Methanoculleus horonobensis sp. nov., a methanogenic archaeon isolated from a deep diatomaceous shale formation

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A methanogenic organism from the domain Archaea, designated strain T10⁷, was isolated from groundwater sampled from a deep diatomaceous shale formation located in Horonobe, Hokkaido, Japan. The strain utilized H₂/CO₂ and formate as substrates for methanogenesis. Cells were strictly anaerobic, Gram-negative-staining, flagellated, irregular coccoids, 0.7–1.6 μm in diameter, and occurred singly. The strain grew at 25–45 °C (optimum 37–42 °C), at pH 5.8–8.2 (optimum pH 6.7–6.8) and in the presence of 0–1.3 M NaCl (optimum 0.1–0.2 M NaCl). The G+C content of the genomic DNA was 62.9 mol%. 16S rRNA gene sequencing revealed that, although the strain is a member of the genus Methanoculleus, it clearly differed from all described species of this genus (95.5–98.3 % sequence similarity). Values for DNA–DNA hybridization with type strains of closely related Methanoculleus species were less than 50 %. Phenotypic and phylogenetic features of strain T10⁷ clearly indicate that it represents a novel species of the genus Methanoculleus, for which the name Methanoculleus horonobensis sp. nov. is proposed. The type strain is T10⁷ (=DSM 21626T=JCM 15517T).

The groundwater sample was collected on 15 January 2005 from the Wakkanai formation, a Miocene diatomaceous shale in northernmost Japan, by using methods described previously (Shimizu et al., 2006). The sample was extracted from 362.4–385.7 m below ground level through the survey borehole HDB-6, which was drilled by the Japan Atomic Energy Agency in the Horonobe area. Based on stable isotopic criteria (Strapoc et al., 2007), the δD value of CH₄ (−197 ‰) and the δ¹³C difference between dissolved CO₂ and CH₄ (Δ¹³C_{CO₂−CH₄} 76 ‰) indicated that the dissolved methane in the groundwater of the Wakkanai formation originated via microbial CO₂ reduction.

Groundwater samples (100 μl) were inoculated into 20 ml JCM 262 medium (http://www.jcm.riken.go.jp/cgi-bin/jcm/jcm_grmd?GRMD=262) in 50 ml serum bottles (Maruemu) sealed with butyl rubber stoppers and aluminium caps in an ANX anaerobic chamber (Hirasawa). The samples were incubated at 37 °C for 4 weeks in a H₂/CO₂ atmosphere (80 : 20, v/v; 200 kPa). The enriched culture, which actively produced methane, was transferred periodically to fresh broth. Strain T10⁷ was successfully isolated after repeated inoculations of a single colony that was serially diluted by using a JCM 262 slant culture containing 200 mg vancomycin l⁻¹ and 1.5 % (w/v) agar in a H₂/CO₂ atmosphere.
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the OD 660 and methane production values (Shimizu et al., 2011), using JCM 262 medium containing 10 mg 2-mercaptoethanesulphonic acid (coenzyme M) l⁻¹. The headspace was filled with either H₂/CO₂ (80 : 20, v/v; 200 kPa) to examine H₂/CO₂ utilization as a methanogenic substrate or with N₂/CO₂ (80 : 20, v/v) to study the utilization of other substrates. Media at different initial pH were prepared by using 20 mM MES (pH 5.5–6.8), PIPES (pH 6.8–6.9), HEPES (pH 6.9–8.0), Tricine (pH 8.2–8.4) and CHES (pH 8.6–9.2) and by varying the ratio of the H₂/CO₂ mixture (200 kPa). The cells were cultured in test tubes (18 × 180 mm) containing 7 ml medium, and the tubes were inclined at 90° to increase the area of contact between the medium and the H₂/CO₂ headspace gas. A minimum of three replicates of the cultures was incubated under each set of conditions for 56 days. The effects of temperature, pH and NaCl concentration on growth of strain T10T are shown in Fig. S2. The strain grew under the following conditions: 25–45°C (optimum 37–42°C), pH 5.8–8.2 (optimum pH 6.7–6.8) and 0–1.3 M NaCl (optimum 0.1–0.2 M).

The growth rate and substrate utilization of strain T10T were determined from the OD₆₆₀ and methane production values (Shimizu et al., 2011), using JCM 262 medium containing 10 mg 2-mercaptoethanesulphonic acid (coenzyme M) l⁻¹. The headspace was filled with either H₂/CO₂ (80 : 20, v/v; 200 kPa) to examine H₂/CO₂ utilization as a methanogenic substrate or with N₂/CO₂ (80 : 20, v/v) to study the utilization of other substrates. Media at different initial pH were prepared by using 20 mM MES (pH 5.5–6.8), PIPES (pH 6.8–6.9), HEPES (pH 6.9–8.0), Tricine (pH 8.2–8.4) and CHES (pH 8.6–9.2) and by varying the ratio of the H₂/CO₂ mixture (200 kPa). The cells were cultured in test tubes (18 × 180 mm) containing 7 ml medium, and the tubes were inclined at 90° to increase the area of contact between the medium and the H₂/CO₂ headspace gas. A minimum of three replicates of the cultures was incubated under each set of conditions for 56 days. The effects of temperature, pH and NaCl concentration on growth of strain T10T are shown in Fig. S2. The strain grew under the following conditions: 25–45°C (optimum 37–42°C), pH 5.8–8.2 (optimum pH 6.7–6.8) and 0–1.3 M NaCl (optimum 0.1–0.2 M).

The specific growth rate for strain T10T under optimal conditions based on the OD₆₆₀ was 0.101–0.110 h⁻¹. The strain used H₂/CO₂ (80 : 20, v/v; 200 kPa) and formate (20 mM) as methanogenic substrates. The strain exhibited good growth with H₂/CO₂ (80 : 20, v/v; 200 kPa), but slow growth with formate (the specific growth rate was 0.038 h⁻¹). Growth and methane formation were not observed on the following substrates: 2-propanol, 2-butanol, cyclopentanol, methanol, monomethylamine, dimethylamine, trimethylamine and acetate (20 mM each). Growth of the strain was stimulated by coenzyme M, acetate and yeast extract.

Genomic DNA was extracted (Marmur, 1961) and purified (Hamamoto & Nakase, 1995). The DNA G+C content was analysed using HPLC (Shimadzu LC-10A) (Katayama-Fujimura et al., 1984). The DNA G+C content of strain T10T was 62.9 ± 0.2 mol% (mean ± SD, n=3).

Phylogenetic analyses were performed as described previously (Shimizu et al., 2006, 2011). The phylogenetic tree, based on the almost-complete (1342 bp) 16S rRNA gene sequence, showed that strain T10T clustered with members of the genus Methanoculleus and was most closely related to Methanoculleus marisnigri JR1T, M. submarinus Nankai-1T and M. chikugoensis MG62T (Fig. 1). Bootstrap analysis indicated a clear branching of strain T10T from these species. BLAST searches using the almost-complete (1432 bp) 16S rRNA gene sequence of strain T10T revealed that the strain was most closely related to strains belonging to the genus Methanoculleus (95.5–98.3 % similarity), and the highest similarity was observed between strain T10T and M. marisnigri JR1T (98.3 %), M. submarinus Nankai-1T (98.3 %) and M. chikugoensis MG62T (98.1 %).

DNA–DNA hybridization was determined according to the method described by Ezaki et al. (1989) using an F0129005 SpectraFluor Plus (GENios). DNA–DNA relatedness between strain T10T and the type strains of the phylogenetically closest related species, M. marisnigri DSM 1498T, M. submarinus DSM 15122T and M. chikugoensis DSM 13459T, was respectively 23.0, 43.0 and 25.0 %. These values were too low to classify strain T10T within one of these existing species of the genus Methanoculleus.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain T10T and members of the genus Methanoculleus and related taxa. The tree was reconstructed using the neighbour-joining method. 16S rRNA gene sequences of Methanomicrobium mobile DSM 1539T (GenBank accession no. AY196679) and Methanogenium cariaci DSM 1497T (M59130) were used to root the tree (not shown). Tree topologies were evaluated by bootstrap analysis (based on 1000 replicates) using the MEGA 5.05 software package (Tamura et al., 2011). Bar, 0.01 substitutions per site.

The colonies were circular, 0.5–1.0 mm in diameter, with entire margins.

The cell morphology of strain T10T was examined under a scanning electron microscope (JEOL JSM6320F), a phase-contrast microscope (Olympus BX-51) and a transmission electron microscope (JEOL JEM-2000EX) (Fig. S1, available in IJSEM Online). The resulting images revealed that cells were irregular cocci, 0.7–1.6 μm in diameter, and that they occurred singly. No motility was observed under phase-contrast microscopy, but flagella were seen under negative-stain transmission electron microscopy. Cells of strain T10T were found to stain Gram-negative, and they lysed in 0.01 % (w/v) SDS.

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<th><strong>Methanoculleus</strong></th>
<th><strong>DSM</strong></th>
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<tr>
<td><strong>M. marisnigri</strong></td>
<td>1539T</td>
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<td><strong>M. submarinus</strong></td>
<td>15122T</td>
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<td><strong>M. chikugoensis</strong></td>
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A comparison of the phenotypic characteristics of strain T10T and the type strains of Methanoculleus species is summarized in Table 1. The main differences were observed in the following parameters: (i) range of growth temperature and pH, which differed from those of all members of the genus Methanoculleus; (ii) range of NaCl concentration for growth, which differed from all other type strains except that of Methanoculleus receptaculi; (iii) optimum temperature and secondary alcohol utilization, which differed from those of the phylogenetically closest related species M. marisnigri, M. submarinus and M. chikugoensis.

In conclusion, on the basis of phenotypic and phylogenetic data, strain T10T represents a novel species of the genus Methanoculleus, for which the name Methanoculleus horonobensis sp. nov. is proposed.

**Description of Methanoculleus horonobensis sp. nov.**

*Methanoculleus horonobensis* (ho.ro.no.ben'sis. N.L. masc. adj. horonobensis of or belonging to Horonobe, a town in Hokkaido, Japan, where the type strain was isolated).

Cells are irregular coccoids, 0.7–1.6 μm in diameter. Strictly anaerobic. Cells stain Gram-negative, are lysed by 0.01 % (w/v) SDS and are non-motile, despite the presence of flagella. Cells can utilize H2/CO2 or formate as substrates, but not 2-propanol, 2-butanol, cyclopentanol, methanol, dimethylamine, trimethylamine, dimethyl sulphide, acetate or monomethylamine. Grows at 25–45 °C (optimum 37–42 °C), at pH 5.8–8.2 (optimum pH 6.7–6.8) and in the presence of 0–1.3 M NaCl (optimum 0.1–0.2 M).

The type strain, T10T (=DSM 21626T=JCM 15517T), was isolated from deep subsurface groundwater from a diatomaceous shale formation in Horonobe, Japan. The genomic DNA G+C content of the type strain is 62.9 ± 0.2 mol%.

**Acknowledgements**

This work was supported by the Ministry of Economy, Trade and Industry (METI) of Japan. Deep subsurface groundwater was provided by the Japan Atomic Energy Agency (JAEA) based on a research partnership programme.

**References**


