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

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**Abstract**

A novel methylotrophic bacterium, strain Zm11$^{T}$, was isolated from reddish brown snow collected in a moor in Japan. Cells of the isolate were Gram-stain-negative, motile and rod-shaped (0.6-0.7×1.2-2.7 μm). Growth was observed at 5–32°C with an optimum growth temperature of 25–28°C. The pH range for growth was 5.4–7.8 with an optimum pH of 6.8. The strain utilized only methanol as carbon and energy sources for aerobic growth. The major cellular fatty acids (>40% of total) were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and $C_{16:0}$. The predominant quinone was Q-8, and major polar lipids were phosphatidylethanolamine and phosphatidylglycerol. The complete genome of strain Zm11$^{T}$ is composed of a circular chromosome (2,800,413 bp), with G + C content of 46.4 mol%. Phylogenetic analyses were conducted based on the 16S rRNA gene sequence and conserved proteins encoded in the genome. The results of analyses indicate that strain Zm11$^{T}$ is a member of the family *Methylophilaceae* but does not belong to any existing genus. On the basis of its genomic and phenotypic properties, strain Zm11$^{T}$ (= DSM111909$^{T}$ = NBRC114766$^{T}$) is proposed as the type strain of a new species in a new genus, *Methyloradius palustris* gen. nov., sp. nov.

## Declarations

### Funding

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### Conflicts of interest

The authors declare that there are no conflicts of interest.

### Availability of data and material

The bacterial strain described in this study, strain Zm11$^{T}$, has been deposited to DSMZ and NBRC, as DSM111909$^{T}$ and NBRC114766$^{T}$, respectively. Its complete genome sequence is available in the GenBank/EMBL/DDBJ, with accession number of AP024110. The accession numbers for the 16S rRNA gene of strain Zm11$^{T}$ is LC647199.

### Code availability

Not applicable

## Introduction

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

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

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

## Materials and methods

### Enrichment and isolation

The novel isolate, strain Zm11$^T$ was obtained from the reddish brown snow collected in Ozegahara moor (N 36° 55' 02'' N 139° 11'26" W) in Japan. To establish the first enrichment, 0.2 mL of melted snow was inoculated into 10 ml of DSMZ medium 921, prepared in a serum bottle (approximately 70 ml volume) sealed with a butyl rubber stopper. Into the head space filled with air, 20 ml of methane was added by using a sterile syringe to make pressurized conditions. The grown culture was transferred to the same medium twice, resulting in enrichment of organisms related to methane-oxidizing bacteria and other methylotrophs. To isolate the enriched methylotroph, carbon source was changed to 1% (v/v) methanol from methane. Finally, a pure culture of strain Zm11$^T$ was obtained by repeated serial dilution in the same medium supplemented 0.1% yeast extract (w/v). In these procedures of enrichment and isolation, the cultures were incubated in the dark at 22°C. The culture purity was checked by microscopy and repeated sequencing of 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% $H_2O_2$ solution onto a pellet of cells. Oxidase activity was carried
95 out using an oxidase test reagent (bioMérieux).

96 For chemotaxonomic analyses, strain Zm11$^T$ 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 $KH_2PO_4$, 2.0 g $(NH_4)_2SO_4$, 0.5 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$,

0.002 g $FeSO_4 \cdot 7H_2O$ (pH was adjusted to 7.4). The medium was supplemented with one of the following substrates (% w/v); glucose (0.1 and 0.5), fructose (0.1), sucrose (0.1), lactate (0.1), malate (0.1), succinate (0.1), acetate (0.1), propionate (0.1), pyruvate (0.1), glycerol (0.1), ethanol (0.1, 0.5 and 1.0), methylamine (0.1 and 0.3), dimethylamine (0.1) and yeast extract (0.1). Methane-dependent growth was tested in a closed bottle whose headspace was filled with mixture of air and methane (30% v/v). Effect of NaCl concentration on growth was tested by altering concentration of NaCl in K medium, to 0, 0.05, 0.5, 1.0, 2.0 and 3.0% (w/v). Effect of pH on growth was tested with modified version of the K medium buffered with 20 mM MES, adjusted to varying initial pH ranging from 4.6 to 8.6 (0.2-unit intervals). Nitrate reduction during aerobic growth was tested by culturing in the medium 921 supplemented with $KNO_3$ and methanol, followed by chrometric detection of nitrite.

Genomic characterization

Genomic DNA of strain $Zm11^T$ was extracted using Wizard Genomic DNA purification kit (Promega). Whole genome sequencing was performed using the Illumina NextSeq and Nanopore GridION platforms. A hybrid assembly was performed using Unicycler (Ver 0.4.7). Completeness of the assembled genome was estimated by CheckM

ver 1.1.2 (Parks et al., 2015). The genome sequence was annotated with DFAST (Tanizawa et al., 2018). In the annotated genome, genes encoding MDH-related proteins were identified by BLASTP searches. The amino acid sequences encoded by the identified genes were aligned with reference sequences of MDHs, by using the MAFFT alignment tool (https://www.genome.jp/tools-bin/mafft) followed by manual inspection. The alignment was imported to the program MEGA X (Kumar et al., 2018), to construct a maximum likelihood tree by using the Le_Gascuel_2008 model with gamma distribution (Le & Gascuel, 2008).

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

Phylogenetic analyses

The 16S rRNA gene sequence of strain Zm11$^T$ identified in the genome was aligned 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$^T$ 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$^T$ 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$^T$ are summarized in Table 1
157 and presented in the species description. Cells of strain Zm11$^T$ 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 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and $C_{16:0}$ were predominant, accounting for 47%
161 and 40% of total, respectively. As other components, $C_{10:0}$ 3-OH (4.3%), $C_{14:0}$ (1.4%)
162 and $C_{20:0}$ (1.2%) were detected. The other minor fatty acids detected (<0.4% of total)

were $C_{16:1}\omega5c$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). The strain had phosphatidylethanolamine and phosphatidylglycerol as major polar lipids (Fig. S2). Diphosphatidylglycerol and lyso-phosphatidylethanolamine were also detected along with some unidentified polar lipids. In the analysis of respiratory quinone, only ubiquinone 8 (Q-8) was detected.

The strain Zm11$^T$ grew at 5–30ºC, with optimum growth at 25–30ºC. Its pH range for growth was 5.4–7.8, and optimum pH was 6.8. The strain grew in the presence of 0.5% or lower concentrations of NaCl, with optimal growth with 0.05% NaCl. No growth was observed in the presence of 1% or higher concentrations of NaCl.

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

Genomic features

To reconstruct the complete genome of strain Zm11$^T$, 6,474,292 DNBSEQ reads and 44,750 GridION reads were subjected to hybrid assembly. As a result, a single

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

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

Among putative proteins encoded in the genome of strain Zm11$^T$, those related to MDHs were subjected to phylogenic analysis. In the genome, four genes encoding putative MDHs were identified. As shown in Fig. S3, one of them encodes MxaF-type MDH and the others encode XoxF-type MDH with distinct amino acid sequences, respectively.

Taxonomic assignment

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

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

On the basis of these results, strain Zm11$^T$ is proposed as the type strain of a novel species of a new genus, with the name *Methyloradius palustris* gen. nov., sp. nov.

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

*Methyloradius* (Me.thy.lo.ra'di.us. N.L. pref. *methylo*-, pertaining to the methyl radical; L. masc. n. *radius*, a staff, rod; N.L. masc. n. *Methyloradius*, methyl-oxidizing rod).

Grows by oxidation of methanol as carbon and energy source. Aerobic and neutrophilic. Gram-stain-negative. Major cellular fatty acids are summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and $C_{16:0}$. Q-8 is the sole respiratory quinone. Phosphatidylethanolamine and phosphatidylglycerol are major polar lipids. Belongs to the family *Methylophilaceae*. The type species is *Methyloradius palustris*.

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

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

In addition to properties listed in the genus description, cells are motile, rod-shaped, 0.6-0.7 μm long and 1.2-2.7 μm wide. Catalase-positive and oxidase-positive. Uses oxygen as electron acceptor. Temperature range for growth is 5–32°C, with an optimum of 25–28°C. Growth occurs at pH 5.4–7.8, with an optimum of pH 6.8. G + C content of genomic DNA of the type strain is 46.4 mol%. Does not grow on glucose, fructose, sucrose, lactate, malate, succinate, acetate, propionate, pyruvate, glycerol, ethanol, methylamine, dimethylamine, yeast extract or methane.

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

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Table 1. Characteristics of strain Zm11$^T$ and those of genera in the family *Methylophilaceae*. ND means not determined. SF3 stands for summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). 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).

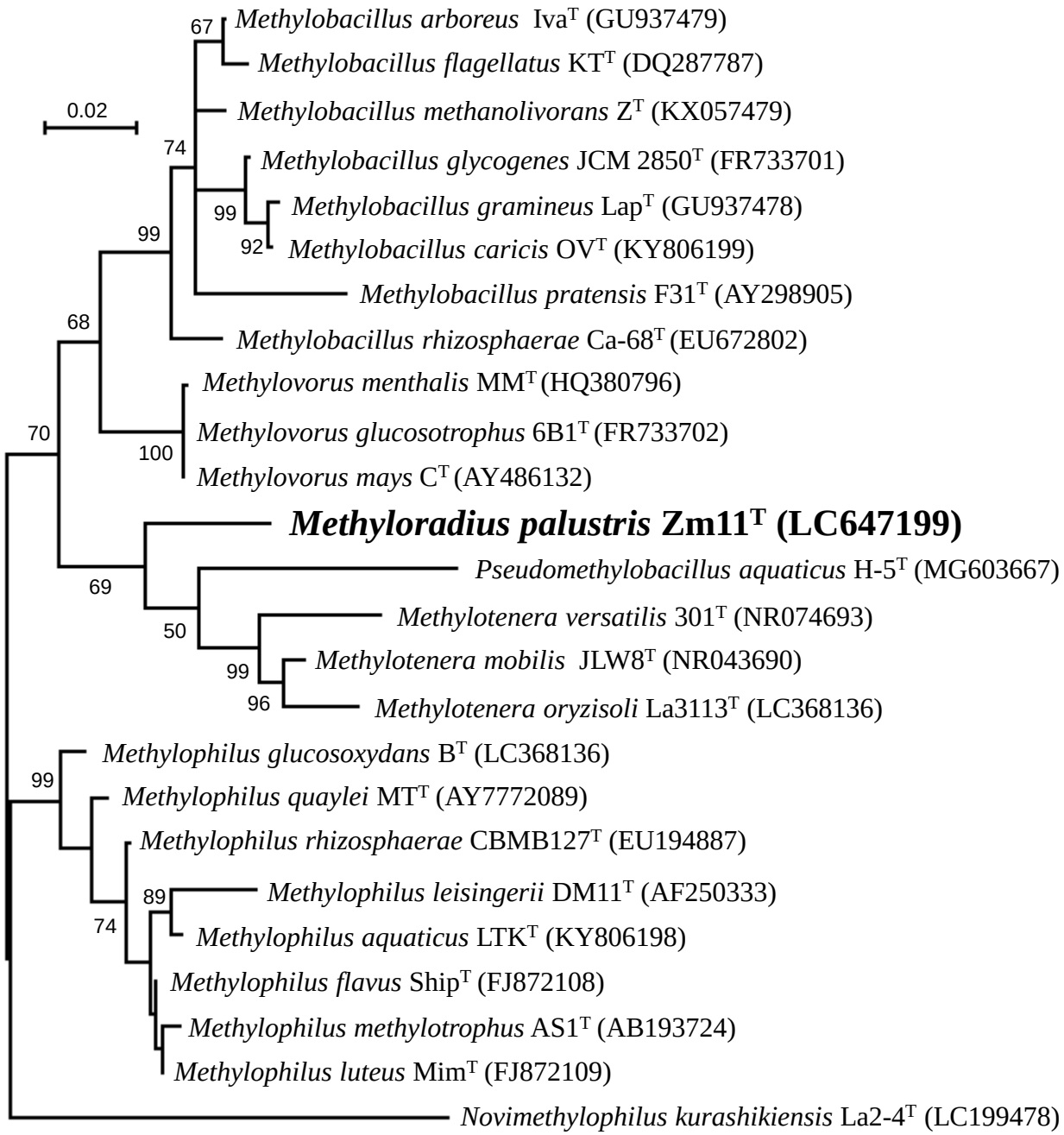
| | | Zm11$^T$ | *Methylobacillus* | *Methylophilus* | *Methylotenera* | *Methylovorus* | *Novimethylophilus* | *Pseudomethylobacillus* |
|---|---|---|---|---|---|---|---|---|
| Temperature for growth (ºC) | Range | 5–32 | 10–52 | 10–37 | 4–30 | 10–45 | 15–40 | 15–30 |
| | Optimum | 25–26 | 19–42 | 19–37 | 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%) | | $C_{16:0}$, SF3 | $C_{16:0}$, SF3 | $C_{16:0}$, SF3, $C_{16:0}$ 2-OH, $C_{10:0}$ 3-OH, $C_{17:0}$ cyclo | $C_{16:0}$, SF3 | $C_{16:0}$, SF3, $C_{17:0}$ cyclo | $C_{16:0}$, SF3 | $C_{16:0}$, 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$^{T}$ and genome-sequenced strains in the family *Methylophilaceae*

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


0.02
67
74
99
92
99
68
70
100
69
50
99
96
99
89
74
*Methylobacillus arboreus* Iva$^T$ (GU937479)
*Methylobacillus flagellatus* KT$^T$ (DQ287787)
*Methylobacillus methanolivorans* Z$^T$ (KX057479)
*Methylobacillus glycogenes* JCM 2850$^T$ (FR733701)
*Methylobacillus gramineus* Lap$^T$ (GU937478)
*Methylobacillus caricis* OV$^T$ (KY806199)
*Methylobacillus pratensis* F31$^T$ (AY298905)
*Methylobacillus rhizosphaerae* Ca-68$^T$ (EU672802)
*Methylovorus menthalis* MM$^T$ (HQ380796)
*Methylovorus glucosotrophus* 6B1$^T$ (FR733702)
*Methylovorus mays* C$^T$ (AY486132)
***Methyloradius palustris* Zm11$^T$ (LC647199)**
*Pseudomethylobacillus aquaticus* H-5$^T$ (MG603667)
*Methylotenera versatilis* 301$^T$ (NR074693)
*Methylotenera mobilis* JLW8$^T$ (NR043690)
*Methylotenera oryzisoli* La3113$^T$ (LC368136)
*Methylophilus glucosoxydans* B$^T$ (LC368136)
*Methylophilus quaylei* MT$^T$ (AY7772089)
*Methylophilus rhizosphaerae* CBMB127$^T$ (EU194887)
*Methylophilus leisingerii* DM11$^T$ (AF250333)
*Methylophilus aquaticus* LTK$^T$ (KY806198)
*Methylophilus flavus* Ship$^T$ (FJ872108)
*Methylophilus methylotrophus* AS1$^T$ (AB193724)
*Methylophilus luteus* Mim$^T$ (FJ872109)
*Novimethylophilus kurashikiensis* La2-4$^T$ (LC199478)

Supplementary material

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

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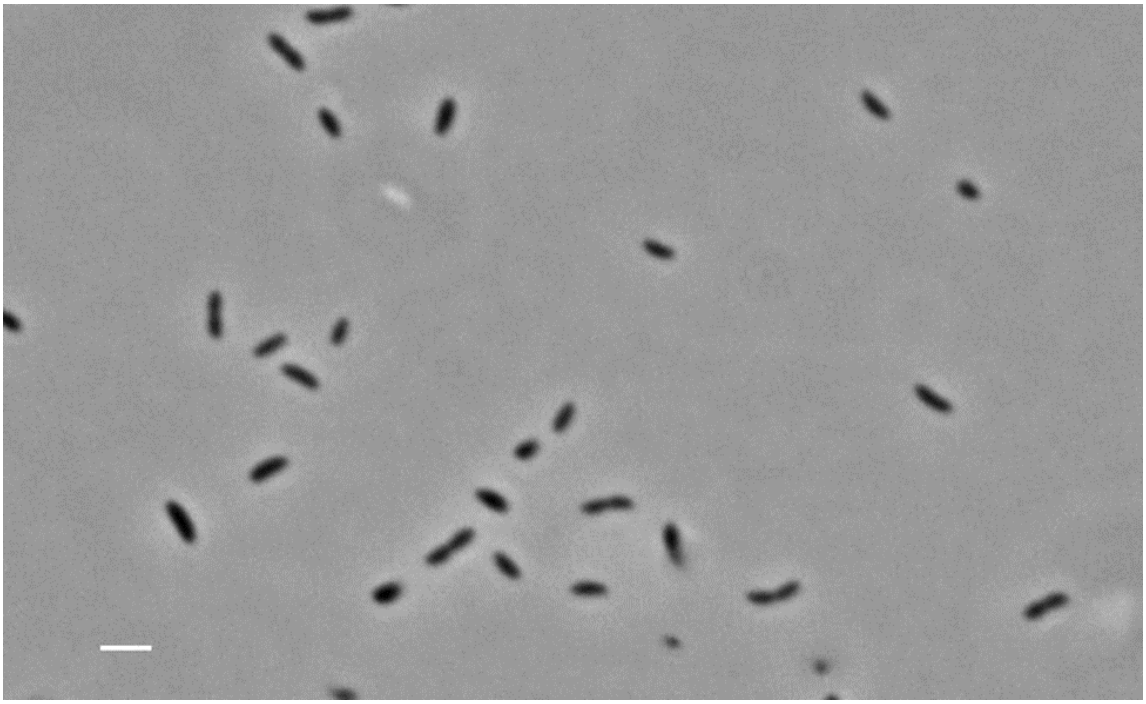

Fig. S1. Phase-contrast micrograph of strain Zm11T. Scale bar represents 2 μm..

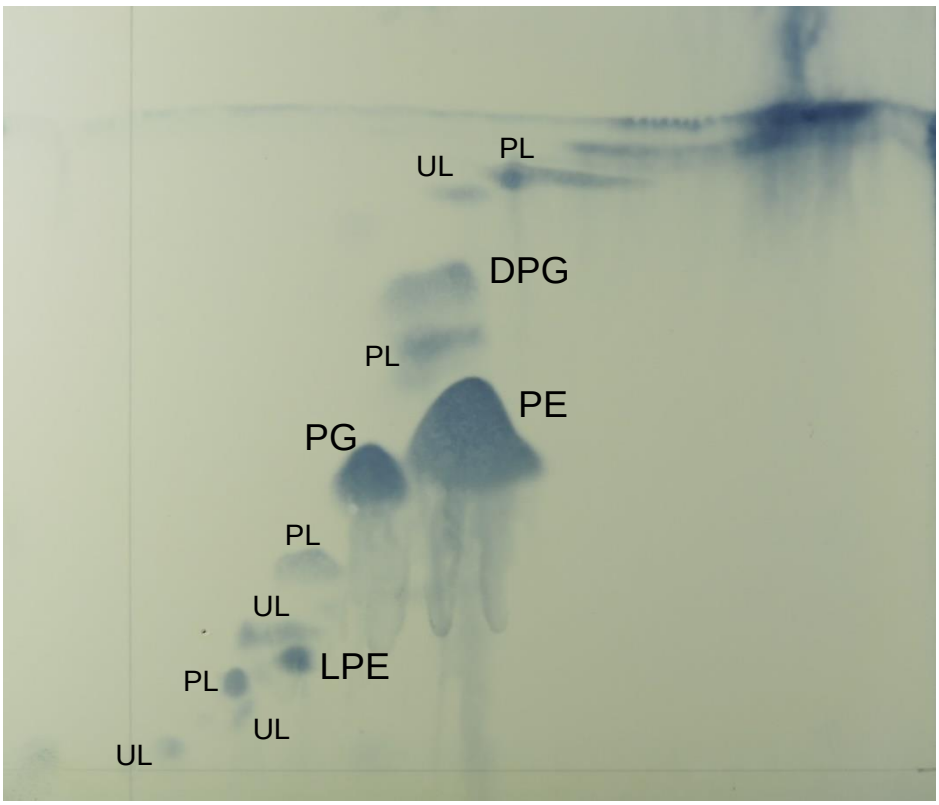


Fig. S2. Polar lipid profile of strain Zm11$^{T}$. PG, phosphatidylglycerol; PE, phosphatidylethanolamine; LPE, lyso-phosphatidylethanolamine; DPG,diphosphatidylglycerol; PL, unidentified phospholipids; UL, unidentified polar lipids.

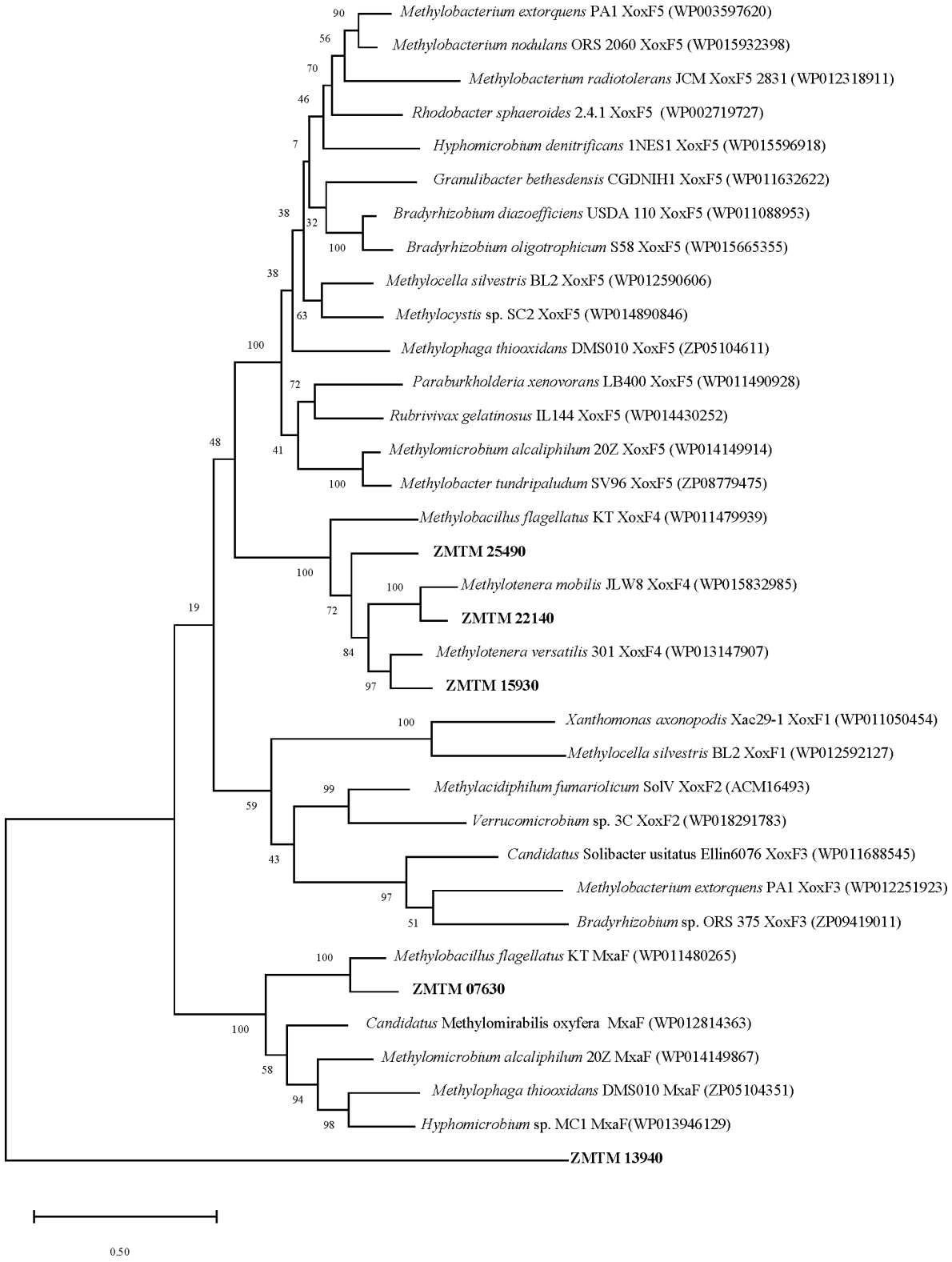


Fig. S3. Phylogenic tree of MDHs encoded by genes predicted in the genome of Zm11$^{T}$ (prefixed with ZMTM). All positions containing gaps and missing data were eliminated and there were a total of 838 positions in the final dataset. Bar, substitutions per site. Numbers on nodes represent percentage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

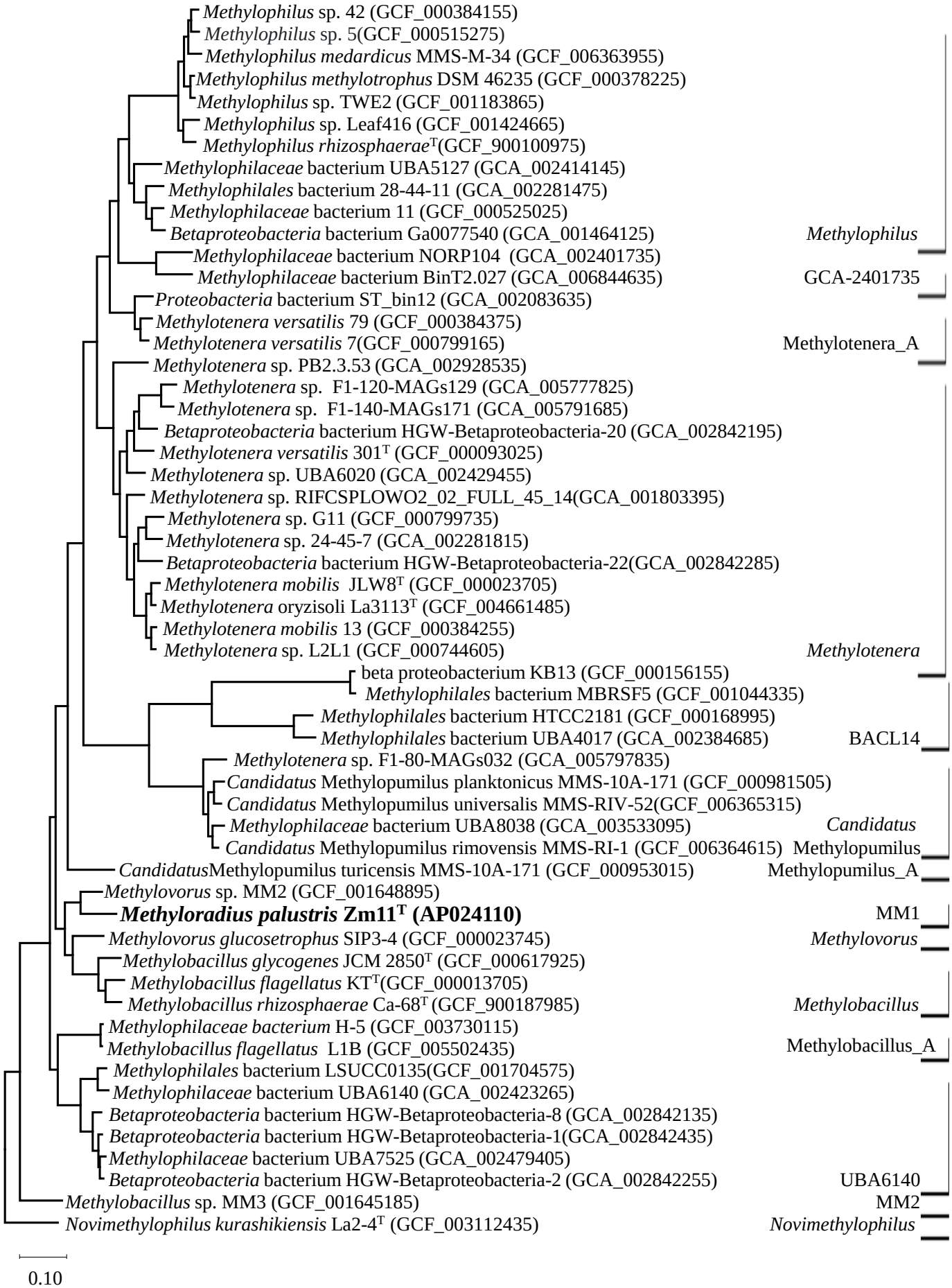


Fig. S4. Position of strain Zm11$^{T}$ in genome-based phylogenic tree, bac120 tree of GTDB. Names of genera are according to the GTDB taxonomy.