NaHCO₃, cysteine.HCl (which was added after the solution was cooled) and sulfide were dissolved in water, boiled and cooled under an oxygen-free atmosphere of N₂/CO₂ (4:1). The medium was distributed into serum bottles (Wheaton Scientific) or Hungate tubes (Bellco 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 N₂:CO₂ ratio in the gas phase and the concentration of NaHCO₃ in the medium were modified to obtain pH values between 6.0 and 8.1.

Enrichment was begun immediately after the samples were brought to the laboratory; the methanogens were isolated and purified as described previously (Lai & Chen, 2001; Lai *et al.*, 2002). Samples (5 ml) from sediments of the marine water aquaculture fishpond and the estuary of Eriln Shi, Taiwan, were inoculated into eight bottles of MB medium (45 ml) that contained methanol, trimethylamine, acetate or formate as methanogenic substrates. After 1 month incubation at room temperature, methanogenesis occurred in all enrichments. Four successive transfers further enriched the formate-grown cells; this culture was then inoculated into roll-tube MB agar medium for further

isolation. Under fluorescent microscopy, three fluorescent colonies with different morphologies were picked and transferred to 5 ml MB medium with formate in a Coy anaerobic chamber. One was a small, white, pinpoint colony with a light-yellow, raised central area (strain K1F9705b^T). The second colony was yellowish, circular and opaque (strain K1F9705c). The third was a circular, round colony (strain O1F9704a). Methane-producing cultures from these single colonies were purified further by combining an increased NaCl concentration (1–10 ‰) in MB medium with serial dilution until contamination by non-methanogens was not detectable. These isolates were deposited in the Oregon Collection of Methanogens, USA, and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Germany, as strains K1F9705b^T (= OCM 772^T = DSM 14646^T), K1F9705c (= OCM 666) and O1F9704a (= OCM 747).

Unless otherwise indicated, phenotypic and morphological characteristics were studied as described by Lai *et al.* (2002). Catabolic substrates tested under an N₂/CO₂ (4:1) atmosphere were sodium formate (100 mM), sodium acetate (50 mM), trimethylamine (40 mM), methanol (50 mM), ethanol (48 mM), propan-2-ol (48 mM), isobutanol (48 mM) and butan-2-ol (48 mM). Utilization of H₂ was tested by pressurizing the culture tubes with H₂ (100%, 200 kPa). Utilization of substrates was determined in MB/W medium by monitoring methane production by GC with flame-ionization detection (Lai *et al.*, 1999). Specific growth rates were calculated from methane production, which was analysed by linear regression of the logarithm of the total amount of methane that accumulated over time. Inocula were grown under conditions that were similar to the experimental conditions.

Sensitivity to ampicillin, penicillin, spectinomycin, kanamycin, tetracycline and chloramphenicol (each at 100 µg ml⁻¹) was tested in MB/W medium with sodium formate (100 mM) plus acetate (20 mM) at 37 °C. Whole-cell proteins were extracted from cell pellets that were lysed by adding loading buffer that contained 4% SDS at a ratio of 1 ml buffer per OD unit. An OD unit was the amount of cells formed in 1 ml culture with an OD₅₄₀ of 1.0. Surface-layer proteins were isolated according to the protocol of König (1995). SDS-PAGE was performed as described by Laemmli (1970) and Coomassie blue R-250 was used to visualize protein. An Olympus BH-2 microscope was used for phase-contrast microscopy. Preparation for negative staining and ultrathin sectioning was performed as described previously (Lai & Shih, 2001). Electron micrographs were taken by using model JEM-1200EX II and 200cx transmission electron microscopes (JEOL).

For phylogenetic analysis, DNA was 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. PCR amplification primers used were 5'-CGACTAAGCCATG-CGAGTC-3' and 5'-GTGACGGGCGGTGTGTGCAAG-3'.

The sequences were checked by the Check-Probe program from the Ribosomal Database Project (Maidak *et al.*, 1996); they corresponded respectively to positions 34–52 and 1340–1320 in the 16S rDNA nucleotide sequence of *M. halotolerans*. The following primers were used for sequencing: 5'-CGACTAAGCCATGCGAGTC-3', 5'-GCT-CAGTAACACGTGGATAACC-3', 5'-GGATTACAGGGT-TTCACTCCTACC-3' and 5'-GTGACGGGCGGTGTGTG-CAAG-3' (*M. halotolerans* SEBR 4845^T, GenBank accession no. AF033672: sequence positions 34–52, 78–99, 609–632 and 1340–1320, respectively). The resulting sequences of strains K1F9705b^T, K1F9705c and O1F9704a were assembled to produce contiguous rDNA sequences of approximately 1254 bp (positions 58–1311), 1251 bp (58–1308) and 1262 bp (58–1319), respectively. Gene sequences of archaea were obtained from the Ribosomal Database Project and GenBank. The similarity matrix obtained was based on the analytical results of the Ribosomal Database Project (<http://rdp.cme.msu.edu/html/>). Multiple sequence alignments were analysed by the CLUSTAL W package at the Biology Workbench (<http://workbench.sdsc.edu/>). Distances were computed with the CLUSTAL tree package at the same website by using the neighbour-joining model and were fed to the program DRAWGRAM of the PHYLIP package version 3.5c (Felsenstein, 1993). Bootstrap confidence analysis was performed with the SEQBOOT program of the PHYLIP package by using 500 replicates.

DNA–DNA hybridization experiments were performed by using the dot-blot technique (Sambrook & Russell, 2000) with the VersiTag fluorescein labelling system (NEN Life Science). The type strain of *M. pumilus*, MHT-1^T, was kindly provided by Dr Koji Mori of Gifu University, Japan, and the type strain of *M. halotolerans*, SEBR 4845^T, was kindly provided by Dr David Boone from the Oregon Collection of Methanogens. *M. taiwanensis* P2F9704a^T and P2F9705 were obtained from our own culture stock. 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 Johnson (1985) and Jarrell *et al.* (1992). Target DNA (500 ng), denatured by 0.8 M NaOH, was blotted on to a Hybond-N⁺ nylon membrane (Amersham Biosciences) and labelled DNA was reassociated in a solution that contained 50 % formamide, 5 × Denhardt's solution (50 × Denhardt's solution contains 1 % Ficoll, 1 % polyvinylpyrrolidone and 1 % BSA) with 0.5 % (w/v) SDS in 5 × SSC buffer (1 × SSC buffer is 0.15 M sodium chloride 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. Triplicate tests were performed for each assay and self-hybridization of the probe with homologous target DNA was set to 100 %.

To determine G+C content, DNA of *Methanocalculus* species was isolated and purified as described above. The melting temperature of purified DNA was measured by using the method of Marmur & Doty (1962) with the slight modification of Jan *et al.* (1999). DNA (50 µg) was dissolved in 1 × SSC buffer (pH 7.0). This DNA solution was then placed in a cuvette and heated from 60 to 95 °C in increments of 2 °C. At each temperature, A_{260} was measured by using a Cary 100 UV-vis spectrophotometer (Varian). The G+C content was computed by using the formula $T_m = 69.3 + 0.41 \times (G + C)$. For comparison and accuracy, T_m values of DNA from *M. pumilus* and *M. halotolerans* were determined by the same method.

Under phase-contrast microscopy, refractive areas were observed in all three new methanogenic isolates (Fig. 1). This is similar to *M. taiwanensis* and *M. pumilus*. Cells of strain K1F9705b^T were non-motile and irregularly coccoid, with a diameter of 1.1–1.6 µm (Fig. 2a). When observed by transmission electron microscopy (TEM) with negative staining, the transparent bag of surface (S)-layers tended to separate from the cells (Fig. 2a). This indicated that the proteinaceous cell-wall structure of strain K1F9705b^T was very sensitive to physical and chemical treatment and lysed easily. Cells of strain K1F9705c were non-motile and irregularly coccoid, with a diameter of 1.0–1.5 µm (Fig. 2b). Several homogeneous, heavily stained granules were observed both in negative and ultrathin preparations (Fig. 2b). It was suggested that these heavily stained granules were polyphosphate (Jacob J. Goldberg, electron microscopist; personal communication). Cells of strain O1F9704a were weakly motile with several flagella and irregularly coccoid, with a diameter of 0.8–1.1 µm (Fig. 2c). Interestingly, cell morphology of strains K1F9705b^T and K1F9705c showed wrinkle bands and heavily stained granules in negative-stain preparations under TEM. Of the two, strain K1F9705c tended to accumulate larger

Fig. 1. Phase-contrast micrograph of strain K1F9705c, showing irregularly coccoid cells with a refractive area. Bar, 2.23 µm.

Fig. 2. Transmission electron micrographs showing: (a) irregularly coccoid cells of strain K1F9705b^T separated from the transparent S-layer envelope and protoplast; (b) ultrathin section of strain K1F9705c with heavy-stained granules; (c) irregularly coccoid cells with flagella of strain O1F9704a. Bars, 0.49 µm (a); 0.18 µm (b); 0.1 µm (c).

amounts of heavily stained granules and cells of this strain tended to clump together.

All three isolates stained Gram-negative and lysed rapidly in the presence of SDS (0.1 g l⁻¹), indicating that the cell envelope consists of a protein S-layer (Boone & Whitman, 1988). Negative-stain TEM of K1F9705b^T and O1F9704a clearly showed a hexagonally arranged pattern of S-layer protein for both strains. The centre-to-centre spacing of the morphological units of the S-layer lattice of strain K1F9705b^T was about 24 nm. Isolation and characterization of the S-layer protein from *Methanocaldococcus* species indicated that *M. taiwanensis* P2F9704a^T, strains K1F9705b^T, K1F9705c and O1F9704a were different from each other and were each composed of two different subunits, whereas the S-layers of *M. halotolerans* and *M. pumilus* were composed of a single subunit. The molecular masses of S-layer proteins of strains K1F9705b^T, K1F9705c, O1F9704a (these were different from the known masses of S-layer proteins in *M. halotolerans*, *M. pumilus* and *M. taiwanensis*) varied from 88 to 102.2 kDa and are summarized in Table 1.

Strains K1F9705b^T, K1F9705c and O1F9704a used formate and H₂/CO₂ as substrates to produce methane. Under

N₂/CO₂ atmospheres, they could not produce methane from acetate, methanol, trimethylamine, ethanol, propan-2-ol, isobutanol or butan-2-ol. Acetate was required for cell growth of all three isolates. The specific growth rates of strain K1F9705b^T in MM and in MB medium (which contained yeast extract and tryptone) with formate plus acetate were the same, indicating that cell growth was not stimulated by yeast extract and tryptone. The specific growth rate of strain O1F9704a in MM was slower than that in MB, indicating that growth was stimulated by yeast extract and tryptone. Addition of tungsten greatly promoted the growth rate of strain K1F9705b^T, from 0.01 h⁻¹ in MB medium to 0.10 h⁻¹ in tungsten-containing MB/W medium. However, addition of tungsten did not affect the growth of strain O1F9704a. Strain K1F9705b^T could grow at 20–45 °C and grew fastest at 37 °C. The pH range for growth of strain K1F9705b^T was broad (5.8–7.7); specific growth rates were >0.10 h⁻¹, with optimal growth at pH 7.2.

Halotolerance was observed in strain K1F9705b^T. Although cells grew optimally in medium that contained 1 % NaCl, these cells tolerated up to 12 % NaCl (Fig. 3). Under optimal growth conditions (temperature, pH and NaCl), the doubling time of strain K1F9705b^T was approximately

Table 1. Differential characteristics of *Methanocalculus* species

Taxa: 1, K1F9705b^T; 2, K1F9705c; 3, O1F9704a (data from this study); 4, *M. taiwanensis* P2F9704a^T; 5, *M. taiwanensis* P2F9705 [data from Lai *et al.* (2001)]; 6, *M. halotolerans* [data from Ollivier *et al.* (1998)]; 7, *M. pumilus* [data from Mori *et al.* (2000)]. All strains are irregularly coccoid, can catabolize formate and H₂/CO₂ and require acetate for growth. ND, Not determined.

Characteristic	1	2	3	4	5	6	7
Size (μm)	1.0–1.6	1.0–1.5	0.7–1.8	0.9–1.4	0.8–1.4	0.8–1.0	0.8–1.0
Flagella	–	–	+	–	–	+	–
Effect of tungsten*	S	ND	NE	E	ND	ND	ND
Growth optimum/range:							
Temperature (°C)	37/20–45	37/20–42	37/20–42	37/25–42	37/28–37	38/25–45	35/24–45
pH	7.2/5.8–7.7	7.2/ND	7.2/6.7–8.0	6.8/6.3–8.3	7.1/ND	7.6/7.0–8.4	6.5–7.5/ND
[NaCl] (%)	1.0/0–12	1.0/0–10	0.5/0–8	0.5/0–4	1/0–3	5.0/0–12	1.0/0–7
M _r of S-layer proteins	101 000, 91 000	102 200, 88 100	101 200, 92 800	95 100, 11 800	ND	91 800	94 300
Habitat	Aquaculture fishpond	Aquaculture fishpond	Estuary	Estuary	Estuary	Oil-producing well	Waste-disposal site

*NE, No effect; S, stimulates growth tenfold; E, extends growth range.

7 h. Optimal temperature and pH for growth of strain K1F9705b and O1F9704a were similar to those of strain K1F9705b^T. There were slight variations in salt tolerance among these three strains; both K1F9705b^T and K1F9705c grew optimally in 1 % NaCl, but the optimal salt concentration for strain O1F9704a was 0.5 % (Fig. 3). They all tolerated high salt concentrations. Among them, strain K1F9705b^T was more halotolerant than the others, as it tolerated up to 12 % NaCl, whereas strains K1F9705c and O1F9704a tolerated up to 10 and 8 %, respectively.

All three new isolates were resistant to ampicillin, penicillin, kanamycin and spectinomycin (each at 100 μg ml⁻¹) and sensitive to chloramphenicol (100 μg ml⁻¹). Tetracycline

Fig. 3. Influence of NaCl concentration on the growth of strains K1F9705b^T (●), K1F9705c (■) and O1F9704a (▲). Cells were grown on MB/W medium with sodium formate as the catabolic substrate and acetate. Specific growth rates were calculated from methane production values and are the means of triplicate cultures.

(100 μg ml⁻¹) inhibited cell growth. Whole-cell proteins from strains K1F9705b^T, K1F9705c, O1F9704a, *M. halotolerans*, *M. pumilus* and *M. taiwanensis* were extracted and analysed; whole-cell protein patterns of the three new isolates were similar, with minor differences (Fig. 4). However, their protein profiles were distinct from those of *M. halotolerans* and *M. pumilus*, and were related more closely to that of the former than to that of the latter (Fig. 4).

Fig. 4. Whole-cell protein profiles of *Methanocalculus* species, as monitored by SDS-PAGE. Lane 1, strain O1F9704a; lane 2, strain K1F9705b^T; lane 3, strain K1F9705c; lane 4, *M. taiwanensis* P2F9704a^T; lane 5, *M. pumilus*; lane 6, *M. halotolerans*. Arrows indicate regions where there is a significant difference between strain K1F9705b^T and other *Methanocalculus* species.

The 16S rDNA sequences of strains K1F9705b^T, K1F9705c and O1F9704a were determined and phylogenetic trees were constructed by using a selection of different methanogen sequences that were obtained from GenBank. These 16S rDNA sequence analyses placed all three new isolates as close relatives of *M. halotolerans* (sequence similarity, 98.1%), *M. pumilus* (98.5%) and *M. taiwanensis* (98.4%) (Fig. 5). The sequence of strain K1F9705c was identical to that of strain K1F9705b^T, and the sequence of strain O1F9704a had 99.9% similarity to those of the other two isolates. Phylogenetic analysis indicated that all known *Methanocalculus* species formed a cluster (>98% similarity), but were distant from other methanogens (<90.7% similarity).

The melting temperatures of DNA from strains K1F9705b^T, K1F9705c and O1F9704a were 90.12, 89.95 and 89.94 °C, respectively. The DNA G+C contents of strains K1F9705b^T, K1F9705c and O1F9704a were 50.8, 50.4 and 50.3 mol%, respectively. These values were close to the reported DNA G+C content of *M. pumilus* (51.9 mol%) (Mori *et al.*, 2000), but were higher than that of *M. halotolerans* (43 mol%) (Ollivier *et al.*, 1998).

In DNA–DNA hybridization experiments, DNA of strain K1F9705b^T exhibited 37% hybridization with that of *M. halotolerans*, 29% hybridization with that of *M. pumilus* and <10% hybridization with the DNA of *M. taiwanensis*. Among the novel strains, the DNA of strain K1F9705b^T exhibited 85% hybridization with that of strain K1F9705c and 80% hybridization with that of strain O1F9704a.

All *Methanocalculus* species reported to date are mesophilic, neutrophilic and hydrogenotrophic, and can only utilize

H₂/CO₂ and formate as catabolic substrates. The three new isolates reported here were similar to the other three known *Methanocalculus* species in catabolic substrates, optimum temperature and pH for growth (Table 1). However, results of the cell morphology, halotolerance, protein profile, S-layer protein, phylogenetic and DNA–DNA hybridization analyses indicated that these three new isolates are distinct from *M. halotolerans*, *M. pumilus* and *M. taiwanensis*. Therefore, we conclude that these three isolates represent a novel species of the genus *Methanocalculus*, for which we propose the name *Methanocalculus chunghsingensis* sp. nov., to include strains K1F9705b^T, K1F9705c and O1F9704a. Strain K1F9705b^T (= OCM 772^T=DSM 14646^T) is designated as the type strain. DNA–DNA hybridization data revealed a high level of DNA relatedness among strains K1F9705b^T, K1F9705c and O1F9704a (up to 80%), which is indicative of strains of the same genospecies. Also, cell morphology, protein profile, molecular mass of S-layer protein, halotolerance and effects of tungsten (Table 1) revealed that they are three separate strains.

All irregularly coccoid methanogens within the order *Methanomicrobiales* possess hexagonal S-layer lattices that consist of glycoprotein subunits with an *M_r* that ranges from 90 000 to 155 000. Among them, the family *Methanocorpusculaceae* has an S-layer glycoprotein with an *M_r* that ranges from 90 000 to 94 000, and those of the family *Methanomicrobiaceae* range from 101 000 to 155 000 (Zellner *et al.*, 1999). There are no discussions of S-layer protein and whole-cell protein profile for *M. halotolerans* or *M. pumilus* (Ollivier *et al.*, 1998; Mori *et al.*, 2000). We have isolated the S-layer protein of all *Methanocalculus* species; the molecular masses of S-layer protein of these strains were different and were located within the range 86 000–112 000 (Table 1). Also, analysis of the whole-cell protein profiles of *M. chunghsingensis*, *M. halotolerans*, *M. taiwanensis* and *M. pumilus* showed different patterns (Fig. 4).

Most halotolerant or halophilic methanogenic species that have been described are methylotrophic 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. Ollivier *et al.* (1998) reported the first hydrogen-oxidizing methanogen, *M. halotolerans*, from an oil-producing well. This organism grows optimally at 5% NaCl (w/v) and tolerates up to 12% NaCl, a range that is the widest reported to date for any hydrogenotrophic methanogen. Recently, Mori *et al.* (2000) reported that *M. pumilus* grew optimally at 1.0% NaCl and did not grow at 10% NaCl. The three new isolates that we report here have an optimal NaCl range of 0.5–1.0%, but they tolerate up to 8–12% NaCl (for strain K1F9705b^T grown in 12% NaCl, the specific growth rate is 0.021 h⁻¹); hence, they are halotolerant, hydrogenotrophic methanogens. Most methylotrophic, halotolerant/halophilic methanogens are adapted for

Fig. 5. Phylogenetic relationship of strains K1F9705b^T, K1F9705c, O1F9704a and other related species, based on distance matrix analysis of 16S rRNA gene sequences. The PHYLIP program was used to estimate phylogenetic distances from distance matrix data. Bootstrap values are shown at nodes (percentage of 500 replicates). Bar, 0.03 base substitutions per 100 bases.

growth over a narrow NaCl concentration range, whereas hydrogenotrophic *Methanocalculus* species, such as *M. halotolerans* and *M. chunghsingensis*, are well-adapted for growth over a broad range of NaCl concentrations (0–2.0 M). This may indicate their better adaptation to an environment where the NaCl concentration fluctuates.

Description of *Methanocalculus chunghsingensis* sp. nov.

Methanocalculus chunghsingensis (chung.hsing.en'sis. N.L. masc. adj. *chunghsingensis* from Chung Hsing University, to honour the university where this research was performed).

Irregularly coccoid cells, non-motile or weakly motile with flagella. Obligately anaerobic cells; Gram-negative. Cell wall is composed of proteinaceous, SDS-sensitive, S-layer subunits with relative molecular masses that range from 88 100 to 102 200. Catabolic substrates used include H₂/CO₂ and formate, but not acetate, methanol, trimethylamine, dimethylamine, ethanol, propan-2-ol, isobutanol or butan-2-ol. Cells are mesophilic; growth occurs at 20–45 °C, with optimum at 37 °C. Cells grow over a wide pH range, from 6.0 to 8.0; optimal pH is 7.2. Cells grow well at 0–12 % NaCl; optimal NaCl concentration for growth is 1.0 %. Acetate is required for growth. Tungsten is highly stimulatory for some strains. Growth is inhibited completely by chloramphenicol and partially by tetracycline, but not by ampicillin, penicillin, kanamycin or spectinomycin. DNA G+C content of cells is about 50.0 mol%.

The type strain is K1F9705b^T (= OCM 772^T = DSM 14646^T). Isolated from a marine water aquaculture fishpond near Wong-Kong, Taiwan. Reference strains are K1F9705c and O1F9704a.

Acknowledgements

The authors would like to thank M.-J. Chuang, T.-Y. Hong, C.-C. Liu, L.-J. Lai and C.-M. Shu for their help in sampling, enrichment and purification. The technical help in electron microscopy of Ms P.-C. Chao and Y.-S. Lin from the Regional Instruments Center and G+C content determination in the laboratory of Dr S. H. Chou at the Biochemistry Institute, National Chung Hsing University, were greatly appreciated.

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