Methylocaldum marinum sp. nov., a thermotolerant, methane-oxidizing bacterium isolated from marine sediments, and emended description of the genus Methylocaldum

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An aerobic, methane-oxidizing bacterium (strain S8T) was isolated from marine sediments in Kagoshima Bay, Japan. Phylogenetic analysis based on 16S rRNA gene sequences indicated that this strain is closely related to members of the genus Methylocaldum (97.6–97.9 % similarity) within the class Gammaproteobacteria. Strain S8T was a Gram-staining-negative, non-motile, coccoid or short rod-shaped organism. The temperature range for growth of strain S8T was 20–47 °C (optimum growth at 36 °C). It required NaCl (0.5 %), tolerated up to 5 % NaCl and utilized methane and methanol. The major cellular fatty acid and major respiratory quinone were C16 : 0 and 18-methylene ubiquinone 8, respectively. The DNA G+C content was 59.7 mol%. Strain S8T possessed mmoX, which encodes soluble methane monooxygenase, as well as pmoA, which encodes the particulate methane monooxygenase. On the basis of this morphological, physiological, biochemical and genetic information, the first marine species in the genus Methylocaldum is proposed, with the name Methylocaldum marinum sp. nov. The type strain is S8T (=NBRC 109686T =DSM 27392T). An emended description of the genus Methylocaldum is also provided.

The methane cycle in the ocean has been the subject of increased attention in recent years. It has been reported that methane is produced not only in anaerobic environments but also in aerobic environments such as the water at the surface of the ocean, possibly through the decomposition of methylphosphonate (Karl et al. 2008) or via methanogenesis (Grossart et al., 2011). On the seafloor, destabilization of methane hydrate due to changes in ocean temperature has been found to occur in the North American margin (Phrampus & Hornbach, 2012), possibly

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Abbreviations: RuMP, ribulose monophosphate; sMMO, soluble methane monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, mmoX, pmoA and cbbL gene sequences of strain S8T are AB894129, AB900160, AB900159 and AB922600, respectively.
releasing methane into the water column. Marine hydrothermal systems distributed widely along mid-ocean ridges and back-arc basins (Van Dover, 2011) are another source of methane in the ocean (Takai & Nakamura, 2010). It is estimated that 10⁸ mol methane is released per year to the open ocean from the Mid-Atlantic Ridge (Reeburgh, 2007). Marine methane-oxidizing bacteria (methanotrophs) play an important role in the ocean’s methane cycle and mitigate the emission of methane, a global warming gas, to the atmosphere. However, the names of only four species have been validly published so far: Methylocaldum marinum (Sieburth et al., 1987; Bowman et al., 1995), Methylobacter marinus (Lidstrom, 1988), Methylocaldum pelagicum (Sieburth et al., 1987; Bowman et al., 1995), and Methylomarinum vadi (Hirayama et al., 2008). The inability to form colonies on conventional solid media (Holmes et al., 1991) or to grow on methanol as sole carbon source (Kester et al., 1967) has prevented the isolation of marine methanotrophs. In this study, we have succeeded in isolating a novel marine methanotroph, strain S8T, from a methane-utilizing mixed culture that originated from marine sediments in Kagoshima Bay, Japan. A novel species, Methylocaldum marinum sp. nov., is proposed to accommodate this isolate.

In Kagoshima Bay, Japan, multiple hydrothermal vents that emit high-temperature water containing methane have been identified at Wakamiko Crater (Ishibashi et al., 2008; Yamanaka et al., 2013). Marine surface sediments from a location north to the Wakamiko Crater (water depth 161 m; 31° 41.09' N 130° 47.124' E) were collected in 2001 and used as an inoculum for the enrichments. A stable methane-utilizing mixed culture was established as described previously (Takeuchi et al., 2014). Strain S8T was isolated from the mixed culture on plates made from NMS medium (Bowman, 2006), which was modified by replacing the distilled water and sodium nitrate with artificial seawater (Kester et al., 1967) and ammonium chloride, respectively, and then solidified by mixing with an equal volume of autoclaved gellan gum solution (final concentration 1.5%; Tamaki et al., 2005, 2009). The plates were then incubated under methanol/air (20:80, v/v) in a desiccator at 35 °C. Isolated colonies were then incubated in a liquid medium. Cloning of the 16S rRNA gene of the original methane-utilizing mixed culture had revealed the existence of three strains (strains S8T, Gela4T and MA2). Strains Gela4T (Methylocacenibacter caenitepidi Gela4T; Takeuchi et al., 2014) and MA2 were also isolated and shown to be, respectively, a methylotroph that grows on a modified NMS medium containing 1 % methanol and solidified with agar, and a heterotroph that grows on marine agar 2216 (Difco). Therefore, the purity of strain S8T was checked by plating the culture onto these two agar media.

The strain was grown in NMS-SP medium, which is NMS medium that has been modified by replacing the distilled water with Daigo’s artificial seawater SP (Wako Chemicals), for further analysis. The cell morphology and motility of the strain were examined under a phase-contrast microscope (Olympus BX51). Heat resistance was assayed by incubating a 7-week-old culture of the strain at 85 °C for 15 min. Desiccation resistance was assayed by drying a 7-week-old culture of the strain for 4 days on a glass slide. The growth substrates tested were methane, methanol, methylamine, dimethylamine, trimethylamine, sodium formate, formamide and methanesulfonic acid. The methane (20 %) was added to the headspace, whereas the other substrates were added to the NMS-SP medium at a concentration of 0.1 %, and the cultures were subsequently incubated at 36 °C. Nitrogen sources tested were sodium nitrate and ammonium chloride. To determine the capacity for nitrogen fixation, cells were incubated in nitrogen-free liquid medium under aerobic conditions. Growth was tested at 9, 16, 20, 25, 35, 36, 38, 40, 45, 47, 48 and 52 °C and pH 5, 6, 7, 8 and 9. The effect of NaCl on growth was examined by using NMS-SP medium supplemented with 2, 3, 5 and 6 % (w/v) NaCl. To obtain lower concentrations of NaCl (0, 0.5 and 1 %), artificial seawater (Kester et al., 1967) with various concentrations of NaCl was used to prepare the NMS medium. Growth of the strain on methanol was examined at 0.01, 0.02, 0.04, 0.1, 0.5, 1, 2, 5, 10 and 20 % (v/v) methanol. Respiratory quinones, cellular fatty acid methyl esters and DNA G+C content were analysed as described previously (Hanada et al., 2002; Kamagata & Mikami, 1991; Zhang et al., 2000). Chromosomal DNA was extracted using the ArchivePure DNA Tissues kit (5 Prime Inc.). The 16S rRNA gene from strain S8T was amplified using primers specific for bacteria, Eub8F (Weisburg et al., 1991) and Eub1389R (Osborn et al., 2000), as described previously (Takeuchi et al., 2011), and sequenced with an ABI 3130xl Genetic Analyzer (Applied Biosystems). The sequencing chromatogram of the 16S rRNA gene of strain S8T was checked to confirm the purity of the strain. The draft genome sequence of strain S8T was determined using 454 pyrosequencing by single-end (SE) and paired-end (PE) data. We generated 137 291 reads by SE and 174 170 reads by PE, and thus obtained 18.6-fold coverage of the genome. Assembly of the obtained sequence data generated 17 scaffolds using Newbler version 2.6. The detailed genome information will be described elsewhere. The 16S rRNA gene sequences of strain S8T and related sequences were aligned using the SILVA Incremental Aligner (SINA; Pruesse et al., 2012). The sequences of the mmoX gene, which encodes the soluble methane monooxygenase (sMMO), and the pmoA gene, which encodes the particulate methane monooxygenase, found in the draft genome, were used to deduce their respective amino acid sequences and aligned with related protein sequences using the Muscle algorithm in MEGA 5 under the default parameters (Tamura et al., 2011). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods using MEGA 5 (Tamura et al., 2011) with the default parameters. The robustness of the tree topology was assessed by bootstrap analysis based on 1000 replications for both neighbour-joining and maximum-likelihood trees.

Cells of strain S8T were Gram-staining-negative and non-motile. Cocci and short rods (0.8–1.7 × 1.0–2.8 μm) and,
Strain S8T grew only on methane and methanol. Growth on a solidified gellan-gum plate was also lost in subsequent incubations. The capacity for colony formation on a gellan-gum plate was observed on the same medium solidified with agar. The capacity for colony formation on a gellan-gum plate was also lost in subsequent incubations. Strain S8T grew only on methane and methanol. Growth occurred at 0.01–2 % methanol. No vitamins were required for growth. Both nitrate and ammonium were used as a nitrogen source. Growth on nitrogen-free liquid medium under aerobic conditions was negative. Growth occurred at 20–47 °C, and no growth was observed above 48 °C or below 16 °C. The optimum growth temperature was 36 °C. Growth occurred at pH 6–8, but not at pH 5 or 9. Optimum growth occurred at pH 7. Strain S8T required NaCl and grew at 0.5–5 % (w/v) NaCl. The optimum NaCl concentration was 2 % (w/v). The specific growth rate of a culture grown on methane (20 %, v/v), calculated from increases in OD660 in the exponential phase of growth under optimal conditions, was 0.53 day−1 (equal to a doubling time of 1.3 days).

The genomic DNA of strain S8T contained 59.7 mol% G+C. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain S8T belonged to the genus Methylocaldum in the class Gammaproteobacteria (Fig. 2). Sequence similarities to other members of the genus Methylocaldum (the type strains of Methylocaldum gracile, Methylocaldum tepidum and Methylocaldum szegediense, Methylocaldum sp. O-12 and Methylocaldum sp. H-11; Bodrossy et al., 1997; Eshinimaev et al., 2004) ranged from 97.6 to 97.9 %. The observation that unexpected 16S rRNA sequences indicated that strain S8T belonged to the genus Methylocaldum (the type strains of Methylocaldum gracile, Methylocaldum tepidum and Methylocaldum szegediense, Methylocaldum sp. O-12 and Methylocaldum sp. H-11; Bodrossy et al., 1997; Eshinimaev et al., 2004) ranged from 97.6 to 97.9 %. The observation that unexpected 16S rRNA genes or apparent duplications of housekeeping genes were not found in the draft genome sequence of strain S8T provided evidence of the purity of the strain. Phylogenetic analysis of the pmoA gene sequence found in the draft genome confirmed that strain S8T belongs to the genus Methylocaldum (Fig. 3a). Deduced amino acid sequence similarities between the pmoA gene of strain S8T and those of strains of other species of the genus Methylocaldum were 97.9 % (Methylocaldum tepidum and Methylocaldum gracile) and 94.4 % (Methylocaldum szegediense). In addition to pmoA, mmoX was found in the draft genome of strain S8T, although previously known species of the genus Methylocaldum do not possess an sMMO (Eshinimaev et al., 2004). The mmoX sequence of strain S8T was most closely related to that of Methylococcus capsulatus Bath, with 90.4 % similarity based on the deduced amino acid sequence (Fig. 3b). The presence of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is characteristic of thermotolerant methanotrophs such as members of the genera Methylococcus and Methylocaldum (Baxter et al. 2002). The presence of key enzymes for both the ribulose monophosphate (RuMP) and serine pathways of formaldehyde assimilation also characterizes the genus Methylocaldum (Bodrossy et al., 1997). In the draft genome of strain S8T, genes encoding the large subunit of RuBisCO (cbbL) and key enzymes of the RuMP pathway (3-hexulose-6-phosphate synthase and 6-phospho-3-hexulonoseisomerase) and serine pathway (serine glyoxylate aminotransferase and hydroxypyruvate reductase) were found. Analysis of the cellular fatty acid composition showed the predominance of C16:0 (59.2 %). Other major fatty acids were C16:1o7c (39.7 %) and C14:0 (1.2 %). The major respiratory quinone was 18-methylene ubiquinone 8.

Differential characteristics of strain S8T and other members of the genus Methylocaldum are listed in Table 1. The absence of motility, the inability to form colonies on agar medium, growth on methanol and the presence of the mmoX gene distinguished strain S8T from other members of the genus Methylocaldum. Furthermore, to our knowledge, strain S8T is the first strain of the genus Methylocaldum isolated from a marine environment, and the only one to require NaCl. Thus, based on these morphological, biochemical, physiological and genetic data, strain S8T is considered to represent a novel species of the genus Methylocaldum. We propose the name Methylocaldum marinum sp. nov. for this novel species.

The genus Methylocaldum is a group of moderately thermophilic or thermotolerant methanotrophs (Bodrossy et al., 1997). The temperature range for growth of strain S8T is 20–47 °C, indicating that strain S8T is also a thermotolerant methanotroph. Although we could not measure the temperature of the sampling point in situ, the temperature of the water at the bottom of the sampling area is known to be around 15 °C (Craig & Horibe, 1994), which is lower than the temperature range for growth of strain S8T. It is suggested that strain S8T originates from the hydrothermal area in Kagoshima Bay.

**Emended description of the genus Methylocaldum**

Cells are aerobic, Gram-staining-negative and may vary in shape from coccoïd to long rods. Moderate thermophiles that grow at temperatures exceeding 40 °C. DNA G+C content varies from 57 to 60 mol%. The major phospholipid
Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences (1242 nt) showing phylogenetic relationships between strain S8\textsuperscript{T} and related methanotrophic strains. A methanotroph of the class Alphaproteobacteria, Methylosinus trichosporium OB3\textsuperscript{b}T, was used as an outgroup. Accession numbers are given in parentheses. Bootstrap values >50 % are shown at nodes. Dots indicate branches that were also found in the maximum-likelihood tree. Bar, 0.02 substitutions per nucleotide position.

Fig. 3. Neighbour-joining trees based on deduced PmoA (a) and MmoX (b) amino acid sequences showing relationships between strain S8\textsuperscript{T} and related methanotrophs. The partial PmoA amino acid sequence of Methylosinus trichosporium BF1 and the BmoX amino acid sequence of an unknown strain of Thauera butanivorans were used as outgroups in (a) and (b), respectively. Bootstrap values >50 % are shown at nodes. Dots indicate branches that were also found in the maximum-likelihood trees. Bars, 0.05 amino acid substitutions per position.
Table 1. Differentiating characteristics of strain S8<sup>T</sup> and other members of the genus *Methylocaldum*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>Temperature range (°C)</td>
<td>30–47</td>
<td>37–62</td>
<td>20–47</td>
<td>30–61</td>
<td>30–59</td>
<td>20–47</td>
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<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>57.2</td>
<td>56.5</td>
<td>59</td>
<td>58.5</td>
<td>58.5</td>
<td>59.7</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rod/pleomorphic</td>
<td>Rod/pleomorphic</td>
<td>Thin rod/coccus</td>
<td>Rod/coccus</td>
<td>Rod/coccus</td>
<td>Rod/coccus</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>1.0–1.2 × 1.0–1.8</td>
<td>0.6–1.2 × 1.2–1.5</td>
<td>0.4–0.5 × 1.0–1.5</td>
<td>0.6–0.8 × 1.8–2.0</td>
<td>0.4–0.6 × 1.4–1.6</td>
<td>0.8–1.7 × 1.0–2.8</td>
</tr>
<tr>
<td>NaCl requirement</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.5</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>Colony formation on agar medium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth on methanol</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>sMMO</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Colour of colonies</td>
<td>Light brown</td>
<td>Brown to dark brown</td>
<td>Brown to dark brown</td>
<td>Cream</td>
<td>Light cream</td>
<td>Brown to dark brown</td>
</tr>
<tr>
<td>Heat resistance</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>Desiccation resistance</td>
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<td>Chain formation</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Major quinone</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Methylene Q-8</td>
<td>Methylene Q-8</td>
<td>Methylene Q-8</td>
</tr>
<tr>
<td>Major fatty acids (% of total)</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>ND</td>
<td>43.4</td>
<td>63.5</td>
<td>65.0</td>
<td>59.2</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;16:1&lt;/sub&gt;</td>
<td>ND</td>
<td>45.9</td>
<td>13.3</td>
<td>12.0</td>
<td>39.7</td>
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</table>
fatty acid is \( \text{C}_{16:0} \). The major quinone type is 18-methylene ubiquinone 8. Do not grow on compounds containing carbon–carbon bonds. They possess key enzymes for both the RuMP and serine pathways of formaldehyde assimilation. They possess particulate methane monooxygenase and produce cysts. Known natural habitats are soil, hot springs and marine sediments. The type species is *Methylocaldum* **szegediense**.

**Description of Methylocaldum marinum** sp. nov.

*Methylocaldum marinum* (ma.r'i'num. L. neut. adj. mar-inum of the sea, marine).

The characteristics are the same as given in the genus description above, with the following additional traits. Cells are non-motile cocci or short rods, 0.8–1.7 \( \times \) 1.0–2.8 \( \mu \)m. Colonies on gellan-gum plates are brown to dark brown, dry and rough-surfaced with entire or irregular margins. Growth occurs at \( \text{pH} \) 6–8, with an optimum at \( \text{pH} \) 7. Temperature range for growth is 20–47 °C, with optimum growth at 36 °C. NaCl is essential for growth. Growth occurs in the presence of 0.5–5% NaCl, with optimum growth at 2%. Growth occurs on methane and methanol. Both nitrate and ammonium are used as a nitrogen source. Growth on nitrogen-free liquid medium under aerobic conditions is negative. It possesses soluble methane monooxygenase in addition to particulate methane monooxygenase.

The type strain is S8T (=NBRC 109685\(^T\)=DSM 27392\(^T\)), which was isolated from marine sediments of Kagoshima Bay, Japan.

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**References**


