**Methanoculleus taiwanensis** sp. nov., a methanogen isolated from deep marine sediment at the deformation front area near Taiwan

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A mesophilic, hydrogenotrophic methanogen, strain CYW4T, was isolated from deep-sea sediment obtained by the Ocean Researcher I cruiser, ORI-961, in 2011. The sediment was from the deformation front area offshore of south-western Taiwan. Here, seismic reflections indicated that methane hydrates were abundant. The methanogenic substrates utilized by strain CYW4T were formate and H2/CO2, but not acetate, secondary alcohols, methylamines, methanol and ethanol. Cells of strain CYW4T were non-motile, irregular cocci and 0.6–1.5 μm in diameter. The S-layer protein had an *M* of 112 000. The optimum growth conditions were at 37 °C, pH 8.1 and 0.08 M NaCl. Growth of the strain was stimulated by acetate. The G+C content of the chromosomal DNA of strain CYW4T was 61 mol%. Phylogenetic analysis revealed that strain CYW4T was most closely related to *Methanoculleus marisnigri* JR1T (96.82 % 16S rRNA gene sequence similarity). Based on the morphological, phenotypic and phylogenetic characteristics presented here, it is evident that strain CYW4T represents a novel species of the genus *Methanoculleus*, and the name *Methanoculleus taiwanensis* sp. nov. is proposed. The type strain is CYW4T (=BCRC AR10043T=NBRC 110782T). The optical density of cultures of strain CYW4T dropped abruptly upon entering the stationary growth phase. During this time numerous particles of approximately 50 nm in diameter were observed on and around the cells. This suggests that strain CYW4T harbours a lytic virus that is induced in the stationary phase, which is of interest because only a few lytic viruses have been reported in methanogens.

The boundary between the Chinese continental margin and the Taiwan orogen offshore of south-western Taiwan (Fig. S1, available in the online Supplementary Material) can be clearly delineated by the deformation front of the contractual structures (Liu et al., 1997, 2006). The deformation front is not only an important tectonic boundary between the compressive orogenic wedge and the adjacent rifted South China Sea continental margin, but also a major structure that traps methane gas (Lin et al., 2013). The high methane concentrations in these regions and the isotopic carbon compositions of selected samples indicate that the methane is mainly from biogenic sources (Chuang et al., 2010). To identify the methanogen involved in deep-sea methane formation that may also be associated with methane seep or methane hydrate environments, methanogens were isolated from deep-sea sediment at the deformation front offshore of south-western Taiwan. In this study, a novel hydrogenotrophic, mesophilic strain, CYW4T, representing a novel species of the genus *Methanoculleus* was enriched from samples from the deformation front and was further purified and characterized.

The piston cores obtained during the ORI-961 cruise on May 17–25, 2011, at station 19 around the deformation front (Fig. S1) offshore of south-western Taiwan were sectioned, and marine sediments from each section were inoculated in MB/W enrichment medium with formate, acetate or methanol (Sigma) as the catabolic substrate. The habitat of strain CYW4T was located at 68–114 cm below the seafloor.

The GenBank accession number for the 16S rRNA gene sequence of strain CYW4T is KM111599.

Five supplementary figures are available with the online Supplementary Material.

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The modified anaerobic technique of Hungate was utilized (Balch et al., 1979; Sowers & Noll, 1995). Sterilized media were prepared under an O2-free N2/CO2 (4:1, v/v) atmosphere. Rich (MB/W) and minimal (MM/W) media were used to enrich the methanogens. MB/W medium was composed of (gl−1) MgCl2·6H2O, 1.0; KCl, 0.5; NaCl, 5.0; CaCl2·2H2O, 0.1; K2HPO4, 0.4; NH4Cl, 1.0; cysteine; HCl, 0.25; NaHCO3, 4.0; yeast extract, 2.0; tryptone, 2.0; and resazurin, 0.001. Vitamin (Wolin et al., 1963) and trace element (Ferguson & Mah, 1983) solutions with tungstate element (Ferguson & Mah, 1983) solutions with tungstate were added to MB/W medium with the same substrate and vancomycin. It was concluded that acetate was replaced species of the genus Methanoculleus sp. nov. Methanoculleus taiwanensis (Wu & Lai, 2011). Inocula were grown under the experimental conditions described in the text.

An Olympus BH-2 microscope was used for phase-contrast microscopy. Preparations for negative staining were performed as described previously (Lai & Shih, 2001). Electron micrographs were taken using JEM-1200EXII and JEM-1400 (JEOL) microscopes. For scanning electron micrographs, samples were prepared as described previously (Lai & Chen, 2001), and cells were sputter-coated with gold and observed with a JSM-7401F (JEOL) scanning electron microscope.

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 (Bio-Rad).

Chromosomal DNA from strain CYW4T was isolated by the general procedure of Jarrell et al. (1992). The PCR amplification primers used for strain CYW4T were 4F (5′-TCCGTTGATCCTGCAG-3′; C.-C; Lin & M.-C. Lai, unpublished data) and 1492NR (5′-GGTTAACCCTGTTACGACTT-3′; Utsumi et al., 2003). All primers were purchased from Mission Biotech (Taipei, Taiwan). The PCR mixtures were prepared according to the instructions of Takara Taq (Takara Bio), and PCR conditions were as follows: 94 °C for 4 min, followed by 25 cycles at 94 °C for 60 s, 50 °C for 30 s, 72 °C for 1 min; followed by 72 °C for 7 min and a 25 °C hold. The cloning of the 16S rRNA gene fragment was carried out by following the technical manual for the pGEM-T vector (Promega).

DNA sequences were sequenced by Mission Biotech. The gene sequences of archaea used in this study were obtained from the GenBank database. Phylogenetic trees were reconstructed with the MEGA6 program (Tamura et al., 2013) using the neighbour-joining method with 1000 bootstrap replicates. To determine the G+C content of the genomic DNA, the chromosomal DNA of strain CYW4T was treated with nuclease P1 at 50 °C for 1 h and then with bacterial alkaline phosphatase at 37 °C for 1 h, according to method of Mesbah et al. (1989). The treated sample was analysed in triplicate by liquid chromatography with a UV detector.

A 0.5 g sample of the sediment core from OR1-961_19A-68-114 cm was added to MB/W medium with acetate as the catabolic substrate on board the ship. Initial enrichments were incubated in triplicate at 4 °C, 37 °C and room temperature. The enrichment at 37 °C grew best and accumulated the most methane; it was transferred into fresh MB/W medium with the same substrate and vancomycin. At this time, PCR amplification of the 16S rRNA gene and sequencing identified two archaea with high similarity to species of the genus Methanoculleus, which uses formate as a methanogenic substrate. Therefore, acetate was replaced with formate in the following isolation steps. Strain CYW4T was purified by serial dilution in MM/W medium with added formate and vancomycin. It was concluded that the culture was axenic, based on microscopic examination revealing the presence of a single morphotype and on the absence of growth in Bacto thioglycollate medium.
Cells of strain CYW4^T were irregular cocci of diameter 0.6–1.5 μm (Fig. 1a, c) and flagella were not observed. In Fig. 1(b), the plane of cell division of strain CYW4^T was observed, and many possible virus-like particles and filaments were attached to cells. When transmission electron micrographs were observed by negative staining, strain CYW4^T had triangular edges (Fig. 1d). Cells were not motile and were lysed with SDS (0.01%, w/v), which indicated that the cell envelope was composed of surface-layer proteins and lacked peptidoglycans, such as pseudomurien. The surface-layer

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**Fig. 1.** Morphology of strain CYW4^T from (a) phase-contrast micrograph, bar, 10 μm; (b) SEM, bar, 100 nm; (c & d) transmission electron microscopes of negatively stained cells (c: bar, 1 μm; d: bar, 250 nm) microscopes of negatively stained cells (c, d).

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**Fig. 2.** Phylogenetic analysis of 16S rRNA gene sequences showed the relationship between strain CYW4^T and related species of the genus *Methanoculleus*. This tree was derived with the MEGA6 software package (Tamura et al., 2013) and reconstructed based on a subset of nearly full-length sequences by the neighbour-joining method. Bootstrap values at the nodes are percentages of 1000 replicates. The accession numbers are shown in parentheses. *Methanohalophilus portucalensis* FDF1^T (AY290717) was used as outgroup. Bar, 0.02 evolutionary distances.
Table 1. Comparison of physiological characteristics of strain CYW4T and related species of the genus *Methanoculleus*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain CYW4T</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.6–1.5</td>
<td>0.8–2.0</td>
<td>1.3</td>
<td>1.0–2.0</td>
<td>1.0–1.3</td>
<td>1.25–2.0</td>
<td>1.0–2.0</td>
<td>0.8–1.7</td>
<td>0.8–2.0</td>
<td>0.7–1.6</td>
<td></td>
</tr>
<tr>
<td>NaCl concn range for growth (M) (optimum)</td>
<td>0–0.34 (0.08)</td>
<td>0–1.3 (0.2)</td>
<td>0–0.69 (0.19)</td>
<td>0–0.3 (0.1)</td>
<td>0.75–0.70 (0.25)</td>
<td>ND</td>
<td>0.17</td>
<td>0–1.3 (0.2)</td>
<td>0.05–0.5 (0.2)</td>
<td>0–1.3 (0.1–0.2)</td>
<td></td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>6.50–8.08 (8.08)</td>
<td>5.0–8.7 (5.0–7.5)</td>
<td>5.6–7.6 (6.2–6.6)</td>
<td>6.7–8.0 (6.7–7.2)</td>
<td>6.18–7.82 (7.0)</td>
<td>6.5–8.0 (6.9–7.5)</td>
<td>5.5–8.0 (6.7)</td>
<td>6.5–8.5 (7.5–7.8)</td>
<td>5.0–8.5 (6.6)</td>
<td>5.8–8.2 (6.7–6.8)</td>
<td></td>
</tr>
<tr>
<td>S-layer protein size (kDa)</td>
<td>112</td>
<td>ND</td>
<td>138</td>
<td>130</td>
<td>120</td>
<td>101</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>DNA G+C content (mol%)†</td>
<td>61 (Lc)</td>
<td>ND</td>
<td>61.2 (Bd)</td>
<td>62.2 (Lc)</td>
<td>59 (Bd)</td>
<td>59 (Tm)</td>
<td>59 (Bd)</td>
<td>55.2 (Tm)</td>
<td>60.2 (Tm)</td>
<td>62.9 (Lc)</td>
<td></td>
</tr>
<tr>
<td>Compounds required or stimulatory for growth‡</td>
<td>A³, A³R, Y³, P³</td>
<td>A³R, A³Y³, p³</td>
<td>TcR³</td>
<td>A³R, Ca³R, Tc³R, Y³R</td>
<td>A³R</td>
<td>A³R, Tc³R, Y³</td>
<td>A³R</td>
<td>A³R, Tc³R, Y³</td>
<td>A³R</td>
<td>A³, Y³</td>
<td></td>
</tr>
</tbody>
</table>

*H, H₂ + CO₂; F, formate; 2P, 2-propanol/CO₂; 2B, butanol/CO₂.

†Determined by: Lc, HPLC; Bd, buoyant density; Tₘ, thermal denaturation.

‡Organic compounds which are required or are greatly stimulatory for growth. Acetate, A; Yeast extract, Y; Trypticase, Tc; Casitone, Ca; Peptone, P.
protein was then isolated and found to have an $M_c$ of 112 000 (Fig. S2).

Strain CYW4$^T$ can use $H_2/CO_2$ or formate as catabolic substrates, but not sodium acetate, methanol, ethanol, 2-propanol or 2-butanol (Fig. S3). Acetate stimulated the growth rate on formate. Growth of strain CYW4$^T$ was resistant to ampicillin, penicillin, kanamycin, spectinomycin and vancomycin, but it was sensitive to chloramphenicol and tetracycline. Strain CYW4$^T$ was able to grow over the temperature range of 25–42 °C, and the optimum growth temperature was 37 °C (Fig. S4a). The strain grew well over the NaCl concentration range of 0 to 0.54 M (Fig. S4b), and optimal growth was seen at 0.08 M NaCl. Strain CYW4$^T$ grew over the pH range of 6.1–8.1 and showed optimum growth at a pH of 8.0 (Fig. S4c). Under optimal conditions of temperature, NaCl concentration and pH, the specific growth rate which calculated from rates of methane production was 0.10 (h$^{-1}$), and the doubling time was 6.7 h. Therefore, strain CYW4$^T$ is a mesophilic, hydrogenotrophic methanogen. The growth of strain CYW4$^T$ was suppressed by high concentrations of sodium sulfide. Thus, the specific growth rate of strain CYW4$^T$ without added sodium sulfide was 0.075 h$^{-1}$, higher than in medium with 0.5 mM sodium sulfide (0.061 h$^{-1}$).

The 16S rRNA gene sequence (1416 nt) of strain CYW4$^T$ was closely related to those of Methanoculleus marisnigri JR1$^T$ (sequence similarity, 96.82 %), and other species of the genus Methanoculleus (Fig. 2). The similarity of the 16S rRNA gene sequence of strain CYW4$^T$ to the sequences from other genera, including Methanofollis, Methanogenium, Methanoplanus and Methanomicrobiurn, within the family Methanomicrobiaeae, was much less (89–93 %). The difference between the sequences of the seven species of the genus Methanoculleus and that of strain CYW4$^T$ was about 3–4 %, which suggests that strain CYW4$^T$ represents a novel species of the genus Methanoculleus. The $G+C$ content of the genomic DNA of strain CYW4$^T$ was 61 mol%.

Nine species of the genus Methanoculleus have been isolated from diverse habitats, such as marine sediments, lakes, rice paddies, river sediments, wetlands and paddy field soil, a high temperature sediment, anaerobic digesters, oilfields and a deep diatomaceous shale formation (Cheng et al., 2008; Dianou et al., 2001; Mitkucki et al., 2003; Ollivier et al., 1986; Rivard & Smith, 1982; Romesser et al., 1979; Shimizu et al., 2013; Zellner et al., 1998). Cell sizes of species of the genus Methanoculleus with validly published names are similar (Table 1). Apart from for Methanoculleus hydrogenotrophicus HC$^T$ (Tian et al., 2010), other species use formate to produce methane. Some strains of species of the genus Methanoculleus, including the Nankai-1$^T$, JR1$^T$, MG62$^T$ and INSUZ$^T$, use secondary alcohols as methanogenic substrates, but CYW4$^T$ did not. Furthermore, the most closely related strain to CYW4$^T$, Methanoculleus marisnigri JR1$^T$, has an optimum growth temperature range of 20–25 °C, but strain CYW4$^T$ grew optimally at 37 °C. In addition, the similarity of the 16S rRNA gene sequence of strain CYW4$^T$ to the sequences of species of the genus Methanoculleus was lower than expected for members of the same species (95.04–96.82 %). Strain CYW4$^T$ also formed a distinctive clade apart from other species of the genus Methanoculleus. Based on the characteristics presented above, it is clear that strain CYW4$^T$ represents a novel species of the genus Methanoculleus, and the name Methanoculleus taiwanensis sp. nov. is proposed.

Free and attached virus-like nanoparticles were apparent in SEMs of strain CYW4$^T$. In addition, the triangular edges of strain CYW4$^T$ were similar to those observed in Sulfolobus islandicus LAL14/1 before release of a rod-shaped virus 2 (SIRV2) (Fig. 2d; Bize et al., 2009). Furthermore, the cell density dramatically decreased at the early stationary phase, and the culture quickly turned transparent (Fig. S5). These results indicate that strain CYW4$^T$ may have been infected and lysed by virus-like nanoparticles.

**Description of Methanoculleus taiwanensis sp. nov.**

Methanoculleus taiwanensis sp. nov. (tai.wan.en’sis. N.L. masc. adj. taiwanensis of Taiwan, indicating the source of the type strain).

Cells are non-motile, irregular cocci, 0.6–1.5 μm in diameter. Lyse rapidly in SDS (0.01%, w/v), and possess an S-layer protein with an $M_c$ of 112 000. Use formate and $H_2/CO_2$, but not acetate, secondary alcohols, methyla- mines, methanol and ethanol. The optimum conditions for growth are 37 °C, pH 8.1 and 0.08 M NaCl. Cell growth is stimulated by acetate and completely inhibited by chloramphenicol and tetracycline, but not by ampicillin, kanamycin, penicillin, spectinomycin or streptomycin.

The type strain CYW4$^T$ (BCRC AR10043$^T$=NBRC 110782$^T$) was isolated from deep-sea sediment collected from the region of the deformation front offshore of south-western Taiwan. The genomic DNA $G+C$ content of the type strain is 61 mol%.

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