**Methanothermobacter tenebrarum** sp. nov., a hydrogenotrophic, thermophilic methanogen isolated from gas-associated formation water of a natural gas field

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A thermophilic and hydrogenotrophic methanogen, strain RMAS\(^T\), was isolated from gas-associated formation water of a gas-producing well in a natural gas field in Japan. Strain RMAS\(^T\) grew solely on \(\text{H}_2/\text{CO}_2\) but required Casamino acids, tryptone, yeast extract or vitamins for growth. Growth of strain RMAS\(^T\) was stimulated by acetate. Cells were non-motile, straight rods (0.5–3.5–10.5 \(\mu\)m) and occurred singly or in pairs. Bundles of fimbriae occurred at both poles of cells and the cell wall was thick (approximately 21 nm, as revealed by ultrathin section electron microscopy). Strain RMAS\(^T\) grew at 45–80 °C (optimum, 70 °C), at pH 5.8–8.7 (optimum, pH 6.9–7.7) and with 0.001–20 g NaCl l\(^{-1}\) (optimum, 2.5 g NaCl l\(^{-1}\)). Phylogenetic analysis revealed that *Methanothermobacter thermautotrophicus* \(\Delta H^T\) was most closely related to the isolate (95.7 % 16S rRNA gene sequence similarity). On the basis of morphological, phenotypic and phylogenetic characteristics, it is clear that strain RMAS\(^T\) represents a novel species of the genus *Methanothermobacter*, for which we propose the name *Methanothermobacter tenebrarum* sp. nov. The type strain is RMAS\(^T\) (=DSM 23052\(^T\)=JCM 16532\(^T\)=NBRC 106236\(^T\)).

Hydrogenotrophic methanogens related to the family *Methanobacteriaceae* (the genera *Methanothermobacter* and *Methanobacterium*) are often found in oil and natural gas fields by culture-based and/or molecular methods and are considered to contribute to CH\(_4\) production in *in situ* subsurface environments (Nazina et al., 2006; Mochimaru et al., 2007; Li et al., 2007; Gray et al., 2009; Gieg et al., 2010; Mayumi et al., 2011). In a previous study, we examined the prokaryotic diversity and ability to produce methane in gas-associated formation water obtained from a natural gas field in Japan and reported that, within the formation water, hydrogenotrophic methanogenesis occurred and an uncharacterized methanogen, which was closely related to *Methanothermobacter thermautotrophicus* \(\Delta H^T\) (95.7 % 16S rRNA gene sequence similarity), dominated the archaeal clone library: the representative clone was designated NAK1-a1 (accession number DQ867048; Mochimaru et al., 2007). Here, the methanogen was isolated and characterized and its distinct ecophysiological features were elucidated.

Gas-associated formation water from a commercial gas-producing well, NAK1, in the natural gas field at Niigata, Japan was collected on 8 March 2005, as described by Mochimaru et al. (2007). The temperature, pH and oxidation/reduction potential of the sample were 53.1 °C, pH 6.8 and less than −300 mV, respectively. Mineral medium (W medium) and saline mineral medium (WS medium) were used to enrich the methanogen. W medium was composed of (l\(^{-1}\)) 0.535 g NH\(_4\)Cl, 0.136 g KH\(_2\)PO\(_4\), 0.204 g MgCl\(_2\)·6H\(_2\)O, 0.147 g CaCl\(_2\)·2H\(_2\)O, 2.52 g...
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NaHCO₃, 1 mg resazurin, 4 µg NaWO₄, 2H₂O, 4 µg NaSeO₃·5H₂O, 1 ml trace element solution and 1 ml vitamin solution. The trace element solution consisted of (L⁻¹) 12.8 g nitrilotriacetic acid, 1.35 g FeCl₃·6H₂O, 0.10 g MnCl₂·4H₂O, 0.024 g CoCl₂·6H₂O, 0.10 g CaCl₂·2H₂O, 0.100 g ZnCl₂, 0.025 g CuCl₂·2H₂O, 0.010 g H₂BO₃, 0.024 g Na₂MoO₄·2H₂O, 1.0 g NaCl and 0.12 g NiCl₂·6H₂O. The vitamin solution was composed of (L⁻¹) 2.0 mg biotin, 2.0 mg folic acid, 10 mg pyridoxine-HCl, 5.0 mg thiamine-HCl·2H₂O, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg D-Ca-pantothenate, 0.1 mg vitamin B₁₂, 5.0 mg p-aminobenzoic acid and 5.0 mg lipoic acid. WS medium consisted of W medium supplemented with (L⁻¹) 2.9 g MgCl₂·6H₂O and 20.5 g NaCl. Both media were prepared as described by Sekiguchi et al. (2000). Na₂S·9H₂O and cysteine-HCl·H₂O (both at final concentration of 0.12 g L⁻¹) were used to reduce the culture conditions and cultures were kept static unless otherwise mentioned. The gases used in this study were H₂/CO₂ (80:20, v/v), N₂/CO₂ (80:20, v/v) and N₂ gas. These gases were deoxygenated using a Deoxized Gas Pressure Injector (IP-8; Sanshin Industrial) unless otherwise indicated.

In our preliminary experiment, we found that the conventional enrichment and subsequent isolation using H₂/CO₂ (c.250 kPa) was unsuccessful, since a methanogen almost identical to Methanothermobacter thermautotrophicus, a minor population in the original sample, always became dominant in the cultures and so the target methanogen could never be isolated. We, therefore, changed our strategy to isolation based on the co-culture technique in which an H₂-supplying partner bacterium was difficult to eliminate. However, after many attempts, we eventually isolated the methanogen in pure culture in a deep gellan gum tube under an atmosphere of H₂/CO₂ (c.250 kPa) using W medium supplemented with (L⁻¹) 1.0 g tryptone (Difco), 1.0 g yeast extract (BBL), 0.82 g sodium acetate, 0.68 g sodium formate, 0.5 g HS-CoM sodium salt, 4 ml vitamin solution, 20 ml fatty acid mixture and 6.0 g gellan gum. The fatty acid mixture consisted of valeric acid, isovaleric acid, 2-methyl butyric acid and isobutyric acid, each at a final concentration of 0.5 g L⁻¹. Colonies of strain RMASᵀ grown in the deep gellan gum medium were yellow, spherical and about 1 mm in diameter after 4 weeks of incubation at 55°C. A colony was picked and cultured in supplemented W medium under an atmosphere of H₂/CO₂ (c.250 kPa). Contaminating cells of T. phaeum PBᵀ or other heterotrophs were not observed within the culture, judging from its subculture in NIH-thioglycolate medium amended with 20 mM pyruvate. The 16S rRNA gene sequence of strain RMASᵀ showed 99.3% similarity with that of clone NAK1-a1, which was the predominant archaeal clone in the environment (Mochimaru et al., 2007). Unless otherwise stated, strain RMASᵀ was cultivated in supplemented W medium under an atmosphere of H₂/CO₂ (c.250 kPa).

Methanothermobacter thermautotrophicus DSM 1053ᵀ was purchased from the DSMZ and used as a reference for growth tests (temperature and organic factor requirement), analyses of susceptibility to lysis, genomic G+C content and polar lipid content. The reference strain was cultured in W medium reduced with Na₂S·9H₂O and cysteine-HCl·H₂O (both at a final concentration of 0.3 g L⁻¹) under an atmosphere of H₂/CO₂ (c.250 kPa) at 65°C.

The morphology of cells of strain RMASᵀ grown at 70°C to the late exponential phase was determined with a phase-contrast microscope (BX51; Olympus). Cells were straight rods (0.5×3.5–10.5 μm) and were found individually or in pairs (Fig. 1a). Cells of strain RMASᵀ did not show motility. The Gram-stain was positive. Cells were negatively stained with 1% uranyl acetate and examined by TEM (75 kV, H-7000; Hitachi). Cells of strain RMASᵀ had fimbriae, which were located at both poles and arranged in bundles (Fig. 1b). Ultrathin sections of cells were also prepared and observed by TEM (Nakamura et al., 2006). Cells of strain RMASᵀ possessed a thicker cell wall, which presumably consists of pseudomurein, with a thickness of about 21 nm (Fig. 1c), which is in contrast with that of Methanothermobacter thermautotrophicus DSM 1053ᵀ, (about 6 nm thickness; Nakamura et al., 2006). A proteinaceous envelope, the S-layer, was not found in strain RMASᵀ.

W medium amended with 1 g tryptone and 0.82 g sodium acetate 1⁻¹ (WA medium) was used for examining temperature, pH and NaCl concentrations for growth. The optical density of cultures was measured at 560 nm. The optimum pH for growth was determined at 70°C and the pH was adjusted at room temperature according to Takai et al. (2003). Concentrated acetate/acetic acid and Tris/HCl buffers adjusted to different pHs were added to the medium to a final concentration of 10 mM to give the desired pH values. Susceptibility to lysis by SDS and hypotonic conditions was examined by microscopically observing the integrity of cells in the presence of 1% SDS and in distilled water. Growth requirements and stimulation tests were performed in W medium amended with each organic factor. Growth with methanogenic substrates (formate, pyruvate, monomethylamine, dimethylamine,
trimethylamine, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, and 2-methylpropanol) was examined in WA medium under an N₂/CO₂ atmosphere (c. 100 kPa) at 70 °C and methanogenesis was observed after 2 months of cultivation by GC, as described by Sekiguchi et al. (2000). All growth tests were performed at least in duplicate.

Strain RMASᵀ grew between 45 and 80 °C (optimum 70 °C). M. thermautotrophicus DSM 1053ᵀ, which has the highest temperature for growth (75 °C), was incapable of growing at 80 °C under our laboratory conditions (Table 1). The upper limit of the growth temperature of strain RMASᵀ exceeded those of the known species belonging to the genus Methanothermobacter (Table 1). The growth curve of strain RMASᵀ at 70 °C is shown in Fig. S1 (available in IJSEM Online), which gave a doubling time of approximately 12 h on the basis of optical density. Strain RMASᵀ grew between pH 5.8 and 8.7 (optimum pH 6.9–7.7) and grew with 0.001–20 g NaCl l⁻¹ (optimum 2.5 g NaCl l⁻¹). The optimum pH and NaCl concentration for growth of strain RMASᵀ were similar to those of species of the genus Methanothermobacter (Table 1). Cells of strain RMASᵀ, as well as those of M. thermautotrophicus DSM 1053ᵀ, did not lyse in the presence of 1 % SDS or in distilled water. Strain RMASᵀ required 0.1 % Casamino acids (Difco), 0.1 % tryptone (Difco), 0.1 % yeast extract (BBL) or 5 ml vitamin solution l⁻¹ for growth, whereas M. thermautotrophicus DSM 1053ᵀ can grow in the absence of growth factors under our laboratory conditions (Zeikus & Wolfe, 1972; Wasserfallen et al., 2000). Acetate (10 mM) stimulated growth, whereas neither coenzyme M nor the fatty acid mixture described above was required or accelerated growth. No growth or methanogenesis was observed with 10 mM acetate, 40 mM formate, 20 mM pyruvate, 10 mM monomethylamine, 10 mM dimethylamine, 10 mM trimethylamine, 20 mM methanol, 5 mM ethanol, 5 mM 1-propanol, 5 mM 2-propanol, 5 mM 1-butanol or 5 mM 2-methylpropanol. Cells of strain RMASᵀ were strictly anaerobic and grew exclusively on H₂/CO₂. Exclusive growth on H₂/CO₂, with some exceptions utilizing formate, is a common feature found in the genus Methanothermobacter, as shown in Table 1.

For polar lipid analysis, cells of strain RMASᵀ were grown in WA medium at 70 °C and harvested at the late exponential phase. Polar lipids were recovered from cells by the acidified Bligh and Dyer method and separated by one-dimensional TLC, as described by Nishihara & Koga (1987). Lipid bands on the TLC plate were visualized using ninhydrin for amino lipids and either 30 % H₂SO₄ or iodine vapour for all lipids. Some lipids visualized using iodine vapour were scraped and analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, AXIMA Resonance, Shimadzu) according to the procedure of Angelini et al. (2010). Cells of M. thermautotrophicus DSM 1053ᵀ at the late exponential phase were also examined as a reference. The visualized lipid bands of strain RMASᵀ and M. thermautotrophicus DSM 1053ᵀ were similar but with some differences between them (Fig. S2). The bands were identified as gentiobiosylcaldarchaetidylinositol (Rᶠ 0.21, both strains) and gentiobiosylcaldarchaetidylethanolamine (Rᶠ 0.40, strain RMASᵀ) on the basis of the MALDI-TOF MS and TLC analyses described by Nishihara & Koga (1987). Both gentiobiosylcaldarchaetidylinositol and gentiobiosylcaldarchaetidylethanolamine have been designated the signature lipids of the genera Methanothermobacter and Methanobacterium within the family Methanobacteriaceae (Koga et al., 1998); hence, this chemotaxonomic trait indicates that strain RMASᵀ belongs to either of these genera.

Fig. 1. Cell morphology of strain RMASᵀ by phase-contrast microscopy (a) and TEM (b and c). Electron micrographs were obtained using negatively stained (b) and ultrathin-sectioned (c) cells. Bars, 10 μm (a) and 1 μm (b and c).
Table 1. Differential characteristics of strain RMAST™ and type strains of species in the genus *Methanothermobacter*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Gas-associated water of natural gas field</td>
<td>Sewage sludge</td>
<td>Anaerobic sludge</td>
<td>Anaerobic sludge</td>
<td>Anaerobic sludge</td>
<td>Mixture of sewage sludge and river sediment</td>
<td>Sewage sludge</td>
</tr>
<tr>
<td>Cell dimensions (μm)</td>
<td>0.5 × 3.5–10.5</td>
<td>0.4 × 3–120</td>
<td>0.40 × 7–20</td>
<td>0.36 × 1.4–6.5</td>
<td>0.42 × 3–6</td>
<td>0.4 × 2.5</td>
<td>0.35 × 3–20</td>
</tr>
<tr>
<td>Gram stain</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth with formate</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Organic factors required for growth</td>
<td>Casamino acids, tryptone, yeast extract, vitamins</td>
<td>−*</td>
<td>−</td>
<td>−</td>
<td>− Coenzyme M</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Optimum temperature (range) (°C)</td>
<td>70 (45–80)</td>
<td>65–70 (40–75*)</td>
<td>55 (45–70)</td>
<td>57 (45–65)</td>
<td>60–65 (45–65)</td>
<td>55–65 (37–74)</td>
<td>65 (45–70)</td>
</tr>
<tr>
<td>Optimum pH (range)</td>
<td>6.9–7.7 (5.8–8.7)</td>
<td>7.2–7.6 (6.0–8.8)</td>
<td>7.9–8.2 (7.5–8.5)</td>
<td>7.6 (7.0–8.5)</td>
<td>6.5–7.0 (6.0–7.5)</td>
<td>7.0–7.5 (6.0–8.2)</td>
<td>6.8–7.4 (5.0–8.0)</td>
</tr>
<tr>
<td>Optimum NaCl (range) (g l⁻¹)</td>
<td>2.5 (0.001–20)</td>
<td>0.6 (0.1–35)</td>
<td>2 (1–30)</td>
<td>ND (0–30)</td>
<td>2 (0.8–20)</td>
<td>&lt;10 (&lt;20)</td>
<td>0.5 (0.1–35)</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>41.5 (HPLC)</td>
<td>51.2 (HPLC)*</td>
<td>55.0 (<em>Tₘ</em>)</td>
<td>44.7 (<em>Tₘ</em>)</td>
<td>62.2 (<em>Tₘ</em>)</td>
<td>61 ± 1 (<em>Tₘ</em>)</td>
<td>48.6 (sequence)</td>
</tr>
</tbody>
</table>

*Confirmed or determined by use of *M. thermautotrophicus* DSM 1053™ in this study.
The G+C content of the genomic DNA of strain RMAS\textsuperscript{T} and \textit{M. thermautotrophicus} DSM 1053\textsuperscript{T} were determined by HPLC (Kamagata & Mikami, 1991). The DNA G+C content was 41.5 and 51.2 mol\%, respectively, which suggests that the two strains belong to different taxa (Table 1).

The 16S rRNA gene of strain RMAS\textsuperscript{T} was amplified using PCR with a slightly modified primer of ARCH21F (DeLong, 1992), namely Ar1otf-kn (5\textsuperscript{\prime}-TCTGGGTAGTCTCGTSRCG-3\textsuperscript{\prime}), and Univ1500R and was sequenced as described by Mochimaru et al. (2007). The almost full-length 16S rRNA gene sequence of strain RMAS\textsuperscript{T} (1414 bp) was aligned with reference sequences using the online alignment tool SINA (http://www.arb-silva.de; Pruesse et al., 2007). The alignment was imported into the \textsc{arb} \textsc{silva} reference database (SSU\textsubscript{Ref}-108) and manually refined using tools available in the \textsc{arb} software package (Ludwig et al., 2004). A total of 35 sequences with 1392 bp valid positions passed through 50\% positional conservatory filter in \textsc{arb} were used for phylogenetic analysis. A neighbour-joining phylogenetic tree was constructed using the Jukes–Cantor correction in \textsc{arb} (Saitou & Nei, 1987; Jukes & Cantor, 1969). Bootstrap resampling analyses with 1000 replicates were performed for the neighbour-joining, maximum-likelihood and maximum-parsimony analyses to estimate the confidence of the tree topologies. \textsc{MEGA} 4.0 (Tamura et al., 2007) was used for the neighbour-joining and maximum-parsimony analyses and \textsc{phylm} 2.4.5 (Guindon & Gascuel, 2003) was used for the maximum-likelihood analysis. The sequence similarity values were calculated using the calculate matrix function of the \textsc{arb} program without a filter.

The phylogenetic analysis based on 16S rRNA gene sequences revealed that strain RMAS\textsuperscript{T} was a member of the genus \textit{Methanothermobacter} within the family \textit{Methanobacteriaceae} (Fig. 2a). This relationship was supported by high bootstrap values (100\%) obtained by the neighbour-joining and maximum-likelihood methods, but not by the maximum-parsimony method. Nonetheless, strain RMAS\textsuperscript{T} showed high sequence similarities with members of the genus \textit{Methanothermobacter} with validly published names, e.g. \textit{Methanothermobacter thermophilus} \textit{HT} (95.7\% 16S rRNA gene sequence similarity), \textit{Methanothermobacter thermoflexus} \textit{IDZ} (95.6\%) and \textit{Methanothermobacter defluvii} \textit{ADZ} (95.5\%), which strongly supports placing strain RMAS\textsuperscript{T} in the genus \textit{Methanothermobacter}. Also, \textit{Methanothermobacter crinale} \textit{Tm} and \textit{Methanobacterium thermagregans} DSM 3266\textsuperscript{T} were found to be closely related to strain RMAS\textsuperscript{T} (98.7 and 95.7\% sequence similarity, respectively); however, we did not include them for comparative analysis because \textit{Methanothermobacter crinale} \textit{Tm} was not validly described at the time of writing (Cheng et al., 2011) and the purity of the culture of \textit{Methanobacterium thermagregans} DSM 3266\textsuperscript{T} is considered doubtful, owing to its purification procedure (Blotevogel & Fischer, 1985), and the reported data are difficult to interpret (Boone et al., 2001). It is also supposed that \textit{Methanobacterium thermagregans} could be placed in the genus \textit{Methanothermobacter} based on thermophilic growth and 16S rRNA gene phylogeny.

\textit{mcrA}, a key gene for methanogenesis, of strain RMAS\textsuperscript{T} was amplified, cloned and analysed using two primer pairs, MCRf and MCRr (Springer et al., 1995) and ME1 and ME2 (Hales et al., 1996). These primer pairs were applied to reduce potential bias of PCR amplification (Lueders et al., 2001). Predicted McrA amino acid sequences were obtained from the FunGene database (http://fungene.cme.msu.edu/index.spr) and aligned with that of strain RMAS\textsuperscript{T} using \textsc{arb}, resulting in an alignment containing 23 sequences with 133 valid positions. A neighbour-joining tree was constructed from a Dayhoff distance matrix (Schwartz & Dayhoff, 1978). Bootstrap resampling analysis was performed as described above.

The deduced amino acid sequences of \textit{mcrA} of strain RMAS\textsuperscript{T} using the MCRf/MCRr and ME1/ME2 primer pairs were 157 and 240 amino acids long, respectively. The McrA sequence of strain RMAS\textsuperscript{T} exhibited high similarities with those of \textit{Methanothermobacter crinale} \textit{Tm} and \textit{Methanobacterium aarhusense} \textit{H2-LRT} (96.7 and 90.1\%, respectively). Very interestingly, the McrA phylogeny (Fig. 2b) revealed that, despite the fact that strain RMAS\textsuperscript{T} and \textit{Methanothermobacter crinale} \textit{Tm} are closely related to \textit{Methanothermobacter thermophilus} \textit{HT} on the basis of 16S rRNA gene sequences (Fig. 2a), they were more related to members of the genus \textit{Methanobacterium} than to \textit{Methanothermobacter}. The reason for the discrepancy between the 16S rRNA gene and McrA phylogenies is not clear, but the physiological and biochemical features, as shown below, support the placement of strain RMAS\textsuperscript{T} in the genus \textit{Methanothermobacter}.

As described earlier, strain RMAS\textsuperscript{T} possesses similar physiological and biochemical features to species of the genus \textit{Methanothermobacter}, which are hydrogenotrophic and thermophilic methanogens. Indeed, strain RMAS\textsuperscript{T} grew with and produced methane from H\textsubscript{2}/CO\textsubscript{2} and had a high optimum growth temperature (70 °C). A polar lipid of strain RMAS\textsuperscript{T} (PNG1L) is generally found in the genera \textit{Methanothermobacter} and \textit{Methanobacterium}, but not in other genera (\textit{Methanobrevibacter} and \textit{Methanosphaera}) within the family \textit{Methanobacteriaceae} (Koga et al., 1998). The 16S rRNA gene-based phylogeny (N) and ML analyses also revealed that strain RMAS\textsuperscript{T} is most closely related to members of the genus \textit{Methanothermobacter} and that the 16S rRNA gene sequence similarities between strain RMAS\textsuperscript{T} and the genus \textit{Methanothermobacter} are higher (95.1–95.7\%) than with the genus \textit{Methanobacterium} (91.3–93.8\%). Because phylogenetic analysis based on the 16S rRNA gene, the most commonly used and reliable phylogenetic marker gene, clearly shows that strain RMAS\textsuperscript{T} is more closely related to the genus \textit{Methanothermobacter} than the genus \textit{Methanobacterium} and because strain RMAS\textsuperscript{T} prefers a high temperature for growth (70 °C), at which all members of the genus \textit{Methanobacterium} cannot grow, we conclude that strain RMAS\textsuperscript{T} belongs to the genus \textit{Methanothermobacter}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Species & G+C content (mol\%) & Growth temperature (°C) \\
\hline
\textit{Methanothermobacter crinale} \textit{Tm} & 51.2 & 70 \\
\textit{Methanothermobacter defluvii} \textit{ADZ} & 55.0 & 70 \\
\textit{Methanobacterium aarhusense} \textit{H2-LRT} & 54.3 & 70 \\
\hline
\end{tabular}
\caption{Physiological and biochemical features of \textit{Methanothermobacter crinale} and \textit{Methanobacterium aarhusense} \textit{H2-LRT}.}
\end{table}
Methanobacterium. In fact, optimum growth temperature is an important physiological trait discriminating the genus *Methanobacterium* (37–45 °C) from the genus *Methanothermobacter* (55–65 °C) (Boone, 2000; Boone et al., 2001).

Although strain RMAS\textsuperscript{T} belongs to the genus *Methanothermobacter*, its physiological features are distinct from species of the genus with widely published names. Strain RMAS\textsuperscript{T} has: (i) the highest temperature for growth (80 °C); *M. thermotutotrophicus* ΔH\textsuperscript{T} has the next highest in the genus (75 °C); (ii) a requirement of organic factors (either Casamino acids, tryptone, yeast extract or vitamins) for growth: no other member of the genus *Methano-thermobacter* requires these factors; and (iii) the lowest G + C content (41.5 mol%): *Methanobacterium therophilus* M\textsuperscript{T} has the next lowest (44.7 mol%) (Table 1). Furthermore, 16S rRNA gene sequence similarities of strain RMAS\textsuperscript{T} to members of the genus *Methanobacterium* were 95.1–95.7%, which are sufficient for differentiating strain RMAS\textsuperscript{T} at the species level (Keswani & Whitman, 2001; Stackebrandt & Goebel, 1994). A recently described species of the genus *Methanobacterium*, *Methanobacterium crinale* Tm\textsuperscript{2}, is phylogenetically closely related to strain RMAS\textsuperscript{T} (98.7% 16S rRNA gene sequence similarity and 96.7% McrA amino acid sequence similarity) and it shares some phenotypic characteristics with strain RMAS\textsuperscript{T}, such as sole growth with H\textsubscript{2}/CO\textsubscript{2}, growth temperature range (45–80 °C) and optimum pH; however, discriminating phenotypic traits between these two strains are also found, such as optimum temperature for growth (65 °C), NaCl for growth (0–40 g NaCl l\textsuperscript{-1}) and requirement of acetate for growth. The differences in their phenotypic properties suggest that these two strains may belong to different species even though they are phylogenetically closely related.

![Fig. 2. Phylogenetic trees showing the position of strain RMAS\textsuperscript{T} in relation to reference taxa of methanogens based on 1392 bp of the 16S rRNA gene sequence (a) and 133 aa of the deduced McrA amino acid sequence (b). Bootstrap values (>70%) based on 1000 replicates using the neighbour-joining/max-imum-likelihood/multiparamsimy algorithms are shown at branch nodes. *Methanococcus vannielii* SB\textsuperscript{V} (GenBank accession number CP000742) and *Methanocaldococcus jannaschii* DSM 2661\textsuperscript{T} (L77117) were used as an outgroup in (a). Methanosarcina barkeri DSM 804 (YP_304447) and *Methanomicrobium mobile* BP\textsuperscript{T} (AF141044) were used as an outgroup in (b). Bars, 0.02 nucleotide substitutions per site (a) and 0.02 amino acid substitutions per site (b).](image-url)

**Table 1.** 16S rRNA gene sequence similarities of strain RMAS\textsuperscript{T} to members of the genus *Methanobacterium*. The lowest G + C content (41.5 mol%) is also indicated (Stackebrandt & Goebel, 1994).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence similarity (%)</th>
<th>G + C content (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methanobacterium alcaliphilum</em></td>
<td>99/99/99</td>
<td>41.5</td>
</tr>
<tr>
<td><em>Methanobacterium arboriphilus</em></td>
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<tr>
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<td><em>Methanobacterium jannaschii</em></td>
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<td><em>Methanobacterium millerae</em></td>
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<tr>
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<tr>
<td><em>Methanobacterium palustrum</em></td>
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<tr>
<td><em>Methanobacterium subterraneum</em></td>
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<tr>
<td><em>Methanobacterium thermautotrophicus</em></td>
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<tr>
<td><em>Methanobacterium therophilus</em></td>
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<td><em>Methanobacterium thermautotrophicus</em></td>
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On the basis of phenotypic, chemotaxonomic and 16S rRNA gene sequence analysis, we propose that a novel species should be created to accommodate strain RMAS\textsuperscript{T} within the genus *Methanobacterium*, with the name *Methanobacterium tenebrarum* sp. nov.
Description of Methanothermobacter tenebrarum sp. nov.

Methanothermobacter tenebrarum (te.ne.bra’rum. L. gen. pl. n. tenebrarum of darkness, of a dark place, referring to the isolation source, a deep terrestrial subsurface).

Cells are Gram-stain-positive, non-motile, straight rods (0.5 × 3.5–10.5 μm), often occurring in pairs. Cells may possess a thick cell wall (c.20 nm). Solely grows on H₂ and CO₂. Acetate, formate, pyruvate, monomethylamine, dimethylamine, trimethylamine, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol and 2-methylpropanol are not utilized for growth or methanogenesis. Non-autotrophic. Requires Casamino acids, tryptone, yeast extract or vitamin mixture for growth. Acetate stimulates growth. Grows at 45–80 °C (optimum, 70 °C), at pH 5.8–8.7 (optimum, pH 6.9–7.7) and with 0.001–20 g NaCl l⁻¹ (optimum, 2.5 g NaCl l⁻¹). Cells are not disrupted by 1% SDS or distilled water. Gentiobiosylcaldarchaetidylinositol and gentiobiosylcaldarchaetidytilhexanolamine are found as polar lipids.

The type strain, RMASᵀ (=DSM 23052ᵀ=JCM 16532ᵀ=NBRC 106236ᵀ), was isolated from gas-associated formation water of a gas-producing well in a natural gas field at Niigata, Japan. The DNA G+C content of the type strain is 41.5 mol% (HPLC).

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References


