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1 ***Methyloradius palustris* gen. nov., sp. nov., a methanol-oxidizing**  
2 **bacterium isolated from snow**

3

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19

20 **Abstract**

21 A novel methylotrophic bacterium, strain Zm11<sup>T</sup>, was isolated from reddish brown  
22 snow collected in a moor in Japan. Cells of the isolate were Gram-stain-negative, motile  
23 and rod-shaped (0.6-0.7 × 1.2-2.7 μm). Growth was observed at 5–32°C with an  
24 optimum growth temperature of 25–28°C. The pH range for growth was 5.4–7.8 with an  
25 optimum pH of 6.8. The strain utilized only methanol as carbon and energy sources for  
26 aerobic growth. The major cellular fatty acids (>40% of total) were summed feature 3  
27 (C<sub>16:1ω7c</sub> and/or C<sub>16:1ω6c</sub>) and C<sub>16:0</sub>. The predominant quinone was Q-8, and major  
28 polar lipids were phosphatidylethanolamine and phosphatidylglycerol. The complete  
29 genome of strain Zm11<sup>T</sup> is composed of a circular chromosome (2,800,413 bp), with  
30 G + C content of 46.4 mol%. Phylogenetic analyses were conducted based on the 16S  
31 rRNA gene sequence and conserved proteins encoded in the genome. The results of  
32 analyses indicate that strain Zm11<sup>T</sup> is a member of the family *Methylophilaceae* but  
33 does not belong to any existing genus. On the basis of its genomic and phenotypic  
34 properties, strain Zm11<sup>T</sup> (= DSM111909<sup>T</sup> = NBRC114766<sup>T</sup>) is proposed as the type  
35 strain of a new species in a new genus, *Methyloradius palustris* gen. nov., sp. nov.

36

37 **Declarations**

38 Funding

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41

42 Conflicts of interest

43 The authors declare that there are no conflicts of interest.

44

45 Availability of data and material

46 The bacterial strain described in this study, strain Zm11<sup>T</sup>, has been deposited to

47 DSMZ and NBRC, as DSM111909<sup>T</sup> and NBRC114766<sup>T</sup>, respectively. Its complete

48 genome sequence is available in the GenBank/EMBL/DDBJ, with accession number of

49 AP024110. The accession numbers for the 16S rRNA gene of strain Zm11<sup>T</sup> is LC647199.

50

51 Code availability

52 Not applicable

53

## 54 **Introduction**

55 Methylotrophic bacteria can grow on organic single-carbon (C1) compounds (e.g.,  
56 methane, methanol and methylated compounds without C-C bond) as sole sources of  
57 energy and carbon. They oxidize methanol to formaldehyde by methanol dehydrogenases  
58 (MDHs), which can be classified into two types: MxaFI-type and XoxF-type. The MxaFI-  
59 type MDH is Ca<sup>2+</sup>-dependent heterotetrameric ( $\alpha_2\beta_2$ ) enzyme, consisting of two large  
60 subunits (MxaF) and two small subunits (MxaI) (Ghosh et al., 1995; Xia et al., 1992). On  
61 the other hand, XoxF-type MDH is dimer of XoxF, distantly related to MxaF (sharing  
62 approximately 50% amino acid sequence identity) (Chistoserdova, 2011). The XoxF-type  
63 MDH was identified as the first rare earth-dependent enzyme (Pol et al., 2014), and  
64 various studies have been carried out to characterize this notable enzyme (Chu &  
65 Lidstrom, 2016; Taubert et al., 2015; Wu et al., 2015).

66 Some bacterial families are known to consist of genera of methylotrophs. One of  
67 such families, the family *Methylophilaceae* currently consists of six genera,  
68 *Methylophilus*, *Methylobacillus*, *Methylovorus*, *Methylotenera*, *Novimethylophilus* and  
69 *Pseudomethylobacillus* (Doronina et al., 2014; Lv et al., 2018; Sheu et al., 2019). All  
70 known members of this family are non-methane-oxidizing methylotrophs that utilize

71 methanol or methylamine aerobically. In this study, a novel methylotrophic bacterium  
72 belonging to the family *Methylophilaceae* was isolated and characterized.

73

## 74 **Materials and methods**

75

### 76 **Enrichment and isolation**

77 The novel isolate, strain Zm11<sup>T</sup> was obtained from the reddish brown snow collected  
78 in Ozegahara moor (N 36° 55' 02'' N 139° 11'26'' W) in Japan. To establish the first  
79 enrichment, 0.2 mL of melted snow was inoculated into 10 ml of DSMZ medium 921,  
80 prepared in a serum bottle (approximately 70 ml volume) sealed with a butyl rubber  
81 stopper. Into the head space filled with air, 20 ml of methane was added by using a sterile  
82 syringe to make pressurized conditions. The grown culture was transferred to the same  
83 medium twice, resulting in enrichment of organisms related to methane-oxidizing bacteria  
84 and other methylotrophs. To isolate the enriched methylotroph, carbon source was  
85 changed to 1% (v/v) methanol from methane. Finally, a pure culture of strain Zm11<sup>T</sup> was  
86 obtained by repeated serial dilution in the same medium supplemented 0.1% yeast extract  
87 (w/v). In these procedures of enrichment and isolation, the cultures were incubated in the  
88 dark at 22°C. The culture purity was checked by microscopy and repeated sequencing of  
89 the 16S rRNA gene fragments amplified with universal PCR primer pairs (Lane, 1991).

90

91 Phenotypical characterizations

92 Cell morphology was examined by phase-contrast microscopy (Axioplan 2; Zeiss).

93 The Gram staining test was carried out using a kit (Sigma-Aldrich). Catalase activity was

94 assessed by pouring 10% H<sub>2</sub>O<sub>2</sub> solution onto a pellet of cells. Oxidase activity was carried

95 out using an oxidase test reagent (bioMérieux).

96 For chemotaxonomic analyses, strain Zm11<sup>T</sup> was grown with DSMZ medium

97 921 supplemented 1% (v/v) methanol at 28°C. The analyses of cellular fatty acids, polar

98 lipids and respiratory quinones were carried out by Techno Suruga Laboratory (Shizuoka,

99 Japan). The cellular fatty profile was obtained with the Sherlock Microbial Identification

100 System (MIDI) version 6.0 (database; TSBA6). Polar lipids and respiratory quinones

101 were analyzed with TLC and HPLC, respectively (Bligh & Dyer 1959; Minnikin et al.

102 1979).

103 Effects of temperature on growth were examined with the medium 921

104 supplemented with methanol, by culturing at 0, 5, 8, 10, 13, 15, 18, 22, 25, 28, 30, 32, 35,

105 37°C. The other tests for phenotypic characterization were all conducted at 22°C.

106 Utilization of growth substrates was tested with K medium, consisting of the following

107 constituents (per litre); 2.0 g KH<sub>2</sub>PO<sub>4</sub>, 2.0 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g NaCl, 0.1 g MgSO<sub>4</sub> · 7H<sub>2</sub>O,

108 0.002 g FeSO<sub>4</sub> · 7H<sub>2</sub>O (pH was adjusted to 7.4). The medium was supplemented with one  
109 of the following substrates (% w/v); glucose (0.1 and 0.5), fructose (0.1), sucrose (0.1),  
110 lactate (0.1), malate (0.1), succinate (0.1), acetate (0.1), propionate (0.1), pyruvate (0.1),  
111 glycerol (0.1), ethanol (0.1, 0.5 and 1.0), methylamine (0.1 and 0.3), dimethylamine (0.1)  
112 and yeast extract (0.1). Methane-dependent growth was tested in a closed bottle whose  
113 headspace was filled with mixture of air and methane (30% v/v). Effect of NaCl  
114 concentration on growth was tested by altering concentration of NaCl in K medium, to 0,  
115 0.05, 0.5, 1.0, 2.0 and 3.0% (w/v). Effect of pH on growth was tested with modified  
116 version of the K medium buffered with 20 mM MES, adjusted to varying initial pH  
117 ranging from 4.6 to 8.6 (0.2-unit intervals). Nitrate reduction during aerobic growth was  
118 tested by culturing in the medium 921 supplemented with KNO<sub>3</sub> and methanol, followed  
119 by chrometric detection of nitrite.

120

## 121 Genomic characterization

122 Genomic DNA of strain Zm11<sup>T</sup> was extracted using Wizard Genomic DNA  
123 purification kit (Promega). Whole genome sequencing was performed using the Illumina  
124 NextSeq and Nanopore GridION platforms. A hybrid assembly was performed using  
125 Unicycler (Ver 0.4.7). Completeness of the assembled genome was estimated by CheckM

126 ver 1.1.2 (Parks et al., 2015). The genome sequence was annotated with DFAST  
127 (Tanizawa et al., 2018). In the annotated genome, genes encoding MDH-related proteins  
128 were identified by BLASTP searches. The amino acid sequences encoded by the  
129 identified genes were aligned with reference sequences of MDHs, by using the MAFFT  
130 alignment tool (<https://www.genome.jp/tools-bin/mafft>) followed by manual inspection.  
131 The alignment was imported to the program MEGA X (Kumar et al., 2018), to construct  
132 a maximum likelihood tree by using the Le\_Gascuel\_2008 model with gamma  
133 distribution (Le & Gascuel, 2008).

134 As genome relatedness indices between Zm11<sup>T</sup> and related strains, values of  
135 average nucleotide identity (ANI) and average amino acid identity (AAI) were calculated  
136 by using tools provided by Kostas lab (<http://enve-omics.ce.gatech.edu/>), with the ANIb  
137 algorithm (Goris et al., 2007). In addition, digital DNA–DNA hybridization (dDDH)  
138 values were calculated using Genome-to-Genome Distance Calculator (GGDC) provided  
139 by DSMZ (Meier-Kolthoff et al., 2013), applying the formula 2.

140

#### 141 Phylogenetic analyses

142 The 16S rRNA gene sequence of strain Zm11<sup>T</sup> identified in the genome was aligned  
143 with those of type strains in the family *Methylophilaceae*, by using CLUSTALW version

144 2.1(Larkin et al., 2003). Based on the resulting alignment, evolutionary distances were  
145 computed by using Kimura's two-parameter model with gamma distribution and invariant  
146 sites, using the MEGA X. Phylogenetic trees were constructed with the maximum-  
147 likelihood, neighbor-joining and minimum evolution methods, available in the MEGA X.

148 A genome-based taxonomic classification of strain Zm11<sup>T</sup> was conducted with the  
149 Genome Taxonomy Database (GTDB), based on 120 conserved proteins (Parks et al.,  
150 2018). Taxonomic position of the strain Zm11<sup>T</sup> in the GTDB (release 95) was identified  
151 using GTDB-Tk (Chaumeil et al., 2020).

152

## 153 **Results and Discussion**

154

### 155 **Physiological and chemotaxonomic characteristics**

156 The fundamental characteristics of strain Zm11<sup>T</sup> are summarized in Table 1  
157 and presented in the species description. Cells of strain Zm11<sup>T</sup> were motile, rod-shaped,  
158 0.6–0.7 μm in width, 1.2–2.7 μm in length (Fig. S1). The cells were oxidase-positive  
159 and weakly positive for catalase. In the cellular fatty acid profile of the strain, summed  
160 feature 3 (C<sub>16:1</sub>ω7*c* and/or C<sub>16:1</sub>ω6*c*) and C<sub>16:0</sub> were predominant, accounting for 47%  
161 and 40% of total, respectively. As other components, C<sub>10:0</sub> 3-OH (4.3%), C<sub>14:0</sub> (1.4%)  
162 and C<sub>20:0</sub> (1.2%) were detected. The other minor fatty acids detected (<0.4% of total)

163 were C<sub>16:1</sub>ω5c and summed feature 8 (C<sub>18:1</sub>ω7c and/or C<sub>18:1</sub>ω6c). The strain had  
164 phosphatidylethanolamine and phosphatidylglycerol as major polar lipids (Fig. S2).  
165 Diphosphatidylglycerol and lyso-phosphatidylethanolamine were also detected along  
166 with some unidentified polar lipids. In the analysis of respiratory quinone, only  
167 ubiquinone 8 (Q-8) was detected.

168           The strain Zm11<sup>T</sup> grew at 5–30°C, with optimum growth at 25–30°C. Its pH  
169 range for growth was 5.4–7.8, and optimum pH was 6.8. The strain grew in the presence  
170 of 0.5% or lower concentrations of NaCl, with optimal growth with 0.05% NaCl. No  
171 growth was observed in the presence of 1% or higher concentrations of NaCl.

172           As sole carbon and energy sources, methanol supported growth of strain  
173 Zm11<sup>T</sup>. The other substrates tested, including sugars, organic acids, amines, glycerol,  
174 ethanol and methane, did not support of the strain. During the aerobic growth on  
175 methanol, nitrate reduction to nitrite was observed, as reported in related organisms  
176 (Table 1).

177

178 Genomic features

179           To reconstruct the complete genome of strain Zm11<sup>T</sup>, 6,474,292 DNBSEQ  
180 reads and 44,750 GridION reads were subjected to hybrid assembly. As a result, a single

181 circular chromosome was generated with coverage of 635-fold. The size of chromosome  
182 was 2,800,413 bp, and its G+C contents was 46.4% (Table 2). The completeness of the  
183 genome was estimated to be 100%.

184 In the genome of strain Zm11<sup>T</sup>, 2576 protein-coding sequences were predicted. The  
185 genome harbors two copies of the 16S rRNA gene with the identical sequence. The 16S  
186 rRNA gene sequence indicated the highest sequence identities to species of  
187 *Methylobacillus* and other genera in the family *Methylophilaceae*, but the identities  
188 were lower than 96%. Phylogenetic position of strain Zm11<sup>T</sup> in this family is shown in  
189 Fig. 1. The values of ANI and AAI between the strain Zm11<sup>T</sup> and genome-sequenced  
190 strains in the family *Methylophilaceae* are shown in Table 2. The ANI values (76–77%)  
191 are clearly lower than 95–96%, the proposed threshold for species delineation (Richter  
192 & Rosselló-Móra, 2009). The low values of dDDH (19–22%) also indicated that strain  
193 Zm11<sup>T</sup> are distinct from the strains listed in Table 2.

194 Among putative proteins encoded in the genome of strain Zm11<sup>T</sup>, those related  
195 to MDHs were subjected to phylogenetic analysis. In the genome, four genes encoding  
196 putative MDHs were identified. As shown in Fig. S3, one of them encodes MxaF-type  
197 MDH and the others encode XoxF-type MDH with distinct amino acid sequences,  
198 respectively.

199

200 Taxonomic assignment

201 Phylogenetic relationships between strain Zm11<sup>T</sup> and type strains of related species  
202 with validly published names are shown in Fig. 1. In the maximum likelihood tree of  
203 16S rRNA gene, strain Zm11<sup>T</sup> represents sister group of a clade consisting of the genera  
204 *Methylotenera* and *Pseudomethylobacillus*. This result was also observed in trees  
205 generated with different approaches, neighbor-joining and minimum evolution methods  
206 (data not shown). The phylogenetic trees consistently indicated that strain Zm11<sup>T</sup> is a  
207 member of the family *Methylophilaceae* but does not belong to any existing genera.

208 To draw conclusion about taxonomy based on whole genomic data, the genome of  
209 strain Zm11<sup>T</sup> was analyzed with the GTDB. As a result, this organism was classified  
210 into a genus distinct from all existing genera with validly published names. The  
211 genome-based phylogenetic tree is shown in Fig. S4. All these results mean that a novel  
212 genus should be created to accommodate strain Zm11<sup>T</sup>.

213 On the basis of these results, strain Zm11<sup>T</sup> is proposed as the type strain of a novel  
214 species of a new genus, with the name *Methyloradius palustris* gen. nov., sp. nov.

215

216 **Description of *Methyloradius* gen. nov.**

217 *Methyloradius* (Me.thy.lo.ra'di.us. N.L. pref. *methylo*-, pertaining to the methyl radical;

218 L. masc. n. *radius*, a staff, rod; N.L. masc. n. *Methyloradius*, methyl-oxidizing rod).

219 Grows by oxidation of methanol as carbon and energy source. Aerobic and neutrophilic.

220 Gram-stain-negative. Major cellular fatty acids are summed feature 3 (C<sub>16:1</sub>ω7c and/or

221 C<sub>16:1</sub>ω6c) and C<sub>16:0</sub>. Q-8 is the sole respiratory quinone. Phosphatidylethanolamine and

222 phosphatidylglycerol are major polar lipids. Belongs to the family *Methylophilaceae*. The

223 type species is *Methyloradius palustris*.

224 **Description of *Methyloradius palustris* sp. nov.**

225 *Methyloradius palustris* (pa.lus'tris. L.masc. adj. *palustris* marshy, swampy)

226 In addition to properties listed in the genus description, cells are motile, rod-shaped, 0.6-

227 0.7 μm long and 1.2-2.7 μm wide. Catalase-positive and oxidase-positive. Uses oxygen

228 as electron acceptor. Temperature range for growth is 5–32°C, with an optimum of 25–

229 28°C. Growth occurs at pH 5.4–7.8, with an optimum of pH 6.8. G + C content of genomic

230 DNA of the type strain is 46.4 mol%. Does not grow on glucose, fructose, sucrose, lactate,

231 malate, succinate, acetate, propionate, pyruvate, glycerol, ethanol, methylamine,

232 dimethylamine, yeast extract or methane.

233 The type strain Zm11<sup>T</sup> (= DSM 111909<sup>T</sup> = NBRC114766<sup>T</sup>) was isolated from snow  
234 collected in Japan. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA  
235 gene and complete genome of strain Zm11<sup>T</sup> are LC647199 and AP024110, respectively.

236

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241

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382

Table 1. Characteristics of strain Zm11<sup>T</sup> and those of genera in the family *Methylophilaceae*. ND means not determined. SF3 stands for summed feature 3 (C<sub>16:1</sub>ω7c and/or C<sub>16:1</sub>ω6c). Data were taken from the following references: *Methylobacillus* (Agafonova et al., 2017; Doronina et al., 2004; Gogleva et al., 2011; Kaparullina et al., 2017; Madhaiyan et al., 2013; Urakami & Komagata, 1986; Yordy & Weaver 1977), *Methylophilus* (Doronina et al., 2012; Doronina et al., 2005; Gogleva et al., 2010; Jenkins et al., 1987; Kaparullina et al., 2018; Madhaiyan et al., 2009), *Methylotenera* (Kalyuzhnaya et al., 2006; Kalyuzhnaya et al., 2012; Lv et al., 2020), *Methylovorus* (Doronina et al., 2005; Doronina et al., 2011; Govorukhina & Trotsenko, 1991), *Novimethylophilus* (Lv et al., 2018); *Pseudomethylobacillus* (Sheu et al., 2019).

		Zm11 <sup>T</sup>	<i>Methylobacillus</i>	<i>Methylophilus</i>	<i>Methylotenera</i>	<i>Methylovorus</i>	<i>Novimethylophilus</i>	<i>Pseudomethylobacillus</i>
Temperature for growth (°C)	Range	5–32	10–52	10–37	4–30	10–45	15–40	15–30
	Optimum	25–26	19–42	19–37	18–40	24–40	28	25
pH for growth	Range	5.4–7.8	6–10.5	4–10	4.2–8.5	4.2–8.5	6–9	6–8
	Optimum	6.8	6.5–9.5	6.5–8.5	5.8–7.5	5.8–7.5	7	7
NaCl conc. for growth (%)	Range	0–0.5	0–3	0–0.50	ND	ND	0–2	0–0.5
	Optimum	0.05	0.05–2.00	0.05	ND	ND	0.05	ND
Nitrate reduction		+	variable	variable	-	ND	+	+
Major fatty acid (>10%)		C <sub>16:0</sub> , SF3	C <sub>16:0</sub> , SF3	C <sub>16:0</sub> , SF3, C <sub>16:0</sub> 2-OH, C <sub>10:0</sub> 3-OH, C <sub>17:0</sub> cyclo	C <sub>16:0</sub> , SF3	C <sub>16:0</sub> , SF3, C <sub>17:0</sub> cyclo	C <sub>16:0</sub> , SF3	C <sub>16:0</sub> , SF3
GC contents (mol%)		46.4	50.5–61.5	47.9–54.5	42.6–54.3	54.5–57.2	56.1	58.3

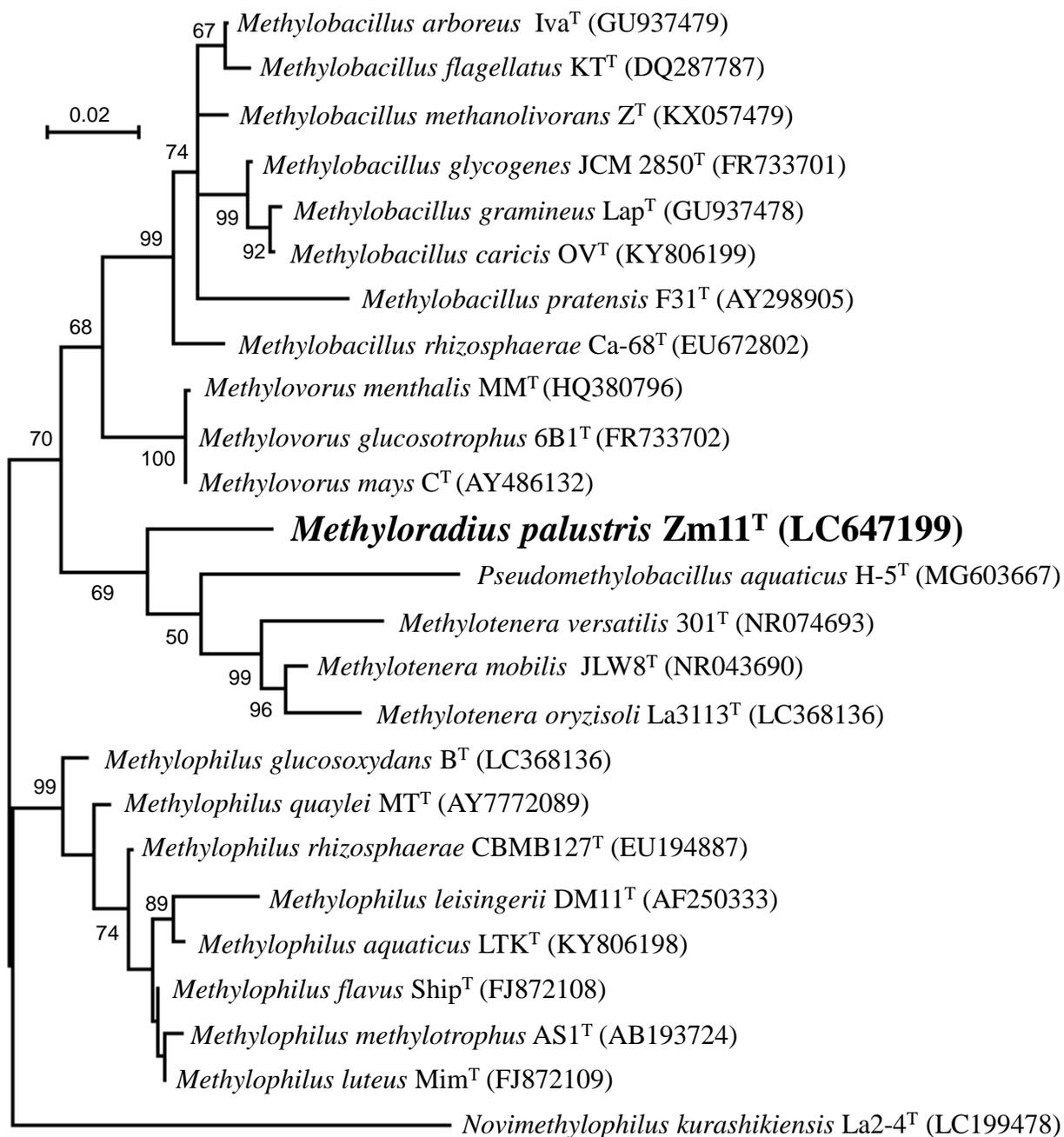
Table 2. Genome relatedness between strain Zm11<sup>T</sup> and genome-sequenced strains in the family *Methylophilaceae*

Strain	Accession number	Genome size (Mbp)	G+C content (%)	ANI (%)	AAI (%)	dDDH (%)
Zm11 <sup>T</sup>	AP024110	2.800	46.4	-	-	-
<i>Methylobacillus flagellatus</i> KT <sup>T</sup>	NC_007947	2.972	55.7	76.88	67.50	19.6
<i>Methylobacillus glycogenes</i> JCM 2850 <sup>T</sup>	NZ_BAMT00000000	3.249	53.4	76.41	64.25	18.7
<i>Methylobacillus rhizosphaerae</i> Ca-68 <sup>T</sup>	NZ_FZOA00000000	2.368	52.4	76.77	67.42	20.3
<i>Methylovorus glucosetrophus</i> SIP3-4	NC_012969	2.996	54.9	77.28	69.16	19.6
<i>Methylotenera mobilis</i> JLW8 <sup>T</sup>	NC_012968	2.548	45.5	76.78	65.50	21.6
<i>Methylotenera versatilis</i> 301 <sup>T</sup>	NC_014207	3.060	42.6	76.25	65.65	20.6
<i>Methylophilus methylotrophus</i> DSM 46235 <sup>T</sup>	NZ_ARJW00000000	2.860	49.6	76.25	61.45	20.3
<i>Methylophilus rhizosphaerae</i> CBMB127 <sup>T</sup>	NZ_FNFX00000000	2.761	51.4	76.47	61.48	19.1
<i>Methylophilus medardicus</i> M-51	NZ_CP040946.1	2.063	49.8	77.37	61.36	21.1
<i>Novimethylophilus kurashikiensis</i> La2-4 <sup>T</sup>	NZ_BDOQ00000000	3.689	56.1	76.25	62.64	19.4
<i>Pseudomethylobacillus aquaticus</i> H-5 <sup>T</sup>	NZ_RJVP00000000	2.550	58.3	77.22	67.57	19.3

## Figure legends

Fig. 1. Maximum likelihood tree showing phylogenetic position of strain Zm11T within the family *Methylophilaceae*, based on the 16S rRNA gene sequences. All positions containing gaps and missing data were eliminated and there were 1295 positions in the final dataset. Bar, substitutions per site. Numbers on nodes represent percentage values of 1000 bootstrap resampling.





***Methyloradius palustris* gen. nov., sp. nov., a methanol-oxidizing bacterium isolated from snow**

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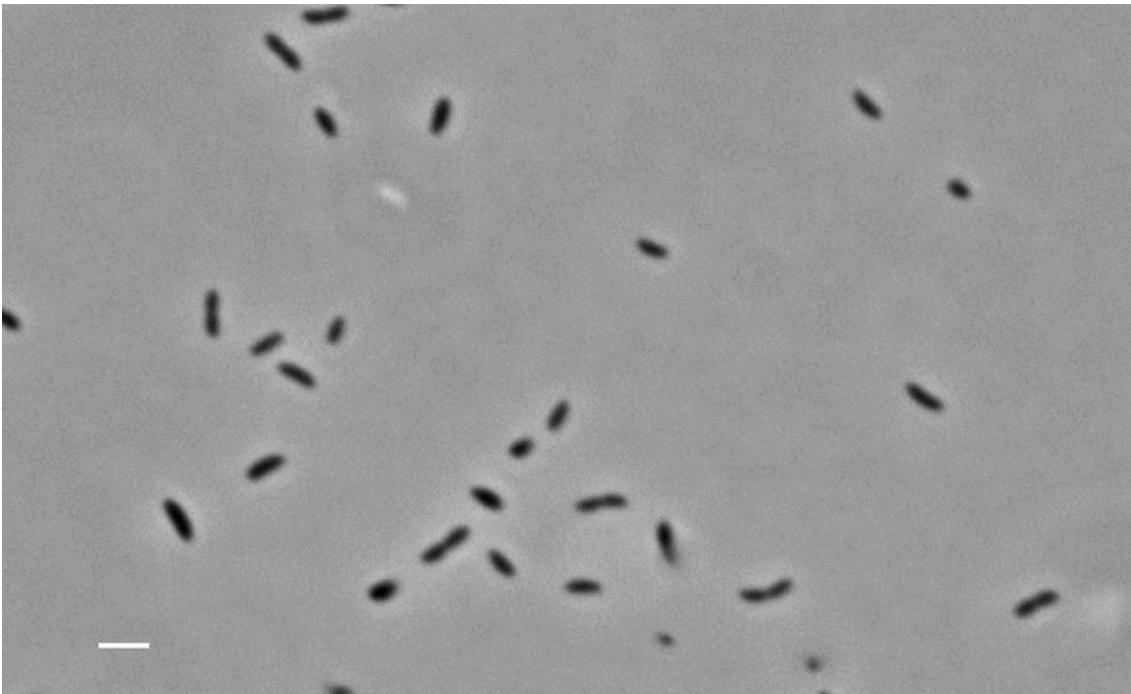


Fig. S1. Phase-contrast micrograph of strain Zm11T. Scale bar represents 2  $\mu\text{m}$ .

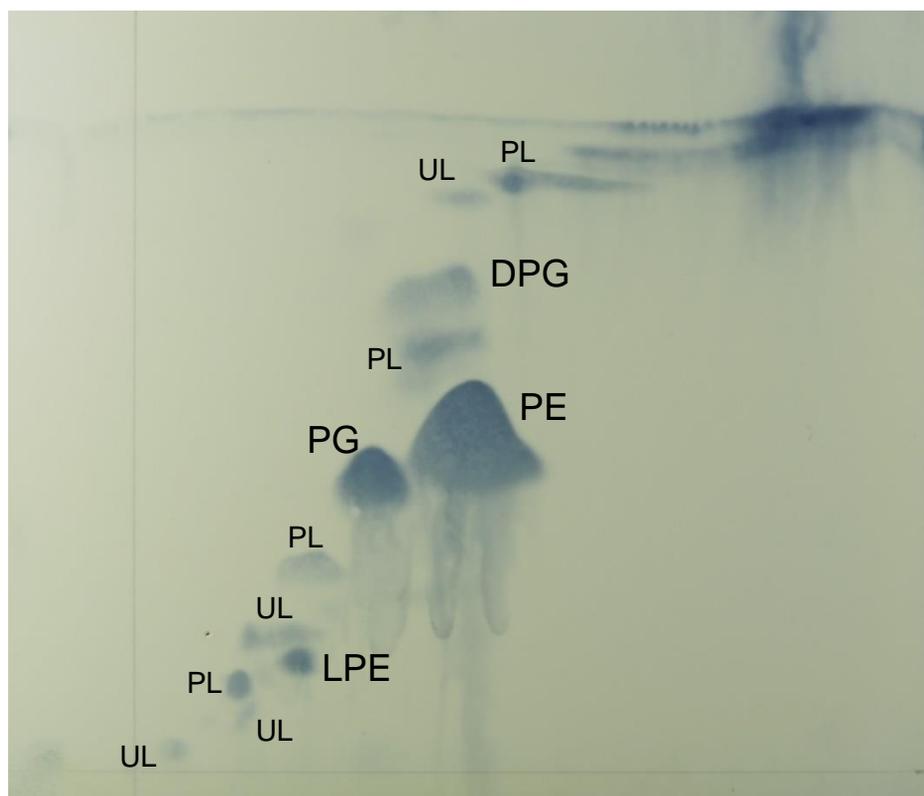


Fig. S2. Polar lipid profile of strain Zm11<sup>T</sup>. PG, phosphatidylglycerol; PE, phosphatidylethanolamine; LPE, lyso-phosphatidylethanolamine; DPG, diphosphatidylglycerol; PL, unidentified phospholipids; UL, unidentified polar lipids.

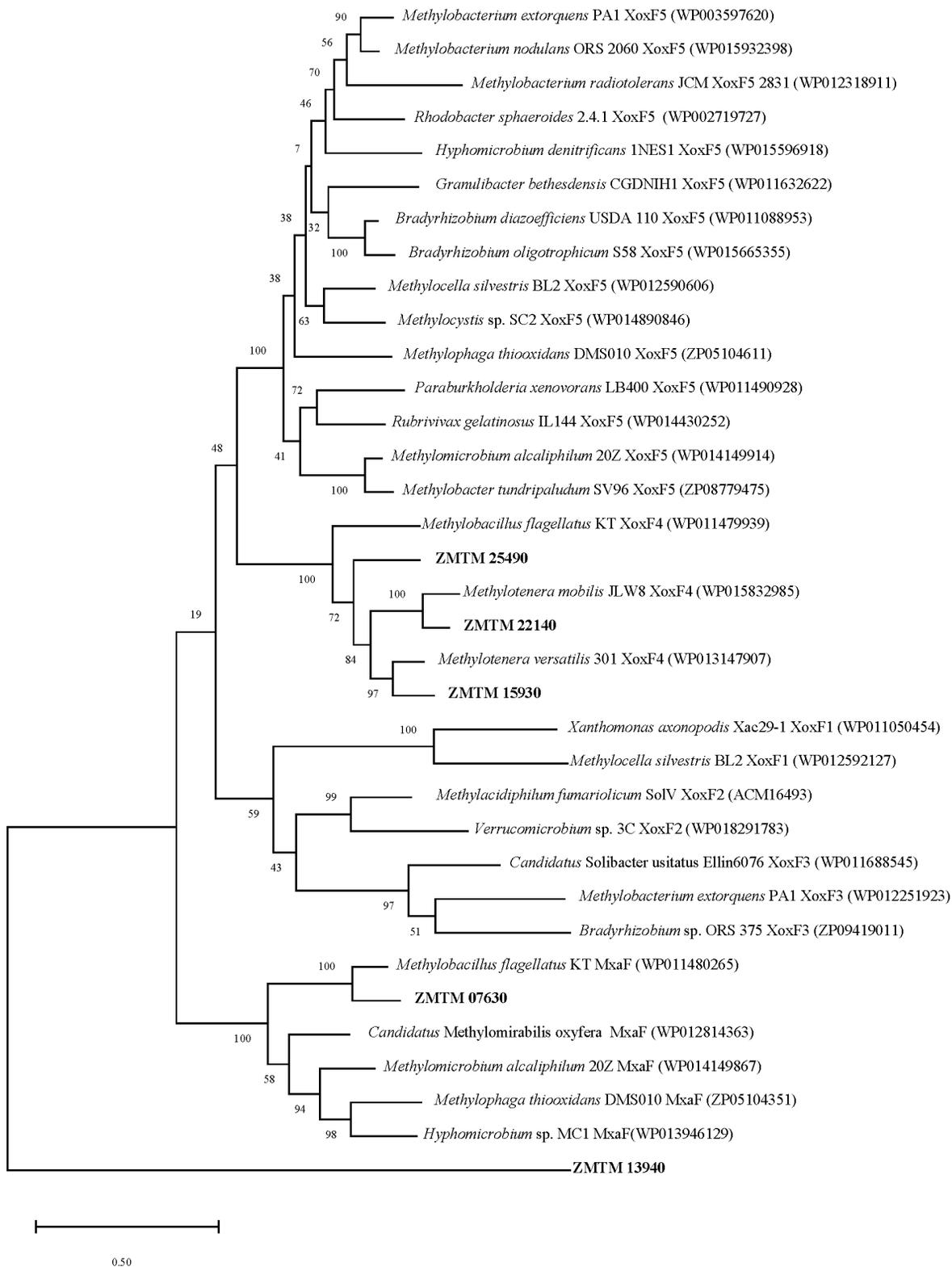
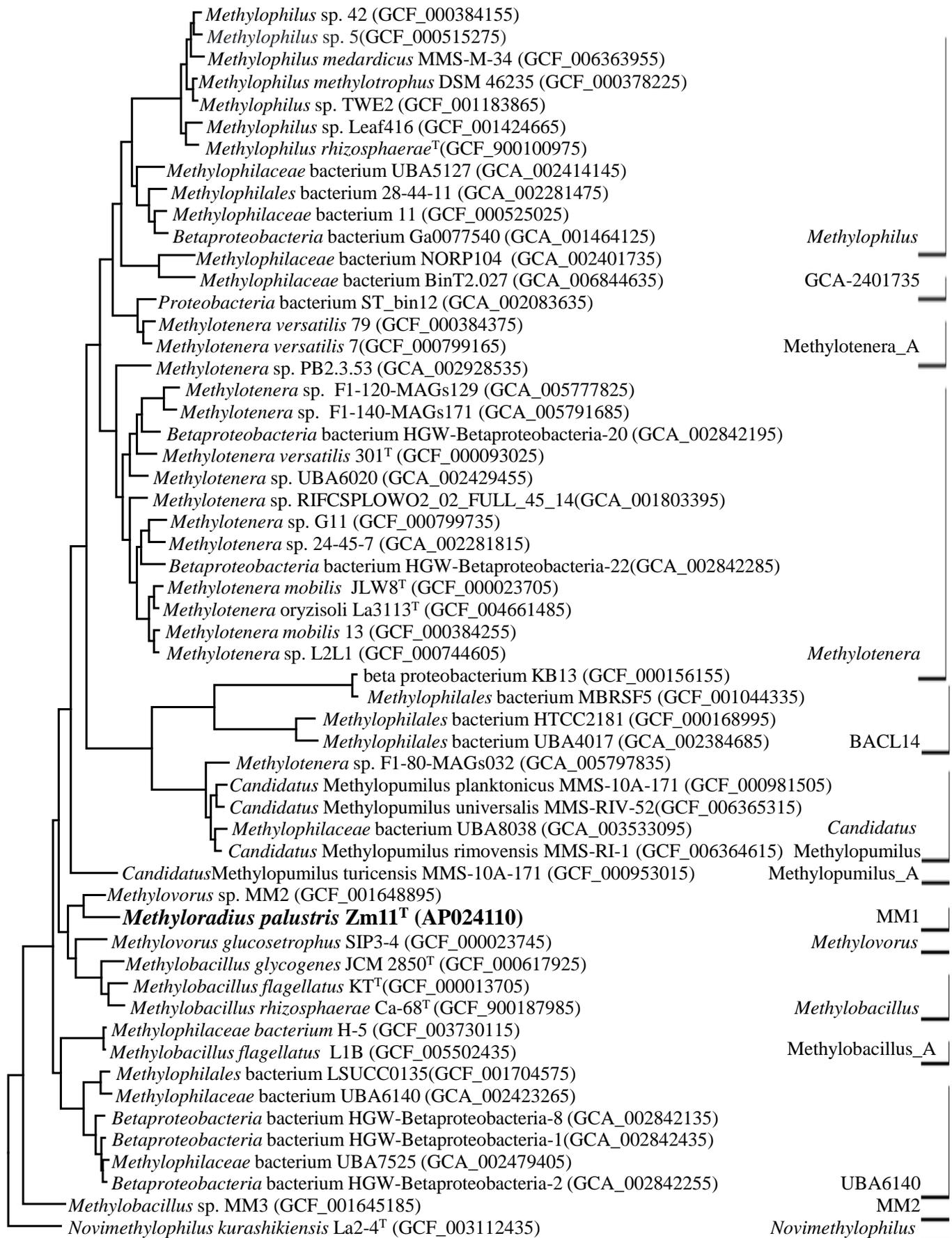


Fig. S3. Phylogenetic tree of MDHs encoded by genes predicted in the genome of *Zm11<sup>T</sup>* (prefixed with ZMTM). All positions containing gaps and missing data were eliminated and there were a total of 838 positions in the final dataset. Bar, substitutions per site. Numbers on nodes represent percentage values of 1000 bootstrap resampling.



0.10

Fig. S4. Position of strain Zm11<sup>T</sup> in genome-based phylogenetic tree, bac120 tree of GTDB. Names of genera are according to the GTDB taxonomy.