**Methanosarcina horonobensis** sp. nov., a methanogenic archaeon isolated from a deep subsurface Miocene formation

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A methanogenic organism, designated strain HB-1T, from the domain Archaea was isolated from groundwater sampled from a subsurface Miocene formation located in Horonobe, Hokkaido, Japan. The strain grew on methanol, dimethylamine, trimethylamine, dimethylsulfide and acetate but not on monomethylamine, H2/CO2, formate, 2-propanol, 2-butanol or cyclopentanol. Cells were Gram-reaction-negative, non-motile, irregular cocci that were 1.4–2.9 μm in diameter and occurred singly or in pairs. The strain grew at 20–42 °C (optimum 37 °C), at pH 6.0–7.75 (optimum pH 7.0–7.25) and in 0–0.35 M NaCl (optimum 0.1 M). The G+C content of the genomic DNA was 41.4 mol%. 16S rRNA gene sequencing revealed that the strain was a member of the genus *Methanosarcina* but that it clearly differed from all recognized species of this genus (93.1–97.9 % sequence similarity). The phenotypic and phylogenetic features of strain HB-1T indicate that it represents a novel species of the genus *Methanosarcina*, for which the name *Methanosarcina horonobensis* sp. nov. is proposed. The type strain is HB-1T (=DSM 21571T =JCM 15518T =NBRC 102577T).

To date, few methanogens have been isolated from deep terrestrial subsurface environments other than oilfields (Christian et al., 2005); these include *Methanobacterium subterraneum*, isolated from deep granitic groundwater (Kotelnikova et al., 1998); *Methanolobus zinderi*, from a deep coal seam (Doerfert et al., 2009); and *Methanolobus profundi*, from a gas field (Mochimaru et al., 2009).

The Wakkanai Formation is a Miocene structure in northernmost Japan. It consists of layers of siliceous mudstone (Fukusawa, 1987). Palaeo-seawater and methane are trapped in the formation (Iwatsuki et al., 2009). Previously, we used 16S rRNA gene sequences to characterize the community structure of resident methanogens by extracting DNA from water taken from 288.7–644.1 m below ground level from the Horonobe area. Our results indicated that the archaeal 16S rRNA gene libraries were dominated by methanogen clones that were mostly related to members of the genera *Methanoculleus* and *Methanosarcina* (Shimizu et al., 2006). However, to our knowledge, no methane-producing strains have yet been isolated from the formation for further characterization.

In this study, we describe the characteristics of a methanogenic archaeon, designated strain HB-1T, which was sampled from an aquifer at the Wakkanai Formation.

A groundwater sample was collected on 15th January 2005 from the Wakkanai Formation by using previously described methods (Shimizu et al., 2006). The sample was extracted 288.7–303.0 m below ground level from survey borehole HDB-6, which was drilled by the Japan Atomic Energy Agency (JAEA) in the Horonobe area.

Groundwater samples were inoculated into modified DSM 120 medium (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium120.pdf), in which the concentrations of L-cysteine-HCl, H2O and Na2S·9H2O were changed to 0.5 g l–1, and incubated at 37 °C in an N2/H2/CO2 (80:10:10, v/v/v) atmosphere. The enrichment culture, which actively produced methane, was periodically transferred to fresh medium. After 14 days of incubation on solid medium, shiny, convex, clear and yellowish colonies were formed. Colonies were circular, with diameters of 0.5–1.0 mm and had entire margins.

An epifluorescence microscope (BX-51; Olympus) was used to perform phase-contrast and autofluorescence...
microscopy of cells in the exponential phase. The resulting images revealed that cells were irregular cocci, 1.4–2.9 μm in diameter and occurred singly or in pairs (Supplementary Fig. S1a, b, available in IJSEM Online). No motility was observed under phase-contrast microscopy and no flagella were seen when negatively stained cells were observed using transmission electron microscopy (JEM-2000EX; JOEL) (Supplementary Fig. S1c). Cells of strain HB-1T were found to be Gram-reaction negative and lysed in a 0.01 % (w/v) SDS solution.

To determine growth conditions and substrate utilization patterns, samples were cultured using DSM 120 medium modified as before, but with the concentration of NaCl changed to 0.1 M. The headspace was filled with H2/CO2 (80 : 20, v/v; 200 kPa) to examine the utilization of H2/CO2 as a methanogenic substrate or N2/CO2 (80 : 20, v/v) to study the utilization of other substrates. The pH of the medium was adjusted by adding HCl or NaOH solution. Cultures were incubated in triplicate under each set of conditions. A UVmini-1240 spectrophotometer (Shimadzu) was used to measure OD660 in order to ascertain growth conditions. A UVmini-1240 spectrophotometer (Shimadzu) was used to measure OD660 in order to ascertain growth rates. Strain HB-1T grew at 20–42 °C and pH 7.0 with an optimum growth temperature of 37 °C (Supplementary Fig. S2a). No growth occurred within 2 months at 15 °C or 45 °C. The optimum pH for growth was 7.0–7.25 (the initial pH of the culture medium), although growth was observed at pH 6.0–7.75; no growth was observed within 2 months at pH 5.5 or 8.0 (Supplementary Fig. S2b). Strain HB-1T grew in the presence of 0–0.35 M NaCl, with an optimum concentration of 0.1 M (Supplementary Fig. S2c). Furthermore, growth was observed in the presence of 0–0.75 M MgSO4, with an optimum concentration of 0–0.2 M (Supplementary Fig. S2d). No growth occurred within 2 months in medium supplemented with 0.4 M NaCl or 0.8 M MgSO4.

Strain HB-1T used (20 mM each) methanol, dimethylamine, trimethylamine, dimethylsulfide and acetate as methanogenic substrates. The strain exhibited good growth and methane formation were not observed on H2/CO2 (80 : 20, v/v; 200 kPa) or on (20 mM each) monomethylamine, formate, 2-propanol, 2-butanol or cyclopentanol. Growth of the strain was stimulated by Bacto yeast extract and Bacto casitone (BD). Furthermore, vitamins and trace minerals were required for growth in the DSM 120 medium.

The sensitivity of strain HB-1T to (100 μg l⁻¹ each) antibiotics such as ampicillin, penicillin G, vancomycin, kanamycin and tetracycline was tested at 37 °C and pH 7.0. Strain HB-1T was resistant to all antibiotics except tetracycline, with which no growth occurred within 2 months.

Cells of strain HB-1T were harvested in late exponential phase and used for DNA isolation according to previously described methods (Marmur, 1961). Crude DNA was purified according to the method of Hamamoto & Nakase (1995). HPLC with an LC-10A component (Shimadzu) was used to determine the G+C content of the DNA (Katayama-Fujimura et al., 1984); this was found to be 41.4 ± 0.4 mol% (mean ± SD, n = 3).

The 16S rRNA gene of strain HB-1T was amplified using the primers A25F (5′-CGGTTGATCCTGCGRG-3′, where Y=C or T and R=A or G) and U1492R (5′-GGTTACCTTGTTACGACTT-3′), according to Dojka et al. (1998). The amplified PCR product was sequenced as previously described (Shimizu et al., 2006), using the following sequencing primers: A25F (as above), U533F (5′-GTCG CGCCGGCGGTA-3′, where Y=C or G; Mikucki et al., 2003), U907R (5′-CGCTCA ATTCCCTTRAGTTT-3′; Dojka et al., 1998), 536-519R (5′-GATTACCGCGGGCTT-3′, where W=A or T; Lane et al., 1985), 388-353r (5′-AKTTTCGCGTTGCT-3′; Kotelnikova et al., 1998) and 0112aR (5′-CCAGGTGTTACTSACC-3′; Achenbach & Woese, 1995).

The BLAST search tool was used to compare the nearly complete (1432 bp) 16S rRNA gene sequence of strain HB-1T to sequences in the DNA database. Searches revealed that strain HB-1T was most closely related to strains belonging to the genus Methanosarcina (93.1–97.9 % 16S rRNA gene sequence similarity) with highest similarity values between strain HB-1T and M.arkeri MST (97.9 %) and M. mazei S-6T (97.2 %), followed by M. acetivorans C2A (96.8 %), M. lacustris ZST (96.1 %), M. thermophila TM1T (96.0 %), M. frisii C16T (95.8 %), M. siciliana T4/M (94.7 %), M. semensis MD1T (94.7 %), M. baltica GS1-A1T (94.0 %) and M. vacuolata Z-761T (93.1 %). Interestingly, the 16S rRNA gene sequence of strain HB-1T was most similar (99.9 %) to that of uncultured clone HDBW- WA05 (accession no. AB237738), which had previously been obtained from deep subsurface groundwater of the Wakkanai Formation in the Horonobe area (Shimizu et al., 2006).

Phylogenetic trees reconstructed using the neighbour-joining, maximum-parsimony and minimum evolution methods showed that strain HB-1T clustered with members of the genus Methanosarcina and was most closely related to M. mazei S-6T and M. frisii C16T (Fig. 1 and Supplementary Fig. S3). M. frisii has been proposed as a synonym of M. mazei since DNA-DNA hybridization values between the type strains were 77 % similar and because the two species have a similar physiology (Maestrojüan et al., 1992). Bootstrap analysis indicated a clear branching of strain HB-1T from these species.

Strain HB-1T had different phenotypic properties and substrate utilization characteristics to those of its closest phylogenetic relative, M. mazei and other related type strains (Table 1), as well as a lower tolerance of NaCl.
Unlike \textit{M. mazei}, strain HB-1\textsuperscript{T} was unable to use monomethylamine or H\textsubscript{2}/CO\textsubscript{2}; the other related type strains could also use monomethylamine. In addition to this, strain HB-1\textsuperscript{T} was tolerant of a much lower concentration of NaCl (0.35 M) than \textit{M. mazei} (1.0 M) and other related type strains ($\geq$0.6 M).

A SpectraFluor Plus (model F0129005; GENios) fluorescence, absorbance and luminescence instrument was used to quantify DNA–DNA hybridization values according to the method described by Ezaki et al. (1989). Strain HB-1\textsuperscript{T} and its closest relative, \textit{M. mazei} S-6\textsuperscript{T}, showed a relatedness value of 14\%. Even lower relatedness values were observed between strain HB-1\textsuperscript{T} and \textit{M. barkeri} MS\textsuperscript{T} (13\%), \textit{M. acetivorans} C2A\textsuperscript{T} (9\%), \textit{M. vacuolata} Z-761\textsuperscript{T} (6\%) and \textit{M. sicilae} T4/M\textsuperscript{T} (3\%). Genomic relatedness between strain HB-1\textsuperscript{T} and these type strains was too low to identify strain HB-1\textsuperscript{T} as belonging to any existing species of the genus \textit{Methanosarcina}.

Therefore, on the basis of the phenotypic and phylogenetic data, strain HB-1\textsuperscript{T} represents a novel species of the genus \textit{Methanosarcina}.

\textbf{Table 1. Characteristics of strain HB-1\textsuperscript{T} and species of the genus \textit{Methanosarcina}}

<table>
<thead>
<tr>
<th>Characteristic</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>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (μm)</td>
<td>1.4–2.9</td>
<td>1.0–3.0</td>
<td>0.9–1.6</td>
<td>1.5–3.0</td>
<td>1.9</td>
<td>1.0–2.0</td>
<td>1.5–2.0</td>
<td>ND</td>
<td>1.5–3.5</td>
<td>1.4±0.2</td>
<td>1.5–3.0</td>
</tr>
<tr>
<td>Gram stain</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>37</td>
<td>40–42</td>
<td>36</td>
<td>40</td>
<td>35–40</td>
<td>37–40</td>
<td>40–42</td>
<td>50</td>
<td>25</td>
<td>30–35</td>
<td>25</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>6.0–7.75</td>
<td>5.5–8.5</td>
<td>6.5–7.2</td>
<td>6.0–7.7</td>
<td>5.5–8.0</td>
<td>5.0–7.0</td>
<td>5.0–8.0</td>
<td>5.5–8.0</td>
<td>4.5–8.5</td>
<td>6.2–8.3</td>
<td>4.0–8.5</td>
</tr>
<tr>
<td>Optimum NaCl for growth (M)</td>
<td>0–0.35</td>
<td>0.1–1.0</td>
<td>0.1–1.0</td>
<td>0–0.17</td>
<td>0.1–1.0</td>
<td>0.1–0.6</td>
<td>0.1–0.8</td>
<td>ND</td>
<td>ND</td>
<td>0–1.0</td>
<td>0.2–1.2</td>
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<tr>
<td>Utilization of: H\textsubscript{2}/CO\textsubscript{2}</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Monomethylamine</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Dimethylsulfide</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Acetate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>41.4</td>
<td>42</td>
<td>38.2</td>
<td>42–43</td>
<td>41</td>
<td>36.3</td>
<td>38.8–43.5</td>
<td>42</td>
<td>43.4</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

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Methanosarcina, for which the name Methanosarcina horonobensis sp. nov. is proposed.

Description of Methanosarcina horonobensis sp. nov.

Methanosarcina horonobensis (ho.ro.no.ben’sis. N.L. fem. adj. horonobensis belonging to Horonobe, the town in Hokkaido, Japan, from which the strain was isolated).

Cells are strictly anaerobic, gram-reaction-negative, irregular cocci that are 1.4–2.9 μm in diameter and lysed by 0.01 % (w/v) SDS. Grows on methanol, dimethylamine, trimethylamine, dimethylsulfide and acetate but not on monomethylamine, H₂/CO₂, formate, 2-propanol, 2-butanol or cyclopentanol. Cells grow at 20–42 °C (optimum 37 °C), at pH 6.0–7.75 (optimum pH 7.0–7.25), in 0–0.35 M NaCl (optimum 0.1 M) and with 0–0.7 M MgSO₄ (optimum 0–0.2 M).

The type strain, HB-1T (=DSM 21571T =JCM 15518T =NBRC 102577T), was isolated in Horonobe, Japan, from deep subsurface groundwater from a siliceous mudstone formation. The DNA G+C content of the type strain is 41.4 ± 0.4 mol%.

Acknowledgements

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References


