Methyloceanibacter caenitepidi gen. nov., sp. nov., a facultatively methylotrophic bacterium isolated from marine sediments near a hydrothermal vent

Mio Takeuchi,1 Taiki Katayama,1 Takao Yamagiishi,1 Satoshi Hanada,2 Hideyuki Tamaki,2 Yoichi Kamagata,2 Kenshiro Oshima,3 Masahira Hattori,3 Katsumi Marumo,4† Munetomo Nedachi,5 Hiroto Maeda,6 Yuichi Suwa7 and Susumu Sakata1

1Institute for Geo-resources and Environments, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8567, Japan
2Bioproduction Research Institute, AIST, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
3The Department of Computational Biology, Graduate school of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwa-no-ha, Kashiwa, Chiba 277-8561, Japan
4Institute of Geology and Geoinformation, AIST, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8567, Japan
5Department of Physics and Geoinformation, AIST, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8567, Japan
6The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-8580, Japan
7Department of Biological Science, Faculty of Science and Engineering, Chuo University, 1-13-27 Kasuga, Bunkyo-ku, Tokyo 112-8551, Japan

A moderately thermophilic, methanol-oxidizing bacterium (strain Gela47) was isolated from methane-utilizing mixed-culture originating from marine sediment near a hydrothermal vent. Phylogenetic analysis of 16S rRNA gene sequences indicated that strain Gela47 was closely related to members of the genus ‘Methyloligella’ (94.7 % similarity) within the class Alphaproteobacteria. Strain Gela47 was a Gram-staining-negative and aerobic organism. Cells were rod-shaped and non-motile. The temperature range for growth of strain Gela47 was 19–43 °C (optimal growth at 35 °C). Strain Gela47 tolerated up to 9 % NaCl with an optimum at 1 %. The organism was a facultative methylotroph that could utilize methanol, methylamine, trimethylamine and a variety of multi-carbon compounds. The major cellular fatty acid and major respiratory quinone were C18:1ω7c and ubiquinone-10, respectively. The predominant phospholipids were phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine. The DNA G+C content was 63.9 mol%. On the basis of the morphological, physiological, biochemical and genetic information, a novel genus and species, Methyloceanibacter caenitepidi is proposed, with Gela47 (=NBRC 109540T=DSM 27242T) as the type strain.

Methylotrophs play an important role in biogeochemical cycling of one-carbon substrates in marine environments. The genus Methylphaga belonging to the class Betaproteobacteria represents the major cultured group of non-methanotrophic methanol-utilizers isolated from marine environments (Janvier et al., 1985; De Zwart et al., 1996; Kim et al., 2007). Other marine methanol-utilizers are ‘Marinosulfonomonas methylotroph’ (Holmes et al., 1997), Methylarcula marina (Doronina et al., 2000), and Hyphomicrobium aestuarii (Garrity et al., 2005) affiliated with the class Alphaproteobacteria. Thus, the number of culturable marine methanol-utilizers is still limited. Furthermore, none of them were isolated from marine sediments. Therefore, methanol-utilizers involved in the cycle of C1 compounds in marine environments remain poorly characterized.
sediments remains to be elucidated. Recently, we established a moderately thermophilic methane-utilizing mixed-culture composed of a methanotroph, a methylotroph and a heterotroph from marine sediments near the hydrothermal area in Kagoshima Bay, Japan, and succeeded in isolating a new member of the marine methanol-utilizing methylotrophs, designated Gela4\textsuperscript{T}, from the mixed-culture. In this study, we describe morphological, physiological, biochemical and genetic characteristics of strain Gela4\textsuperscript{T}, and propose a novel genus and species, \textit{Methyloceanibacter caenitepidi} gen. nov., sp. nov., for the isolate.

In Kagoshima Bay, Japan, hydrothermal vents that emit high-temperature fluid containing methane were identified within the Wakamiko crater (Ishibashi \textit{et al.}, 2008; Yamanaka \textit{et al.}, 2013). Marine surface sediment located to the north of the Wakamiko crater (water depth of 161 m, 31° 41’ 09” N 130° 47’ 124” E) was collected in 2001 and used as an inocula to the enrichments. For the initial enrichment, NMS medium (Bowman, 2006) prepared with aged seawater instead of distilled water was used as a medium. Then, a stable methane-utilizing mixed-culture was established using NaCl-NMS liquid medium, i.e. NMS medium supplemented with 2 % NaCl, with headspace gas of methane and air (20:80, v/v) after repeated dilution to extinction treatments for 3 years. This mixed-culture is moderately thermophilic and grows best at 39 °C. A 16S rRNA gene clone library revealed that the culture contained three bacterial species. Strain Gela4\textsuperscript{T} was isolated using NaCl-NMS agar medium with 1 % methanol. We also succeeded in isolating two other members of the mixed-culture, a heterotrophic strain, MA2, belonging to the class \textit{Alphaproteobacteria} and a methanotrophic strain, S8, belonging to the class \textit{Gammaproteobacteria}. Taxonomic features of these two strains will be described in separate papers (M. Takeuchi and others, unpublished).

Cells were investigated after growth in NaCl-NMS medium with 1 % methanol. Cell morphology and motility were examined by using a phase-contrast microscope (Olympus BX51) and a transmission electron microscope (Hitachi h7000) after negative staining with 1 % aqueous uranyl-acetate. The intracellular inclusions were stained with the dye Nile red (Spiekermann \textit{et al.}, 1999). Heat resistance was assayed by incubating one-month-old culture of strain Gela4\textsuperscript{T} at 85 °C for 15 min. Desiccation resistance was assayed by drying one-month-old culture of strain Gela4\textsuperscript{T} for 3 days on a glass slide. Biochemical tests were carried out using API 20NE, API 50CH and APIZYM strips (bioMérieux). For API 20NE and APIZYM tests, cells were suspended in 2 % NaCl solution. Two different culture media were used for the API 50CH and part of the API 20NE assay: CHB/E medium with 2 % NaCl and NaCl-NMS medium. Strips were incubated in a desiccator at 35 °C for 3 weeks. Growth substrates tested in vials were methane, methanol, methylamine, dimethylamine, trimethylamine, methanesulfonic acid, dimethylsulfide, methylsucinic acid, ethanol, acetate, lactate, succinate, propionate, acetone, tolune, vanillin, aspartate, choline, betaine, oxalic acid, glycolate, pyruvate, formate, sarcosine, arginine, serine, histidine, valine, methionine, alanine, asparagine, glucose, sucrose, maltose, lactose and yeast extract. These substrates were added to the NaCl-NMS medium at concentrations of 0.3 and 0.03 % (except for methane, which was added at 20 % in the head space) and incubated at 35 °C. Growth on marine broth 2216 (Difco) was also tested.

Growth of strain Gela4\textsuperscript{T} in NaCl-NMS medium with 1 % methanol was tested at various temperatures (9, 14, 19, 30, 35, 37, 39, 41, 43, 45 and 55 °C), pH (5, 6, 7, 8, 9 and 10) and NaCl concentrations (0, 1, 1.5, 3, 4, 5.5, 9, 12 and 15 %). Nitrate reduction in the presence or absence of oxygen was examined using $^{15}$NO$_3$ according to the previously described method (Katsuyama \textit{et al.}, 2008; Yoshinaga \textit{et al.}, 2011). In brief, nitrate was added to the vial to a concentration of 15 mM, and N$_2$ and N$_2$O concentrations in the headspace gas were monitored after 8 and 15 days of incubation as described previously (Katsuyama \textit{et al.}, 2008; Yoshinaga \textit{et al.}, 2011).

Respiratory quinones, cellular fatty acid methyl esters and the DNA G+C content were analysed as described previously (Hanada \textit{et al.}, 2002, Kamagata & Mikami, 1991, Zhang \textit{et al.}, 2000). Cellular lipids were extracted following the method of Bligh & Dyer (1959) and analysed by two-dimensional TLC. Chloroform/methanol/7 M ammonium hydroxide (65:25:5, by vol.) and chloroform/methanol/acetic acid/water (170:25:25:2, by vol.) were used for the first and second direction of development, respectively (Nichols & James, 1964).

Chromosomal DNA was extracted using an ArchivePure DNA Tissues kit (5 Prime). The genome sequence was determined using 454 pyrosequencing that generated 512 001 reads by single-end sequencing and 192 793 reads by paired-end sequencing and gave 76.4-fold coverage of the genome. The assembly of the obtained sequence data generated two scaffolds by using Newbler v.2.5. The detailed genome information will be described in a separate publication. The 16S rRNA gene sequence found in the genome of strain Gela4\textsuperscript{T} was aligned with its relatives by \textit{MOthur} v.1.29 (Schloss \textit{et al.}, 2009) referring to the \textit{silva} database (Pruesse \textit{et al.}, 2007). The sequence of the \textit{mxf} gene, encoding the large subunit of methanol dehydrogenase, found in the genome was translated to the amino acid sequence and aligned with its relatives by \textit{CLUSTAL W} software. Neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) trees were reconstructed using \textit{MEGA} 5.1 (Tamura \textit{et al.}, 2011) and the \textit{treefinder} version published in March 2011 (Jobb \textit{et al.}, 2004), respectively. The robustness of tree topology was assessed by bootstrap analysis based on 1000 replications.

Cells of strain Gela4\textsuperscript{T} were Gram-negative, non-motile and colourless rods, 0.7–1.1 x 2.8–10.4 μm in size (Fig. 1a, b). Reproduction mainly occurred by binary fission. Poly-$\beta$-hydroxybutyrate-like inclusions were often observed at both ends of cells (Fig. 1c). One-month-old culture of strain Gela4\textsuperscript{T} was not heat-resistant but was desiccation-resistant. When grown on agar medium with methanol.

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When growth substrates were added at 0.3%, strain Gela4T grew in the presence of 0–9% (w/v) NaCl. No growth was observed at temperatures higher than 45 °C or lower than 14 °C. The optimum growth temperature and pH were 35 °C and pH 6–8, respectively. Strain Gela4T grew in the presence of 0–9% (w/v) NaCl. The optimum NaCl concentration was 1% (w/v). Growth was not observed at NaCl concentrations above 12% (w/v). Doubling time was 15 h at 35 °C and pH 7. Nitrate reduction to nitrite, nitrogen or nitrous oxide was not observed under either anaerobic or aerobic conditions.

Analysis of the cellular fatty acid composition after growth on methanol showed the predominant fatty acid to be C_{18:1}ω7c (60.2%). Other main fatty acids were C_{18:0} (36.6%), C_{19:0} (1.6%) and C_{16:0} (1.6%). Strain Gela4T contained phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids, an unidentified aminolipid and an unidentified glycolipid (Fig. S1, available in IJSEM Online). These two unidentified phospholipids were neither phosphatidyl(dimethylethanol)amine, diphasphatidylglycerol nor phosphatidylinositol. The major respiratory quinone was ubiquinone-10. The genomic DNA of strain Gela4T contained 63.9 mol% G+C.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain Gela4T was affiliated with the order Rhizobiales in the class Alphaproteobacteria (Fig. 2). The most closely related strains were ‘Methyloligella halotolerans’ C2^T (94.7% sequence similarity) and ‘Methyloligella solikamskensis’ SK12^T (94.7%), isolated from saline soils (Doronina et al., 2013). Other closely related bacterial species were Hyphomicrobium hollandicum (93%), Dichotomicrobium thermostabilis (92%), Hyphomicrobium aestuarii (92%), Hyphomicrobium vulgaris (92%), and Methylcystis parvus (91%). A phylogenetic tree based on 16S rRNA gene sequences showed that strain Gela4T obviously formed a distinct cluster with several environmental clones retrieved from marine environments. As shown in Fig. 2, this clade was clearly separated from the above-mentioned genera, suggesting that a new genus should be created for the isolates in this cluster. The mxaF gene of strain Gela4T was also similar to that of the species of the genus ‘Methyloligella’ (88–89% similarity at the amino acid level), but strain Gela4T formed a distinct branch in the phylogenetic tree (Fig. 3). The draft genome sequence of strain Gela4T contained a gene encoding serine hydroxymethyltransferase, but not 3-hexulose-6-phosphate synthase, which suggested the ability of the organism to assimilate C_{1} carbon via the serine pathway in the same way as other alphaproteobacterial methylotrophs (Holmes et al., 1997; Doronina et al., 2000).

The differential characteristics of strain Gela4T and related genera are listed in Table 1. Strain Gela4T could be distinguished from species of the genus ‘Methyloligella’ by its cell size, inability to form rosettes, and negative reactions for oxidase and catalase. Strain Gela4T was a facultative methylotroph that could utilize a variety of multi-carbon compounds in addition to some C_{1} compounds while

![Fig. 1.](image-url)
**Fig. 2.** Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain Gela4\(^\top\) and closely related species and environmental clones. Bootstrap values >50% are shown at nodes. Filled circles indicate branches that were also found in the maximum-likelihood tree. Bar, 0.01 substitutions per nucleotide position.

**Fig. 3.** Neighbour-joining tree based on MxaF amino acid sequences showing the relationship between strain Gela4\(^\top\) and related species. Bootstrap values >50% are shown at nodes. Filled circles indicate branches that were also found in the maximum-likelihood tree. Bar, 0.02 amino acid substitutions per position.
members of the genus 'Methyloligella' are obligate methylo-
trophs unable to grow on any multi-carbon compounds. 
Nitrate was used as a nitrogen source by strain Gela4T but 
not by members of the genus 'Methyloligella'. Growth 
temperature range and NaCl tolerance also differentiated 
strain Gela4T from the genus 'Methyloligella'. Although the 
predominant fatty acid was the same (C 18 : 1
v 7c), the 
proportion of saturated fatty acid (C18 : 0) was clearly higher 
(37%) in strain Gela4T compared with those of the genus 
'Methyloligella' (17%). The strains of the genus 'Methyloligella' 
contained phosphatidyldimethylethanolamine and dipho-
sphatidylglycerol, while strain Gela4T did not. Furthermore, 
strain Gela4T originated from marine sediment while species 
of the genus 'Methyloligella' were isolated from terrestrial 
saline soils. Strain Gela4T could be easily differentiated from 
the genus Hyphomicrobium by hyphae formation, motility, 
and tolerance to high NaCl concentrations. Based on 
morphological, biochemical, physiological and genetic data, 
we propose a novel genus and species for strain Gela4T, w h i c h 
represents the first methanol-utilizing bacteria obtained from 
marine sediment.

Environmental 16S rRNA gene clones sharing high sequence 
similarities (98–99%) with strain Gela4T have been obtained 
from marine sediments worldwide as follows: clone 8bav-
H9_arb retrieved from Santa Barbara Basin, USA (EU181478), 
clone CI75 cm.2.18 from Kane‘ohe Bay, Hawaii, USA 
(EF208711), clone CK_1C4_20 from Thalassia sea, Greece 
(EU488046), clone SZB47 from mangrove sediment of Shenzhen City, China (AM176849), and clone HSS103 from Gujarat coast, India (HQ397447). Clone SHFH439 (FJ203406) derived from coral, Montastraea faveolata, also showed high sequence similarity to strain Gela4T. The findings indicate that this organism is ubiquitous in marine surface sediments, and possibly plays an important role in the cycle of C1 compounds.

**Description of Methyloceanibacter gen. nov.**

*Methyloceanibacter* (Me.thyl.o.ce.a.ni.bac’ter. N.L. neut. n. methylum the methyl radical, the methyl group; L. masc. n. oceanus the sea; N.L. masc. n. bacter rod; N.L. masc. n. *Methyloceanibacter* a marine methylotrophic rod).

Gram-negative, non-motile rods. Aerobic and reproduce by binary fission. Facultative methylotrophs able to use a variety of multi-carbon compounds in addition to some C1 compounds. Assimilate C1 compounds via the serine pathway. Do not grow on marine broth 2216. Utilize nitrate and ammonia as a nitrogen source. The major fatty acids are C18:1ω7c and C18:0. The major respiratory quinone is ubiquinone-10. The predominant phospholipids are phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine. Habitats are marine environments. Phylogenetically, a member of the order *Rhizobiales* in the class *Alphaproteobacteria*. The type species is *Methyloceanibacter caenitepidi*.

### Table 1. Main differentiating characteristics of strain Gela4T and related genera

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Gela4T</th>
<th>‘Methyloligella’</th>
<th>Hyphomicrobium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell length (µm)</td>
<td>2.8–10.4</td>
<td>1.8–2.0</td>
<td>0.5–5.0</td>
</tr>
<tr>
<td>Hyphae formation</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Rosette formation</td>
<td>–</td>
<td>+</td>
<td>+/–</td>
</tr>
<tr>
<td>Flagellation</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Reproduction by:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Budding</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Division</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Reduction of nitrate</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Growth using:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methylamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetate</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>–</td>
<td>–</td>
<td>+/–</td>
</tr>
<tr>
<td>Utilization of NO3⁻ as a N source</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth temp. range (optimal)</td>
<td>19–43 (35)</td>
<td>10–40 (29)</td>
<td>5–45 (28–35)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>64</td>
<td>60–61</td>
<td>59–65</td>
</tr>
<tr>
<td>Major polar lipids</td>
<td>PC, PG, PE</td>
<td>PC, PG, PE, PDE, DPG</td>
<td>PC, PG, PE, PDE</td>
</tr>
<tr>
<td>Major quinone</td>
<td>UQ-10</td>
<td>UQ-10</td>
<td>UQ-9</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>9</td>
<td>16</td>
<td>5.5</td>
</tr>
<tr>
<td>Reference</td>
<td>This paper</td>
<td>Doronina <em>et al.</em> (2013)</td>
<td>Goldfine &amp; Hagen (1968); Garrity <em>et al.</em> (2005)</td>
</tr>
</tbody>
</table>
Description of Methyloceaniobacter caenitepidi sp. nov.

Methyloceaniobacter caenitepidi (cae.mi.te’pi.di. L. neut. n. caenum mud; L. adj. tepidus lukewarm; N.L. gen. neut. n. caenitepidi of lukewarm mud).

Characteristics are the same as described for the genus with the following additions. Cells are 0.7–1.1 × 2.8–10.4 µm in size. Colonies on agar medium with methanol are 0.5–0.8 mm in diameter, white, round and convex with entire edges. Does not reduce nitrate to nitrite. Able to grow at 19–43 °C and with 0–9% NaCl. Optimal conditions for growth are 35 °C, pH 6–8 and 1.0% NaCl. Utilizable C₆ compounds are methanol, methylamine and trimethylamine. Vitamins are not required for growth.

The type strain is Gel4T (=NBRC 109540T=DSM 27242T), which was isolated from marine sediments of Kagoshima Bay, Japan. The G+C content of the type strain is 63.9 mol%.

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