Methyloradius Palustris Gen. Nov., Sp. Nov., a Methanol-oxidizing Bacterium Isolated From Snow

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1 ***Methyloradius palustris* gen. nov., sp. nov., a methanol-oxidizing**
2 **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 ($0.6\text{--}0.7 \times 1.2\text{--}2.7\text{ }\mu\text{m}$). 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 ($\text{C}_{16:1}\omega 7c$ and/or $\text{C}_{16:1}\omega 6c$) and $\text{C}_{16:0}$. The predominant quinone was Q-8. 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.

37 **Declarations**

38 Funding

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40 Fukui.

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^T, has been deposited to
47 DSMZ and NBRC, as DSM111909^T and NBRC114766^T, respectively. Its complete
48 genome sequence is available in the GenBank/EMBL/DDBJ, with accession number of
49 AP024110.

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^{2+} -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

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% H₂O₂ 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.

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

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

104 37°C. The other tests for phenotypic characterization were all conducted at 22°C.

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

106 constituents (per litre); 2.0 g KH₂PO₄, 2.0 g (NH₄)₂SO₄, 0.5 g NaCl, 0.1 g MgSO₄ · 7H₂O,

107 0.002 g FeSO₄ · 7H₂O (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 KNO₃ 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).

Phylogenetic analyses

The 16S rRNA gene sequence of strain Zm11^T was aligned with those of type strains in the family *Methylophilaceae*, by using CLUSTALW version 2.1 (Larkin et al., 2003). Based on the resulting alignment, evolutionary distances were computed by using Kimura's two-parameter model with gamma distribution and invariant sites, using the MEGA X. Phylogenetic trees were constructed with the maximum-likelihood, neighbor-

joining and minimum evolution methods, available in the MEGA X.

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

Accession numbers

Genome sequence of strain strain Zm11^T is available under accession number AP024110.

Results and Discussion

Physiological and chemotaxonomic characteristics

The fundamental characteristics of strain Zm11^T are summarized in Table 1 and species description. Cells of strain Zm11^T were motile, rod-shaped, 0.6–0.7 µm in width, 1.2–2.7 µm in length (Fig. 1). The cells were oxidase-positive and weakly positive for catalase. In the cellular fatty acid profile of the strain, summed feature 3 (C_{16:1}ω7*c* and/or C_{16:1}ω6*c*) and C_{16:0} were predominant, accounting for 47% and 40% of total, respectively. As other components, C_{10:0} 3-OH (4.3%), C_{14:0} (1.4%) and C_{20:0}

(1.2%) were detected. The other minor fatty acids detected (<0.4% of total) were C₁₆:
1 ω 5 c and summed feature 8 (C_{18:1} ω 7 c and/or C_{18:1} ω 6 c). The strain had
phosphatidylethanolamine and phosphatidylglycerol as major polar lipids (Fig. S1).
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 5–30°C, with optimum growth at 25–30°C. 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. 2. 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).

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. S2, one of them encodes MxaF-type MDH and the others encode XoxF-type MDH with distinct amino acid sequences, respectively.

199

200 Taxonomic assignment

201 Phylogenetic relationships between strain Zm11^T and type strains of related species
202 with validly published names are shown in Fig. 2. In the maximum likelihood tree of
203 16S rRNA gene, strain Zm11^T represents sister group of a clade consisting of the genera
204 *Methylothera* 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^T 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^T 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. S3. All these results mean that a novel
212 genus should be created to accommodate strain Zm11^T.

213 The methanol-oxidizing bacterium isolated in this study, strain Zm11^T, turned out to
214 be a novel member of the family *Methylophilaceae*. It does not belong to any existing
215 genera in the family, as revealed by analyses of the 16S rRNA gene and whole genome.

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

218

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

220 *Methyloradius* (Me.thy.lo.ra'di.us. N.L. neut. n. methylum, the methyl radical; from
221 French masc. n. méthyle; from French masc. n. méthylène; from Gr. neut. n. [μέθυ] methy,
222 wine; from Gr. fem. n. [ῥύλη] hylê, wood; N.L. pref. methylo-, pertaining to the methyl
223 radical; L. masc. n. *radius*, a staff, rod; N.L. masc. n. *Methyloradius*, methanol-oxidizing
224 rod).

225 Grows by oxidation of methanol as carbon and energy source. Aerobic and neutrophilic.
226 Gram-stain-negative. Major cellular fatty acids are summed feature 3 (C_{16:1}ω7c and/or
227 C_{16:1}ω6c) and C_{16:0}. Belongs to the family *Methylophilaceae*. The type species is
228 *Methyloradius palustris*.

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

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

231 Cells are motile, rod-shaped, 0.6-0.7 μm long and 1.2-2.7 μm wide. Catalase-positive and
232 oxidase-positive. Uses oxygen as electron acceptor. Q-8 is the sole respiratory quinone.

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 complete genome sequence of strain Zm11^T is AP024110.

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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}ω7c and/or C_{16:1}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), *Methylostenora* (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	<i>Methylobacillus</i>	<i>Methylophilus</i>	<i>Methylostenora</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 _{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 to Zm11 ^T (%)	AAI to Zm11 ^T (%)
Zm11 ^T	--	2.800	46.4	100	100
<i>Methylobacillus flagellatus</i> KT ^T	NC_007947	2.972	55.7	76.88	67.50
<i>Methylobacillus glycogenes</i> JCM 2850 ^T	NZ_BAMT000000000	3.249	53.4	76.41	64.25
<i>Methylobacillus rhizosphaerae</i> Ca-68 ^T	NZ_FZOA000000000	2.368	52.4	76.77	67.42
<i>Methylovorus glucosetrophus</i> SIP3-4	NC_012969	2.996	54.9	77.28	69.16
<i>Methylothermobacter mobilis</i> JLW8 ^T	NC_012968	2.548	45.5	76.78	65.50
<i>Methylothermobacter versatilis</i> 301 ^T	NC_014207	3.060	42.6	76.25	65.65
<i>Methylophilus methylotrophus</i> DSM 46235 ^T	NZ_ARJW000000000	2.860	49.6	76.25	61.45
<i>Methylophilus rhizosphaerae</i> CBMB127 ^T	NZ_FNFX000000000	2.761	51.4	76.47	61.48
<i>Methylophilus medardicus</i> M-51	NZ_CP040946.1	2.063	49.8	77.37	61.36
<i>Novimethylophilus kurashikiensis</i> La2-4 ^T	NZ_BDOQ000000000	3.689	56.1	76.25	62.64
<i>Pseudomethylobacillus aquaticus</i> H-5 ^T	NZ_RJVP000000000	2.550	58.3	77.22	67.57

Figure legends

Fig. 1. Phase-contrast micrograph of strain Zm11^T. Scale bar represents 2 μ m.

Fig. 2. Maximum likelihood tree showing phylogenetic position of strain Zm11^T 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.

Supplementary Files

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