**Methylophilus rhizosphaerae** sp. nov., a restricted facultative methylotroph isolated from rice rhizosphere soil

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Three facultative methylotrophic bacterial strains, designated CBMB127T, CBMB145 and CBMB147, were isolated from the rhizosphere soil of rice and characterized. The strains produced indole-3-acetic acid and siderophores, had 1-aminocyclopropane-1-carboxylate deaminase activity and sulfur oxidation property and also methanol dehydrogenase. Phylogenetic analysis based on the 16S rRNA and methanol dehydrogenase (mxaF) gene sequences showed that Methylophilus methylotrophus was their close relative. The results of the phenotypic, phylogenetic and genotypic analyses showed that strains CBMB127T and CBMB145, with 99.4 % 16S rRNA gene sequence similarity and 99 % DNA–DNA hybridization, could be distinguished from recognized species of Methylophilus. Therefore strain CBMB127T and CBMB145 are considered to represent a novel species of Methylophilus, for which the name Methylophilus rhizosphaerae sp. nov. is proposed, with CBMB127T (KACC 13099T = NCCB 100233T) as the type strain. Strain CBMB147 represents a novel strain of the species Methylophilus methylotrophus.

Methylotrophic bacteria comprise a diverse group of microbes capable of metabolizing single carbon compounds such as methanol or methylamine as sole source of carbon and energy. Based on the carbon source utilized, methylotrophs are classified into three subgroups: obligate methylotrophs that use only single carbon compounds; restricted facultative methylotrophs that can also utilize a limited range of more complex organic compounds; and the less-restricted facultative methylotrophs that can grow on a wide range of more complex carbon compounds as sole carbon and energy source (Jenkins et al., 1987). To date, three genera have been described as belonging to the restricted facultative methylotrophic group, Methylobacillus (Yordy & Weaver, 1977; Urakami & Komagata, 1986), Methylophilus (Jenkins et al., 1987) and Methyllovorus (Govorukhina & Trotsenko, 1991) classified in the Betaproteobacteria, whereas the most common less-restricted facultative methylotrophic genus Methyllobacterium belongs to the Alphaproteobacteria. Members of the genus Methylophilus have been shown to be clearly separated from other groups, forming a distinct branch that includes two recognized species, Methylophilus methylotrophus and Methylophilus leisingerii, with Methyllovorus methylotrophus (http://www.bacterio.cict.fr/m/methylphilus.html) as the type species (Jenkins et al., 1987; Doronina & Trotsenko, 1994). Cells are strictly aerobic, Gram-negative rods and utilize the ribulose monophosphate pathway for formaldehyde assimilation. Recent studies in our laboratory focusing on the methylotrophic bacterial population associated with different parts of the rice ecosystem collected from Chungbuk Provincial Agricultural Research and Extension Services (Cheongwon, Republic of Korea), allowed us to isolate a large number of bacteria. In the present study we describe a novel species of Methylophilus and one novel strain of the species Methylophilus methylotrophus isolated from rice rhizosphere soil, with multiple plant-growth promoting characteristics such as production of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) and indole-3-acetic acid (IAA).

**Abbreviations:** ACCD, 1-aminocyclopropane-1-carboxylate deaminase; AMS, ammonium mineral salts; IAA, indole-3-acetic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and mxaF gene sequences of strain CBMB127T are EU194887 and EU194904, respectively, and those for strain CBMB145 are EU194883 and EU194902 and those for CBMB147 are EU194892 and EU194905, respectively.

A neighbour-joining phylogenetic tree based on deduced amino acid sequences of mxaF products is available as supplementary material with the online version of this paper.
The novel strains, designated CBMB127\textsuperscript{T}, CBMB145 and CBMB147, isolated from rhizosphere soils of rice cultivars (\textit{Oryza sativa} L. cv O-daee and Nam-pyeoung, respectively) on selective ammonium mineral salts (AMS) medium (Whittenbury et al., 1970) with 0.5\% (v/v) methanol were studied in detail. Cells were maintained on nutrient agar (NA; Difco) with 1\% (v/v) methanol, or on AMS medium with 0.5\% (v/v) methanol. Morphological properties, and nutritional and phenotypic characterization were studied according to protocols described by Gerhardt \textit{et al.} (1994) and Green & Bousfield (1982). The carbon source utilization pattern was studied using Biolog GN2 Microplates (Madhaiyan \textit{et al.}, 2007). Other physiological and biochemical characteristics were tested using the API ZYM, API 20NE and API 32GN galleries (bioMérieux), according to the manufacturer’s instructions. \textit{Methylophilus methylotrophus} DSM 5691\textsuperscript{T} was included as a positive control in these experiments. Sample preparation and other procedures for scanning electron microscope (SEM) observations were performed as described by Madhaiyan \textit{et al.} (2007) and the samples were visualized using a Hitachi S-2500C SEM with GEMINI column (Hitachi) equipped with a field emission source. The quantitative assay for the estimation of IAA and plate assays for detecting the presence of siderophore production, ACCD activity and sulfur oxidation were performed as described by Poonguzhali \textit{et al.} (2006) and Anandham \textit{et al.} (2007). Quantitative estimation of methanol dehydrogenase enzyme activity was carried out according to Dunfield \textit{et al.} (2003). Cellular fatty acids were extracted from cultures grown on NA with 1\% (v/v) methanol at 28\,°C, for 4 to 5 days, derivatized to methyl esters and analysed by using a gas chromatograph (Hewlett Packard 6890) with the Microbial Identification System (MIDI; Microbial ID) software package, according to the standard protocols (Sasser, 1990).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
\textbf{Characteristic} & \textbf{1} & \textbf{2} & \textbf{3} & \textbf{4} & \textbf{5} \\
\hline
Flagellation & – & – & – & + & – \\
Cell diameter (\textmu m) & 0.5–1.2 × 0.25–0.3 & 0.4–1.2 × 0.2–0.3 & 0.6–1.2 × 0.2–0.3 & 0.5–0.6 & ND \\
Motility & + & +/w & + & + & + \\
Oxidase & + & +/w & + & + & + \\
Catalase & +/w & + & + & + & + \\
Growth at 45 \degree C & – & w & – & – & – \\
Optimum pH & 6.8–7.5 & 6.8–7.5 & 6.5–7.2 & 6.5–7.2 & 6.8–7.2 \\
Carbon sources for growth & & & & & \\
Glucose & – & + & + & + & + \\
Fructose & + & + & – & – & – \\
Dichloromethane & w & – & – & + & – \\
Ethanol & w & – & w & + & + \\
Trimethylamine & w & + & – & + & w \\
Methyamine & w & w & – & w & – \\
Diethanolamine & w & + & + & – & – \\
Formaldehyde & – & w & w & – & – \\
Acetate & – & – & – & w & – \\
Formate & – & – & w & w & – \\
Methane & –/w & –/w & – & – & – \\
DNA G+C content (\textit{T}m) (mol\%) & 47.9 & 48.7 & 42.0 & 50.3 & 50.2 \\
\hline
\end{tabular}
\caption{Differential phenotypic characteristics of strains CBMB127\textsuperscript{T}, CBMB145 and CBMB147 and type strains of related \textit{Methylophilus} species.} \\
Strains: 1, CBMB127\textsuperscript{T}; 2, CBMB145; 3, CBMB147; 4, \textit{Methylophilus methylotrophus} DSM 5691\textsuperscript{T}; 5, \textit{Methylophilus leisingeri} DSM 6813\textsuperscript{T}. All are Gram-negative, rod-shaped, restricted facultative and utilize methanol and succinate as sole source of carbon and energy but not dimethylamine. +, Positive; –, negative; w, weakly positive; ND, not determined.
\end{table}
Cells of the novel strains were aerobic, motile, non-endospore forming, Gram-negative rods, occurring singly or in pairs on solid AMS medium, forming white, translucent to opaque colonies with regular, convex edges. An SEM photomicrograph of cells of strain CBMB127T is shown in Fig. 1. The strains grew with methanol, diethanolamine and succinate as carbon sources. Strains CBMB127T and CBMB145 showed growth on fructose whereas strain CBMB147 and other species tested showed no growth. Strain CBMB127T could not utilize glucose, which differentiated it from the other strains used. The nutritional and physiological characteristics of strain CBMB127T are given in the species description and the differential characteristics of its closest relatives are given in Table 1. All three strains possessed ACCD activity and sulfur oxidation when examined using plate assays. Strains CBMB127T, CBMB145 and CBMB147 produced, respectively, 6.32, 8.55 and 10.39 μg IA A ml⁻¹ and methanol dehydrogenase (mxaF) gene was checked using PCR with the primer pair mxaF f1003 and mxaF r1561, as described by McDonald & Murrell (1997), and the products were sequenced. The sequences of the 16S rRNA genes or the deduced amino acid sequences from the mxaF gene were screened against sequences in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov.proxy.lib.siu.edu/blast/), and were then aligned with a set of representative genes from the same and related genera using CLUSTAL W software (Thompson et al., 1994). Phylogenetic trees were constructed by using the neighbour-joining method with MEGA 3.1 (Kumar et al., 2004).

The 16S rRNA gene phylogenetic tree obtained placed the strains with Methylophilus, forming two clusters with strains CBMB127T and CBMB145 grouping together in a separate cluster. The 16S rRNA gene sequence similarities for the strains were 99.01 % with Methylophilus methylotrophus DSM 6813T and ranged from 97.56 to 97.92 % with Methylophilus leisingeri DSM 6813T (Fig. 2). Analysis of the mxaF genes, which codes for the β-subunit of methanol dehydrogenase, of the three strains confirmed the 16S rRNA sequencing results, showing close similarity to Methylophilus methylotrophus. Strains CBMB127T and CBMB145 showed 99.4 % similarity with each other and 98.2 and 98.8 % similarity with Methylophilus methylotrophus, respectively. Strain CBMB147 showed 99.4 % similarity with M. methylotrophus and 97.6 and 98.2 % similarity with strains CBMB127T and CBMB145, respectively. A phylogenetic tree showing the mxaF genes of the three strains and representatives of close relatives is shown in Supplementary Fig. S1 (available in IJSEM Online).

DNA–DNA hybridization was carried out using nitrocellulose membrane filters according to the method of Seldin & Dubnau (1985). The DIG-High prime system and DIG luminescent detection kit (Roche Diagnostics GmbH) were used for labelling the probe DNA and visualization. Hybridization temperatures were 60 and 65 °C. The G + C content of the genomic DNA was determined by HPLC using a reversed-phase column (Supelcosil LC-18S) of individual nucleosides, as described by Mesbah et al. (1989).

Strains CBMB127T and CBMB145 showed a high level of DNA–DNA relatedness (97–99 %) with each other, whereas they showed a low level of relatedness with strain CBMB147 (30 and 29 %, respectively). Strain CBMB127T

<table>
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<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>C10:0</td>
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<td>0.48</td>
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<tr>
<td>C14:0</td>
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<td>0.71</td>
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<td>0.63</td>
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<tr>
<td>C16:0</td>
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<td>30.0</td>
<td>37.89</td>
<td>27.76</td>
<td>36.82</td>
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<tr>
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<td>0.17</td>
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<td>–</td>
<td>0.52</td>
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<tr>
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<tr>
<td>C9:0:3-OH</td>
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<tr>
<td>C10:0:3-OH</td>
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<tr>
<td>iso-C15:0:3-OH</td>
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<tr>
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<td>0.22</td>
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<td>C17:0 cyclo</td>
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<td>13.77</td>
<td>18.87</td>
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<tr>
<td>C16:1o5c</td>
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<td>–</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>C17:1o7c</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C18:1o5c</td>
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<td>0.10</td>
<td>–</td>
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<td>C18:1o7c</td>
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<td>33.85</td>
<td>29.25</td>
<td>41.55</td>
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<tr>
<td>4</td>
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<td>0.42</td>
<td>7.26</td>
<td>–</td>
<td>–</td>
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<tr>
<td>8</td>
<td>–</td>
<td>4.24</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Unknown 14,502 (ECL)</td>
<td>–</td>
<td>0.13</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contains C16:1o7c and/or iso-C15:0:2-OH or C16:1o6c; summed feature 4 contains iso-C17:1:1 and/or anteiso-C17:0:1B; summed feature 8 contains C18:1o7c and/or C18:1o6c.

Table 2. Cellular fatty acid compositions (%) of strain CBMB127T and related species of the genus Methylophilus

Strains: 1, CBMB127T; 2, CBMB145; 3, CBMB147; 4, Methylophilus leisingeri DSM 6813T; 5, Methylophilus methylotrophus DSM 5691T.

Values are percentages of total fatty acids. Fatty acids representing less than 0.1 % in all strains were omitted. All strains do not contain C12:0, C15:0, C17:0, C18:1, iso-C13:0 and C16:1o7c. –, Not detected; ECL, equivalent chain-length.
showed DNA–DNA relatedness of only 28 and 29%, respectively, with the closely related species Methylophilus methylotrophus and Methylophilus leisingeri. Strain CBMB147 had a high percentage (91%) level of DNA–DNA relatedness with Methylophilus methylotrophus. Based on the DNA–DNA hybridization data, it was shown that strains CBMB127T and CBMB145 do not belong to any of the recognized species of Methylophilus, when the recommendation of a threshold value of 70% DNA–DNA relatedness for species definition is considered (Wayne et al., 1987), and strain CBMB147 represents a novel strain of the species Methylophilus methylotrophus. The DNA G+C content of strain CBMB127T was 47.9 mol%, which falls within the range described for the genus Methylophilus (Jenkins et al., 1987). On the basis of the results given, strains CBMB127T and CBMB145 are considered to represent a novel species of the genus Methylophilus, for which the name Methylophilus rhizosphaerae sp. nov. is proposed.

Description of Methylophilus rhizosphaerae sp. nov.

Methylophilus rhizosphaerae (rhi.zo’spha.e’rae. Gr. fem. n. rhiza root; L. fem. n. sphaera -ae ball, any globe, sphere; N.L. gen. fem. n. rhizosphaerae of the rhizosphere).

Gram-negative, non-endospore forming, strictly aerobic, motile rods (0.5–1.2 × 0.25–0.3 μm), occurring singly or in pairs. Colonies are white, convex, and translucent with regular edges, slow-growing and 0.3–0.6 mm in diameter after 96 h at 28 °C on AMS. Growth occurs on NA supplemented with 1% methanol, R2A, NFb medium (Baldani & Döbereiner, 1980) with 1 g ammonium chloride l⁻¹ as combined nitrogen source, and Colby and Zathman medium (Colby & Zatman, 1973) supplemented with 0.2% methanol and 2% MH medium. Does not grow in the presence of 2.0% NaCl or higher. Growth occurs at 20–30 °C (optimum, 28 °C) and pH 4.0–10.0 (optimum, pH 6.8). Does not grow at 4 or 45 °C. Catalase, oxidase and urease are positive. Pectinase, cellulase, protease, arginine dihydrolase and β-galactosidase are absent. Nitrate reduction and indole production are positive. Starch, glycerol tributyrate, casein and aesculin are not hydrolysed; hydrolysis of gelatin is weak. Methanol, dichloromethane, ethanol, trimethylamine, methylamine, succinate and diethanolamine are utilized as sole carbon sources. Ammonium sulfate, sodium nitrate, ammonium chloride, potassium nitrate, 1-aminocyclopropane-1-carboxylate, L-alanine, L-glutamine, glycine, urea, methyamine, potassium thiocyanate and trimethylamine are utilized as sole nitrogen sources. The following compounds are not utilized as nitrogen sources: L-glutamate, potassium cyanate, L-tryptophan, L-aspartic acid, diethylamine and diphenylamine. The following compounds are utilized as sole carbon and energy sources (Biolog): α-cyclodextrin, D-fructose, formic acid and D-glucose 6-phosphate. In the
API 20NE and API 32GN tests, no single carbon/nitrogen source was assimilated. In API ZYM assays, esterase (C4), leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase are present, but alkaline phosphatase, esterase lipase (C8), trypsin, acid phosphatase, lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase are not. Major fatty acids are C16:0, C17:0 cyclo and summed feature 3.

The type strain, CBMB127T (=KACC 13099T=NCCB 100233T) was isolated from rhizosphere soil of rice (Oryza sativa L. cv O-dae) collected at the vegetative stage (V10) of the plant. The DNA G+C content of the type strain is 47.9 mol%. Strain CBMB145 is a second strain of the species.

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References


