Oharaeibacter diazotrophicus gen. nov., sp. nov., a diazotrophic and facultatively methylotrophic bacterium, isolated from rice rhizosphere

Haoxin Lv,1 Sachiko Masuda,1,2 Yoshiko Fujitani,1 Nurettin Sahin3 and Akio Tani1,*

Abstract
A novel facultatively methanol-utilizing bacterial strain, SM30T, was isolated from rice rhizosphere. Strain SM30T was Gram-stain-negative, aerobic, motile, short rods, and grew optimally at pH 7 and at 28 °C. It could tolerate 0 to 2% (w/v) NaCl. Based on 16S rRNA gene sequence comparisons, strain SM30T was most closely related to Pleomorphomonas oryzae DSM 16300T, with a low similarity of 94.17%. One of the lanthanide metals, lanthanum, could enhance its growth slightly on methanol. Phylogenetic trees, based on the mxaF, xoxF and cpn60 genes of SM30T showed its distinct phylogenetic position with respect to species with validly published names. Polymerase chain reaction (PCR) amplification of the nifH and growth on nitrogen-free medium indicated that strain SM30T is a diazotroph. The major cellular fatty acids were summed feature 8 (containing 18:1ω7c and 18:1ω6c) and cyclo 19:0ω8c. The major quinone was ubiquinone 10. The DNA G+C content was 74.6 mol%. Based on the genotypic and phenotypic characteristics, strain SM30T represents a novel genus and species, for which the name Oharaeibacter diazotrophicus gen. nov., sp. nov. is proposed with the type strain SM30T (=DSM 102969T).

Large amounts of methane are produced on the earth, most of which is released into the atmosphere contributing to global warming, and some of which remains in ecosystems [1]. In these ecosystems, methylotrophs, including methanotrophs, play an important role in consuming methane, so alleviating the greenhouse effect. Methylotrophs are organisms that can use one-carbon compounds (methane and methanol for example) as a sole carbon and energy source [2]. Methanol is the first intermediate in the process of methane oxidation in methanotrophs, and the oxidation of methanol catalyzed by methanol dehydrogenase (MDH) is a key step for all methylotrophs [3, 4]. Gram-negative methylotrophic bacteria possess pyrroloquinoline-quinone-dependent MDH. The function and structure of calcium-dependent MxaFI-type MDH is well understood [5–7]. Most methylotrophic Gram-negative bacterial genomes contain the xoxF gene, which encodes another type of MDH [8]. XoxF shares approximately 50% amino acid identity with the large subunit of MxaF, and was recently found to contain lanthanides (light lanthanides: La, Ce, Pr, and Nd; hereafter Ln) as cofactors [3, 4, 9–11]. Ln are included in the rare-earth elements, but their abundance is not rare in the Earth’s crust; their content is similar to that of copper and zinc (La 39 ppm; Cu, 60 ppm; and Zn, 70 ppm) [12]. Only in the presence of Ln is the robust activity of XoxF evident [13]. Therefore, methylotrophs may exist that have never been isolated in Ln-free laboratory conditions, and the discovery of such microorganisms would contribute to both the understanding of how Ln affects the activity of microorganisms as-yet not isolated, and to helping mitigate global warming.

Since the rice rhizosphere is one of the largest sources of methane emissions, we screened methylotrophs in the presence of Ln [we used lanthanum (La)] from rice rhizosphere soil samples. In this study, we report on the characterization of a novel isolate, designated SM30T, using a polyphasic approach. On the basis of the results obtained in this study, strain SM30T represents a novel genus and species, for

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Keywords: Oharaeibacter diazotrophicus gen. nov., sp. nov.; methanol dehydrogenase; Lanthanum.
Abbreviations: Ln, lanthanide; MALDI-TOF/MS, matrix assisted, laser desorption/ionization time-of-flight mass spectrometry; MDH, methanol dehydrogenase; PES, phenazine ethosulfate.
The GenBank/EMBL/DBJ accession numbers are as follows: SM30T 16S rRNA (LC153750), mxaF (LC154793), xoxF (LC154794), cpn60 (LC160005) and nifH (LC154795); Hartmannibacter diazotrophicus LMG27460T xoxF (LC163943), and cpn60 (LC171325), and Pleomorphomonas diazotrophica DSM25022T cpn60 (LC171326).
Six supplementary figures and one supplementary table are available with the online Supplementary Material.
which the name *Oharaeibacter diazotrophicus* gen. nov., sp. nov. is proposed.

A mud sample (ca. 50 mg) collected from rice roots was inoculated with 20 ml nitrate mineral salts (NMS) medium [14] supplemented with 30 µM LaCl₃ (NMS+La) prepared in a 70 ml vial, which was capped with a rubber seal. Methane gas (final concentration 20%, v/v) was injected into the vials. After several rounds of enrichment cultivation, the cultured samples were spread onto NMS+La plate media solidified with 1.5% (w/v) agar. The plates were incubated at 28°C for 7 to 10 days under 20% (v/v) methane. Colonies with distinct morphology were selected and purified by several rounds of streaking. The isolate, named SM30ᵀ, could not grow on methane in liquid cultures, while it grew very weakly on solidified NMS+La in 20% (v/v) methane. Interestingly, SM30ᵀ could grow well on NMS+La with 0.5% (v/v) methanol. Later it was found that mineral medium (MM) [15] containing 0.5% (v/v) methanol with 30 µM LaCl₃ was the best medium for SM30ᵀ growth. In addition, R2A medium was also suitable for SM30ᵀ. Therefore, strain SM30ᵀ was cultured in MM medium supplemented with 0.5% (v/v) methanol, NMS medium with 0.5% (v/v) methanol, or in R2A medium (Becton Dickinson) at 28°C for further analysis. When necessary, 30 µM LaCl₃ was added aseptically.

The cell morphology and Gram-staining [16] of SM30ᵀ were evaluated by light microscopy using an Olympus BX43 microscope (×1000) using colonies grown for 72 h. The motility of SM30ᵀ was tested by stabbing the cells with a needle into a semisolid (0.3%, w/v) agar NMS+La with 0.5% (v/v) methanol medium and observing the cells spread. NaCl tolerance, pH and temperature optimums were determined according to the methods of Smibert & Krieg [17] in triplicate. Salt tolerance was checked in R2A liquid medium containing 0.1, 0.2, 0.5, 1, 2, 5 and 10% (v/v) NaCl at 28°C for one week. Optimum pH conditions were determined by culturing in R2A medium adjusted to pH 3, 4, 5, 6, 7, 8, 9 and 10. The following buffers were used to adjust the pH values: citric acid/sodium citrate (pH 4.0–6.0), Na₂HPO₄/NaH₂PO₄ (pH 6.0–8.0), and Na₂CO₃/NaHCO₃ (pH 8.0–10.0). Growth at different temperatures (5, 10, 15, 20, 25, 28, 35, and 40°C) was also checked in R2A liquid medium. Nitrate reduction was tested in liquid R2A medium containing 0.2% (v/v) KNO₃. Urease activity was checked by urea agar medium [all 1⁴ in sterile water: 1 g tryptone, 5 g NaCl, 1 g glucose, 1.2 g Na₂HPO₄, 0.8 g KH₂PO₄, 0.03 g neutral red, 15 g agar, with 50 ml 40% (w/v) urea solution added]. Nitrogen fixation was confirmed by using modified NFB medium with methanol as the carbon source instead of DL-malic acid [18]. Other physiological and biochemical characteristics were determined by using API 20NE strips (bioMérieux) and Biolog GN2 Microplates. Utilization of different carbon sources was tested with Biolog GN2 Microplates (obtained from CSC) by following the manufacturer’s instructions and the OD₅₉₅ was read using a microplate reader (Powerscan HT, DS Pharma) every day for 8 days. The morphological and physiological characteristics of strain SM30ᵀ were compared with the most closely related type strains of species of the genera *Pleomorphomonas*, *Hartmannibacter*, *Methyllobrevis* and *Labrenzia* (Table 1). Further detailed information strain SM30ᵀ is given in the species description.

Both NMS and MM medium supplemented with 0.5% (v/v) methanol or 0.1% (v/v) methanol in the presence and absence of La³⁺ were adopted for testing the utilization of methanol or methylamine as the sole carbon source. The growth of strain SM30ᵀ and related type strains on different media is shown in Fig. S1 (available in the online Supplementary Material). Although strain SM30ᵀ could grow in the absence of La³⁺, the presence of La³⁺ promoted its growth in both media (Fig. S1a, b). Only *H. diazotrophicus* LMG 27460ᵀ exhibited La³⁺-dependent growth on methanol in NMS medium, which was likely due to the existence of xoxF (we sequenced the PCR-amplified fragment, Table 1), however, its methylotrophy was unknown [19]. *mxaF* and *xoxF* were not sequenced in the genomes of other strains (we sequenced the PCR-amplified fragment, Table 1), but no products were obtained (Table 1). These results indicate that these three species of the genus *Pleomorphomonas* are not methylotrophic, and that they are critically different to SM30ᵀ. The growth of SM30ᵀ in MM containing 0.1% (w/v) methylamine was La³⁺-independent, whereas La³⁺ could promote its growth in NMS (Fig. S1c, d). *H. diazotrophicus* LMG 27460ᵀ was able to utilize methylamine in NMS medium only in the presence of La³⁺ (Fig. S1d). No strains of species of the genus *Pleomorphomonas* could utilize methylamine as a carbon source in either media regardless of La³⁺ (Fig. S1c, d). Methylamine could be converted to formaldehyde by methylamine dehydrogenase, and XoxF-MDH was reported to be capable of formaldehyde oxidation [20, 21], which might explain the better growth of SM30ᵀ and *H. diazotrophicus* LMG 27460ᵀ on methylamine in the presence of La³⁺.

Quinone and fatty acid analyses were carried out by TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). SM30ᵀ cells (5 g and 0.1 g wet weight) collected from seven day-old cultures of MM methanol medium were used for major quinone and cellular fatty acid methyl esters analyses, respectively. Ubiquinones were extracted from the cells [22] and were analyzed by HPLC (ACQUITY UPLC system) according to Tamaoka et al. [23]. The quinone components were ubiquinone-10 (98.7%) and ubiquinone-9 (1.9%), which is similar to related species. Cellular fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The major cellular fatty acids were 16:00 (0.71%), 17:00 (1.32%), 18:00 (6.69%), 11-methyl 18:1ω7c (4.23%), cyclo 19:0ω8c (12.94%), 18:0 3OH (1.93%), 20:1ω7c (1.54%), summed feature 2 (comprising 14:0 3OH and 16:1 iso; 20.3%), and summed feature 8 (containing 18:1ω7c...
Table 1. Differential characteristics of strain SM30\textsuperscript{T} from species of the genera Pleomorphomonas, Hartmannibacter, Methylobrevis and Labrenzia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<td>Motility</td>
<td>+</td>
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<td>Catalase activity</td>
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<td>Nitrate reduction to nitrite (NO\textsubscript{3}\textsuperscript{-})</td>
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<td>N-Acetylglosamine</td>
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<td>+\textsuperscript{*, †}</td>
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<td>–</td>
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<td>Major fatty acids (&gt;10%)</td>
<td>SF\textsubscript{8}, C19 : 0 cyclo \textomega\textsubscript{8c}</td>
<td>SF\textsubscript{8}, C16 : 0</td>
<td>SF\textsubscript{8}, C19 : 0 cyclo \textomega\textsubscript{8c}, C16 : 0</td>
<td>SF\textsubscript{8}, C19 : 0 cyclo \textomega\textsubscript{8c}</td>
<td>SF\textsubscript{8}, C19 : 0 cyclo \textomega\textsubscript{8c}</td>
<td>SF\textsubscript{8}</td>
<td>SF 2, SF8</td>
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<td>Hydroxy fatty acids</td>
<td>C14\textsubscript{0} 3-OH, C18\textsubscript{0} 3-OH</td>
<td>C14\textsubscript{0} 3-OH, C18\textsubscript{0} 3-OH</td>
<td>C14\textsubscript{0} 3-OH, C18\textsubscript{0} 3-OH</td>
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<td>C14\textsubscript{0} 3-OH, C18\textsubscript{0} 3-OH</td>
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<tr>
<td>Quinone type</td>
<td>Q-10 (98.7 %), Q-9 (13 %)</td>
<td>Q-10 (86 %), Q-8 (14 %)</td>
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<td>Q-10</td>
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<td>Isolation source</td>
<td>Rhizospheric soil of rice (cv Norin 18)</td>
<td>Root tissue of Jatropha curcas</td>
<td>Contaminated culture of Rhodopseudomonas palustris</td>
<td>Roots of rice plants</td>
<td>Rhizospheric soil of Plantago winteri</td>
<td>Saline hot spring</td>
<td>Marine sediment</td>
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<td>DNA G+C content (mol %)</td>
<td>74.6 (HPLC)</td>
<td>63.2 (HPLC)</td>
<td>65.1 (HPLC)</td>
<td>63\textsuperscript{†}</td>
<td>59.9</td>
<td>68.9\textsuperscript{†}</td>
<td>59.1\textsuperscript{†}</td>
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* Present study.
† Genome data.
‡ La-dependent.
§ Data obtained from under the respective strain entry at www.ccug.se.
and 18: Ln6c; 68.62%), the pattern of which could be totally distinguished from related type strains (Table S1).

MDH activity was assayed with a dye-linked MDH assay method, using phenazine ethosulfate (PES) as the electron acceptor [24]. SM30™ cells were grown in 100 ml liquid MM and NMS medium containing 0.5% (v/v) methanol (0.02% w/v yeast extract was added for better growth) with different metallic conditions (without CaCl$_2$ and LaCl$_3$, only CaCl$_2$, only LaCl$_3$, and both CaCl$_2$ and LaCl$_3$, all added at 30 µM). After cultivation at 28°C for 5 days, the cells were harvested by centrifugation (200 × g; 5 min, 4°C). The cells were disrupted with a MINI-BEADBEATER (BioSpec Products, Bartlesville, OK), and the samples were centrifuged at 15000 r.p.m., 4°C for 10 min. The supernatant was used as a cell-free extract, and subjected to an MDH activity assay. The MDH activity of cells grown in MM containing 0.5% methanol (+ Ca + La) was 0.09 ±0.012 U mg$^{-1}$ (mean and standard deviation, n=3) in the presence of ammonium ions, which was much higher than that in the absence of ammonium ions (0.02±0.006 U mg$^{-1}$, n=3). When grown in NMS methanol medium (+ Ca + La), the MDH activity was very low [less than 0.01 U mg$^{-1}$ (0.003±0.0004 U mg$^{-1}$, n=3)]. This may explain the better growth on MM medium than on NMS medium (Fig. S1a, b). We could not detect measureable MDH activity of the cells grown on either media with only Ca$^{2+}$ or La$^{3+}$ whereas the cells could grow in these conditions.

Whole-cell protein mass spectrometry analysis with matrix-assisted, laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) [25] showed that strain SM30™ could be distinguished from related species (Fig. S2).

Genomic DNA of strain SM30™ was extracted with a DNeasy Blood and Tissue kit (QIAGEN). The G+C content of the genome was measured using HPLC, according to Blood and Tissue kit (QIAGEN). The G+C content of the strain SM30™ was 47.6 mol%, which was higher than those of related type strains (Table S1).

The 16S rRNA gene fragment was amplified by PCR with the universal primer set (Eub8f, 5′-AGAGTTTGGTACCTTGGCAC-3′ and Eu1492r, 5′-GGCTACCTTGTTACGACTT-3′). The 16S rRNA gene sequence was screened for similarities with those from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned with close relatives by using CLUSTAL W. All phylogenetic trees were reconstructed using MEGA 5 software ([38]; Fig. S3–S6).

Phylogenetic analysis of the 16S rRNA gene sequence showed that strain SM30™ was separately branched and distinctly related to members of the genera Pleomorphomonas [39–41], with 93.5 to 94.2% similarities, H. diazotrophicus LMG 27460T [19], with 93.8% similarity, Methylobrevis pamukkalensis PK2T with 93.6% similarity and members of the genus Labrenzia with 92.1 to 92.7% similarity. In general, a minimum 16S rRNA gene sequence identity value of lower than 94.9±0.4% may lead to a novel genus circum-scription [42]. The gene sequence of strain SM30™ 16S rRNA formed a monophyletic group with a bootstrap value of >90% that of an uncultured bacterium, SNR59, from rice paddy soil (AB608674); these results were also supported with the maximum-likelihood and maximum-parsimony trees (Fig. 1).

The strain SM30™ mxaF gene was positioned distinctly distant from those of members of the genera Hyphomicrobiurn, Methylophila and Hansschelegelia (with 89.8, 90.8 and 89.9% similarity, respectively) (Fig. S3). The strain SM30™ xoxF gene may belong to the XoxF5 family [35], but was distinctly distanced from those from Methylosinus, Methylocella, Azooarcus and Hyphomicrobiurn (with 82.8, 82.7, 74.4 and 74.7% similarity, respectively) (Fig. S4). The SM30™ cpn60 gene had 93, 91.4, 90.6 and 92.4% similarity to those from P. koreensis NBRC 100803T, P. oryzae DSM 16300T, P. diazotrophica DSM 25022T and H. diazotrophicus LMG 27460T, respectively (Fig. S5). The similarity of the SM30™ nifH gene was also low compared to those from species of the genus Pleomorphomonas (90.2, 89.3 and 89.7% similarity to P. diazotrophica DSM 25022T, P. koreensis NBRC 100803T and P. oryzae DSM 16300T, respectively) and Hartmannibacter diazotrophicus LMG 27460T (89.1% similarity) (Fig. S6). The phylogenetic positions in the mxaF, xoxF, cpn60, and nifH sequence-based trees (Fig. S3–S6, respectively) also support the classification of strain SM30™ as both a novel genus and species.

Based on the results described above, strain SM30™ represents a novel genus within the Alphaproteobacteria; therefore, the name Oharaeibacter diazotrophicus gen. nov. sp. nov., is proposed.

**DESCRIPTION OF OHARAEBACTER GEN. NOV.**

Oharaeibacter [O.h.a.ra.e.i.ba’c’ter. N.L. masc. n. bacter (from Gr. n. bakterion) a rod-shaped bacterium; N.L. masc. n. Oharaeibacter referring to Megasaburo Ohara (1880–
Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the relationship of the novel genus within the Rhizobiales, class Alphaproteobacteria. The evolutionary distances were computed using the Kimura 2-parameter method. Circles indicate consensus bootstrap values from neighbour-joining, maximum-likelihood and parsimony analyses. Nodes supported at ≥90% in the majority of analyses are indicated by filled circles. Nodes supported at 70-90% in most analyses are indicated by open circles. Unsupported nodes (<70%) have no circle. There were a total of 1261 positions in the final dataset. Bar, 0.01 substitutions per site.
1943), a Japanese entrepreneur and philanthropist, in recognition of his promotion of welfare for farmers and agriculture, and foundation of the Ohara Institute for Agriculture (present Institute of Plant Science and Resources, Okayama University) in Japan.

Aerobic, Gram-stain-negative, non-spore-forming, motile rod-shaped bacterium forming smooth, translucent, white colonies on MM containing 0.5% (v/v) methanol and 30 µM La$^{3+}$. Catalase- and oxidase-positive. Major fatty acids are summed feature 8 (C18:1ω7c/C18:1ω6c) and cyclo C19:0ω8c. The major respiratory quinone component is Q-10. The DNA G+C content is 74.6 mol%. Phylogenetically, the genus is a member of the class Alphaproteobacteria, order Rhizobiales. The type species is Oharaeibacter diazotrophicus.

**DESCRIPTION OF OHARAEBACTER DIAZOTROPHICUS SP. NOV.**

*Oharaeibacter diazotrophicus* (dia. zo. tro’phi. cus. Gr. pref. di two, double; Fr. n. azote nitrogen; Gr. adj. trophikos nursing, tending, or feeding; N.L. masc. adj. diazotrophicus feeding on dinitrogen, diazotrophic).

Cells are 1.5–2.1 µm×1.1–1.4 µm and occur singly. Colonies are 1 to 2 mm in diameter, smooth, raised, translucent, viscous and white on MM containing 0.5% (v/v) methanol and 30 µM La$^{3+}$. Utilizes methanol and methylamine as sole carbon and energy sources. Lanthanum can promote growth on methanol. In the BIOLOG GN2 system, the following substrates are oxidized: Tween 40, β-methyl-d-glucosamine, maltose, gluconate and 30 µM La$^{3+}$. Catalase- and oxidase-positive. Major fatty acids are summed feature 8 (C18:1ω7c/C18:1ω6c) and cyclo C19:0ω8c. The major respiratory quinone is Q-10. The DNA G+C content is 74.6 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


