Exploration of lanthanide-dependent methylotrophic bacteria

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Contents

Abstract

Plants release large amount of methane and methanol into the atmosphere as by-products of their metabolism. Methane and methanol are important C1 compounds widespread in nature and they are essential intermediates in the global carbon cycle. These compounds can be utilized by methylotrophs, which are defined as a group of microorganisms capable of utilizing C1 compounds as the sole carbon and energy source. In Gram-negative methylotrophs, oxidation of methanol is catalyzed by methanol dehydrogenase (MDH). It has been found that two types of MDHs exist. One is well-studied Ca^{2+}-dependent MDH, encoded by *mxaF* gene. MxaF-MDH contains Ca^{2+} in its active site. The function of another MDH, XoxF, encoded in all methylotrophs, had been a mystery for a long time. Recently it was found to be a lanthanide (Ln^{3+})-dependent MDH, sharing 50% amino acid sequence identity with that of MxaF. The unexpected dependency of XoxF on Ln^{3+} has not only shed light on the unexplored bacterial methylotrophy but also expanded the importance of the new metal in biology. Although (meta)genomic analyses suggested the existence of large number of methylotrophs, the members so far isolated are very limited, and in general, most soil microorganisms are known to be un-culturable. Thus, Ln^{3+} can be a possible growth stimulation factor for such uncultured methylotrophs. In this study, in order to explore the variety and function of as-yet unisolated methylotrophs, I aimed to isolate and characterize Ln^{3+}-dependent methylotrophs.

A new aerobic facultative methylotrophic and diazotrophic strain SM30^T was isolated from rice rhizosphere by Dr. Sachiko Masuda with nitrate mineral salts (NMS) medium containing 20% methane and 30 μM lanthanum (La^{3+}). Its growth on methanol was not dependent on Ln^{3+} but enhanced by Ln^{3+}. The strain was most closely related to *Pleomorphomonas oryzae* DSM 16300^T, with low 16S rRNA gene similarity of 94.17%. Due to its low 16S rRNA gene identity, strain SM30^T was considered to belong to a novel genus. Thus, I characterized the phenotypes of the strain and published it as a new genus and new species, and the name *Oharaeibacter diazotrophicus* gen. nov., sp. nov. was proposed (type strain SM30^T = NBRC 111955^T = DSM 102969^T). Further, I determined the complete genome of the strain using PacBio sequencer. The genome consists of one chromosome and two plasmids, comprising a total of 5,004,097 bp, and the GC content was 71.6 mol%. A total of 4497 protein-coding sequences (CDSs), 67 tRNA, and 9 rRNA were encoded. Typical alpha-proteobacterial

methylotrophy genes were found: pyrroloquinoline quinone (PQQ)-dependent MDH (*mxaF* and *xoxF1-4*), methylotrophy regulatory proteins (*mxbDM* and *mxcQE*), PQQ synthesis, tetrahydrofolate (H_4F) pathway, tetrahydromethanopterin (H_4MPT) pathway, formate oxidation, serine cycle, and ethylmalonyl-CoA pathway. SDS-PAGE and subsequent LC-MS analysis, and qPCR analysis revealed that MxaF and XoxF1 were the dominant MDH in the absence or presence of La^{3+}, respectively. The growth of MDH gene-deletion mutants on alcohols and qPCR results indicated that *mxaF* and *xoxF1* were involved in the oxidation of methanol, ethanol, and propanol; *xoxF2* was involved in methanol oxidation in the presence of La^{3+}; *xoxF3* was associated with methanol and ethanol oxidation in the absence of La^{3+}. Four Ln^{3+} such as La^{3+}, cerium (Ce^{3+}), praseodymium (Pr^{3+}), and neodymium (Nd^{3+}) served as cofactors for XoxF1 by supporting *ΔmxaF* growth on methanol. Recently, *Mongoliimonas terrestris* from desert soil and *Chthonobacter albigriseus* from grass-field soil showing 96.3% and 96.28% 16S rRNA gene identity to that of strain SM30^T, respectively, have been published as new genus and new species after the publication of *O. diazotrophicus* SM30^T. The phylogenetic analysis based on 16S rRNA gene and multilocus sequence analysis (MLSA) combined with the digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) values indicated that strain SM30^T was totally different from the two new genus and species and confirmed the novel phylogenetic location of strain SM30^T. Additionally, strain SM30^T can fix nitrogen, which may offer nitrogen source for plants to promote the growth of plants.

On the other hand, I myself also tried to isolate novel Ln^{3+}-dependent methanotroph and methylotroph with NMS medium supplemented with 20% methane as the sole carbon source and 30 μM La^{3+} or 30 μM Ho^{3+} as an essential growth factor from various sources. Among ca. 300 isolates, two isolates belonging to *Methylomonas koyamae* could grow on methane, however, their growth on methane was not Ln^{3+}-dependent. The growth of strain La2-4^T on methanol, which was isolated from rice rhizosphere soil, was strictly Ln^{3+}-dependent. Its 16S rRNA gene sequence showed only 93.4% identity to that of *Methylophilus luteus* Mim^T, and the name *Novimethylophilus kurashikiensis* gen. nov. sp. nov. was proposed (type strain La2-4^T = NBRC 112378^T = KCTC 62100^T). Its draft genome (ca. 3.69 Mbp, G+C content 56.1 mol%) encodes 3579 putative CDSs and 84 tRNAs. The genome harbors five *xoxF*s but no *mxaFI*. XoxF4 was the major MDH in the cells grown on methanol and methylamine, evidenced by protein

identification and quantitative PCR analysis. Methylamine dehydrogenase gene was absent in the La2-4^{T} genome, while genes for the glutamate-mediated methylamine utilization pathway were detected. The genome also harbors those for the tetrahydromethanopterin and ribulose monophosphate pathways. The methylotrophy genes were that of beta-proteobacteria. Additionally, as known species, isolates of *Burkholderia ambifaria*, *Cupriavidus necator*, and *Dyadobacter endophyticus* exhibited Ln^{3+}-dependent growth on methanol.

In this research, new Ln^{3+}-dependent methylotrophs were obtained from rice rhizosphere by the addition of Ln^{3+} via enrichment cultivation. I also measured methanol emission from rice roots and Ln^{3+} concentration in the field soil of the institute. The data suggested that Ln^{3+}-dependent methylotrophy takes place in the agricultural environment. This study contributes to understanding the bacterial methylotrophy, in which Ln^{3+} plays an important role in MDH activity and regulation, and suggests that Ln^{3+} participate in life activities much more actively beyond our envision. The discovery of the two new bacteria enriches the variety of life in the world.

Abbreviations

ANI	average nucleotide identity
CDSs	protein-coding sequences
Ce^{3+}	cerium
CRISPR	clustered regularly interspaced short palindromic repeats
dDDH	digital DNA-DNA hybridization
GGDC	Genome-to-Genome Distance Calculator
GIs	genomic islands
H_4F	tetrahydrofolate
H_4MPT	tetrahydromethanopterin
Ho^{3+}	holmium
HSP	high-scoring segment pair
HTH	helix-turn-helix
L	lipid
La^{3+}	lanthanum
Ln^{3+}	lanthanide
MADH	methylamine dehydrogenase
MALDI-TOF/MS	matrix-assisted, laser-desorption/ionization time-of-flight mass spectrometry
MDH	methanol dehydrogenase
MFS	major facilitator superfamily
MLSA	multilocus sequence analysis
MM	mineral medium
MMO	methane monooxygenase
Nd^{3+}	neodymium
NMS	nitrate mineral salts
NMS+Ho	NMS supplemented with 30 μM Ho^{3+}
NMS+La	NMS supplemented with 30 μM La^{3+}
NMS+M	NMS with 20% (v/v) methane
NMS+MA	NMS with 0.1% methylamine

NMS+MALa	NMS with 0.1% methylamine and 30 µM La^{3+}
NMS+MeLa	NMS containing 0.5% methanol and 30 µM La^{3+}
NMS+MHo	NMS with 20% (v/v) methane and 30 µM Ho^{3+}
NMS+MLa	NMS with 20% (v/v) methane and 30 µM La^{3+}
PE	phosphatidylethanolamine
PEP	phosphoenol pyruvate
PES	phenazine ethosulfate
PG	phosphatidylglycerol
PL	phospholipids
pMMO	particulate methane monooxygenase
PNL	phosphoaminolipid
PQQ	pyrroloquinoline quinone
Pr^{3+}	praseodymium
REE	Rare Earth Elements
RND	resistance-nodulation-division
RuMP	ribulose monophosphate
sMMO	soluble methane monooxygenase
SQR	sulfide/quinone reductase
TCA	tricarboxylic acid

General introduction

Methane is one of the important greenhouse gases affecting the global climate and its emission into the atmosphere is estimated to account for 580 Tg per year, among which rice field contributes to 5-19% of the total amount (IPCC, 2007). Although anaerobic oxidation of methane has been reported in many anoxic sediments, aerobic oxidation accounts for more than 90% of total emitted methane from rice field (Holzapfel-Pschorn et al., 1986; Krüger and Frenzel, 2003). The paddy rice root and rhizosphere are partially oxic, allowing the growth of aerobic methanotrophs that can utilize methane and methanol as the sole source of carbon and energy (Hanson and Hanson, 1996). Numerous studies focusing on methanotrophs of rice paddy ecosystems have been reported (Liesack et al., 2000; Shrestha et al., 2010; Bridgham et al., 2013; Mills et al., 2013). Knowledge on methanotrophs will contribute to understand their actual roles in the ecosystems and efficient control of methane emission from agriculture.

Research on methanotrophs have begun since Kaserer (1905) and Söhngen (1906) discovered the existence of methane-oxidizing bacteria for the first time. Söhngen designated the first methanotroph as "*Bacillus methanicus*" (1906), however, this microbe was reisolated and renamed *Pseudomonas methanica* (Dworkin and Foster, 1956; Leadbetter and Foster, 1958) 50 years later. *Methylococcus capsulatus* strain Texas, the second methanotroph, was isolated in 1966 (Foster and Davis, 1966). The times for methanotroph research came in 1970 since Whittenbury et al. isolated and described more than 100 new aerobic methanotrophs from a variety of terrestrial and freshwater environments (Whittenbury et al., 1970). Based on physiological, morphological, ultrastructural and chemotaxonomic traits, methanotrophs were divided into two major groups, type I and type II methanotrophs (Whittenbury and Dalton, 1981). There are certain distinctive characteristics between type I and type II methanotrophs, such as vesicular discs of the arrangement of internal membranes for type I, paired membranes aligned to the cell periphery for type II; carbon fixation mechanism via the ribulose monophosphate pathway for type I, serine cycle for type II and the predominance of specific fatty acids C_{16} for type I, C_{18} for type II (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008). In addition, type X methanotrophs were further separated from type I methanotrophs due to the presence of ribulose-1,5-bisphosphate carboxylase, differences in nitrogen fixation capability or preference for higher growth temperatures (Green, 1992; Hanson and Hanson, 1996; Bowman, 2006). The

diversity of aerobic methanotrophs has been expanded since three different studies in 2007 and 2008 discovered methanotrophs within the phylum *Verrucomicrobia* (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008). These methanotrophs were further reported to represent distinct species of the genus *Methylacidiphilum* (Op den Camp et al., 2009). Recently, *Methylacidimicrobium*, a second genus within the novel methanotrophic family *Methylacidiphilaceae* was published (van Teeseling et al., 2014). To date, the physiology and biochemistry of aerobic methanotrophy have been characterized in pure cultures within Alphaproteobacteria (Type II), Gammaproteobacteria (Type I and X), and Verrucomicrobia (Chistoserdova and Lidstrom, 2013).

Methanotrophs can use methane as the sole carbon and energy source, and the initial oxidation of methane into methanol is catalyzed by methane monooxygenase (MMO). Two forms of MMOs are known: the cytoplasmic or soluble MMO (sMMO) and the membrane associated or particulate methane monooxygenase (pMMO) (Stanley et al., 1983; Nielsen et al., 1996). The sMMO, encoded by a six-gene operon *mmoXYBZDC*, consists of three components: (1) a 250-kDa hydroxylase with an $\alpha_2\beta_2\gamma_2$ structure (the α subunits, MmoX, harboring the binuclear iron active center in which the substrate oxygenation happens), (2) a 39-kDa NAD(P)H-dependent reductase (MmoC) with flavin adenine dinucleotide (FAD) and Fe_2S_2 prosthetic groups, and (3) a 16-kDa component (MmoB) known as protein B or coupling/gating protein that does not contain prosthetic groups or metal ions (Green and Dalton, 1985; Liu and Lippard, 1991). In contrast, the pMMO, encoded by the genes *pmoCAB*, harbors three polypeptides of approximately 49, 27 and 22 kDa (Semrau et al., 1995). Some methanotrophs, such as *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b, can produce both forms of MMO. While most known methanotrophs possess only pMMO, for example, *Methylomonas methanica*, *Methylomicrobium album* BG8, *Methylocystis parvus* OBBP, and the verrucomicrobial and NC10 methanotrophs, only a few methanotrophs within the *Beijerinckiaceae* family such as *Methylocella silvestris* and *Methyloferula stellata*, have sMMO but not pMMO (Dunfield et al., 2003; Vorobev et al., 2011). The *pmoA* gene encoding the β subunit of pMMO is the most frequently used hallmark for methanotrophs, as it is present in most aerobic methanotrophic bacteria (Knief, 2015). A cultivation-independent survey based on the *pmoA* gene revealed thousands of "unknown methanotrophic bacterial" sequences (Knief,

2015). This indicates that a large number of methanotrophs are uncultivated due to unknown factors that may be necessary for them to grow but absent in the laboratory experimental conditions.

Besides their important role in the global methane cycle, methanotrophs are of biotechnological interest for long time. They are capable of biodegradation of organic pollutants such as trichloroethylene owing to the oxidation catalysis by methane monooxgenase (MMO) (Hanson and Hanson, 1996; Smith and Dalton, 2004; Dalton, 2005; Jiang et al., 2010; Semrau et al., 2010; Strong et al., 2015). Additionally, their potentials converting methane to valuable chemicals such as organic acids, vitamins, pigments or lipids have been investigated (Strong et al., 2015). Further applications for biosynthesis processes, for example, epoxide production via the conversion of propene to epoxypropane through the co-metabolic activities of MMO, have been reported (Hanson and Hanson, 1996; Dalton, 2005).

Methanol is the first intermediate in the process of methane oxidation in methanotrophs, and its annual emission is about 150 Tg, among which 100 Tg is from plants (Galbally and Kirstine, 2002). The phyllosphere especially leaf surface is a well-studied habitat of methanol-consuming methylotrophs, such as the genera *Methylobacterium*, *Methylophilus*, *Methylibium* and *Hyphomicrobium* (Lopez-Velasco et al., 2011; Reisberg, et al., 2012, 2013). *Methylobacterium* species, representing the major genus among the leaf microbial community, have mutualistic relationship with plants; for example, they promote plants growth due to their ability to synthesize phytohormones (Ivanova et al., 2000; Koenig et al., 2002; Schauer and Kutschera, 2011). Therefore, methylotrophs including *Methylobacterium* species are expected as bio-fertilizer. In addition, methylotrophs are also important for industrial wastewater-treatment facilities because of the conversion of methanol into valuble products such as formaldehyde and esters (Weissermel and Arpe, 2008). Since these C1 compounds (methane and methanol) are cheap feedstocks for valuable microbial fermentation products, discovery of novel methylotrophs may contribute to more efficient synthesis of chemicals, or to engineering microbial processes.

Methylotrophic bacteria can oxidize methanol to formaldehyde by the catalysis of methanol dehydrogenase (MDH). In gram-negative methylotrophic bacteria, MDH possesses pyrroloquinoline quinone (PQQ) as a prosthetic group (Anthony and Zatman, 1964; Williams et al., 2005). Ca^{2+}-dependent MxaFI MDH, heterotetrameric ($\alpha_2\beta_2$) enzyme, consisting of two large

(MxaF) and two small (MxaI) subunits, has been well studied within different species (Xia et al., 1992; Ghosh et al., 1995; Nojiri et al., 2006; Choi et al., 2011). Additionally, Kalyuzhnaya et al. demonstrated a different MDH, named MDH2, in four *Burkholderiales* strains (2008a). MDH2 showed different properties from MxaFI and it was composed of a single subunit with less than 35% amino acid identity to MxaF protein. Furthermore, genomic data indicated the presence of XoxF proteins, the homolog protein of MxaF, sharing approximately 50% of its amino acid identity with MxaF (Harms et al., 1996; Chistoserdova and Lidstrom, 1997; Kane et al., 2007; Kalyuzhnaya et al., 2008a; Chistoserdova et al., 2009; Vuilleumier et al., 2009; Schmidt et al., 2010; Chistoserdova, 2011).

The first discovery of XoxF protein in methyloroph was in 1996 (Harms et al., 1996), but its real function had remained a mystery. Deletion of *xoxF* genes of methylotrophs that harbors *mxaF* or *mdh2* as well was shown to have no effect on their ability to oxidize methanol (Harms et al., 1996; Chistoserdova and Lidstrom, 1997; Kalyuzhnaya et al., 2008a). A *xoxF* mutant of *Rhodobacter sphaeroides* containing neither *mxaF* nor *mdh2*, was not able to utilize methanol as a sole carbon source, indicating that XoxF was possibly incorporated in methanol oxidation (Wilson et al., 2008). Compared with those of MxaFI proteins, the expression level of *xoxF* in both *M. extorquens* AM1 and *Methylobacillus flagellatus* grown on methanol or methylamine were low (Bosch et al., 2008; Hendrickson et al., 2010). Despite of the little expression of XoxF in *M. extorquens* AM1 under laboratory culture conditions, it was unexpectedly found that *xoxF* genes were highly expressed in *Methylobacterium* residing on natural plant surface, suggesting an important physiological role of XoxF during plant colonization (Delmotte et al., 2009). Based on proteomic analysis, XoxF proteins were found to be highly abundant in *Methylotenera mobilis* that does not harbor MxaFI proteins (Bosch et al., 2009). It was shown that mutations of *xoxF* in *M. extorquens* AM1 resulted in 30% decrease in methanol growth rate and the reduction of methanol uptake (Schmidt et al., 2010). In addition, the XoxF purified from *M. extorquens* AM1 showed high affinity toward methanol but lower methanol oxidation rate compared with that of MxaF, however, the overexpression of *xoxF* didn't allow the growth of *mxaF* mutant in *M. extorquens* AM1 (Schmidt et al., 2010). Later it was reported that if both *xoxF* homologs were absent, *M. extorquens* AM1 couldn't grow on methanol and didn't show any MDH activity, which were due to the loss of gene expression from the *mxa* promoter in spite of the strong

xoxF1 promoter activity, implying that XoxF is required for the expression of *mxaFI* genes (Skovran et al., 2011).

On the other hand, lanthanides (Ln^{3+}), a group of metals with atomic numbers 57 through 71, along with chemically similar scandium and yttrium, are regarded as 'Rare Earth Elements' (REE). REE are widely used for high-tech products, such as solar cells, mobile phones, and computers. Despite the deceptive name, Ln^{3+} are relatively rich in the Earth's crust and as common as copper or zinc (Taylor and McLennan 2003; Krishnamurthy and Gupta, 2004; Tyler, 2004). Due to their low solubility, they were considered to be biologically inert. In 2011, Hibi et al. found that the addition of lanthanum (La^{3+}) or cerium (Ce^{3+}) to the methanol medium remarkably increased MDH activity of *Methylobacterium* sp. MAFF211642, and the addition of La^{3+} to *Methylobacterium* spp. culture resulted in a several folds increase in MDH activity. They further purified MDH protein from *Methylobacterium radiotolerans* NBRC15690 grown in the presence of La^{3+}, and identified the protein as a product of *xoxF* (Hibi et al., 2011). At the same time, Fitriyanto et al. discovered that addition of Ce^{3+} could increase MDH activity of *Bradyrhizobium* sp. MAFF211645 and the purified MDH was comprised of two identical subunits of XoxF-type MDH (Fitriyanto et al., 2011). Pol et al. (2014) revealed that Ln^{3+} were the essential growth factors for a methanotroph, *Methylacidiphilum fumariolicum* SoIV and reported crystal structure of the purified XoxF-MDH that contains Ln^{3+} in its active site. Thus, XoxF is now recognized as the first Ln^{3+}-dependent enzyme involved in bacterial methylotrophy. In addition, the ability of ExaF, a PQQ-dependent ethanol dehydrogenase in *M. extorquens* AM1 that uses Ln^{3+} as a cofactor enlarged the importance of Ln^{3+} to multicarbon metabolism (Good et al., 2016). The finding of PedH, Ln^{3+}-dependent PQQ-alcohol dehydrogenase in a non-methylotrophic bacterium, *Pseudomonas putida* KT2440, expanded the range of Ln^{3+}-utilizing bacteria beyond the methylotrophs (Wehrmann et al., 2017).

A survey in coastal marine environments revealed the existence of *xoxF* in a high number of unknown bacterial species (Taubert et al., 2015). Comparative metagenomics also showed that uncultured methylotrophs in the ocean and freshwaters of North America (Ramachandran and Walsh, 2015) and uncultured lineages of two novel Type I methanotrophs from North Sea sediments (Vekeman et al., 2016) possessed only XoxF-MDH but not MxaFI-MDH. A quantity of culturable facultative methylotrophic bacteria from a cactus *Neobuxbaumia macrocephala*

showed positive hybridization with a *xoxF* probe, but not with a *mxaF* probe, while all these isolates showed methylotrophic growth in the presence of Ce^{3+} or Ca^{2+} (Bustillos-Cristales et al., 2017). Thus, bacteria that harbor only *xoxF* but not *mxaF* may only be isolated in the presence of Ln^{3+}.

Based on the information above, the studies on the variety of XoxF-containing bacteria and their roles in the ecosystems have just begun. Since all the methylotrophs so far isolated and identified have been obtained in Ln^{3+}-free laboratory conditions, Ln^{3+}-dependent bacteria, if any, might have been overlooked by microbiologists. What if Ln^{3+} is present in the screening for methylotrophs, novel methylotrophs can be obtained? Many studies have shown that light Ln^{3+}, such as La^{3+}, Ce^{3+}, Pr^{3+} and Nd^{3+} can support the activity of XoxF-MDH (Pol et al., 2014; Vu et al., 2016); is there any XoxF MDH dependent on heavier Ln^{3+}? The objectives of this study are to isolate new Ln^{3+}-dependent methanotrophs and methylotrophs using light and heavy Ln^{3+} (La^{3+} and holmium [Ho^{3+}]) as essential factors from plants, and to characterize the novel isolates.

Chapter 1 *Oharaeibacter diazotrophicus* gen. nov., sp. nov., a diazotrophic and facultatively methylotrophic bacterium

1.1 Introduction

Molecular ecological and metagenomic analyses have detected large amounts of yet-uncultured methylotrophs in various natural ecosystems (Radajewski et al., 2002; Kalyuzhnaya et al., 2008b). The unculturability might be due to the unavailability of Ln^{3+} under experimental laboratory conditions. Therefore, Dr. Sachiko Masuda tried to isolate novel methanotrophs and methylotrophs from rice rhizosphere in the Institute of Plant Science and Resources (IPSR), Okayama University, Okayama, Japan, with 20 % (v/v) methane as the sole carbon and energy source in the absence or presence of 10 mM KNO_3 or 30 μM La^{3+}, respectively. Among the 44 isolates obtained, a strain named as SM30 showed only 93.5% to 94.2% identity of 16S rRNA gene to the *Pleomorphomonas* species in the family *Methylocystaceae,* and was regarded as a novel genus and species at that time (Masuda et al., unpublished). Therefore, I characterized strain $SM30^T$ by polyphasic approach and the name *Oharaeibacter diazotrophicus* gen. nov., sp. nov. was proposed for it (Lv et al., 2017).

Strain $SM30^T$ could not grow on methane but showed enhanced growth on methanol in the presence of La^{3+}, suggesting the presence and importance of *xoxF* (Lv et al., 2017). In addition, the strain is a diazotroph, which is one of the common characteristics in the subgroup that includes the *Pleomorphomonas* species, none of which are methylotrophs. Interestingly, partial 16S rRNA sequences homologous to that of the *Pleomorphomonas* species have been reported to be enriched in rhizocompartments metagenomics (Edwards et al., 2015). Thus, the relevance of *O. diazotrophicus* to methylotrophy, diazotrophy, and symbiosis with plants has yet to be investigated.

Recently, two related new genera and species, *Mongoliimonas terrestris* MIMtkB18 from desert soil and *Chthonobacter albigriseus* ED7 from grass-field soil have been described, and they have 96.3% and 96.28% 16S rRNA gene identity to that of strain $SM30^T$, respectively (Kim et al., 2017; Xi et al., 2017). Similar to strain $SM30^T$, both strains can utilize methanol and methylamine. In order to differentiate strain $SM30^T$ from these new species, and to figure out its methylotrophy modules as well, the genomic information of strain $SM30^T$ is needed.

In this chapter, I describe on the characterization of *O. diazotrophicus* SM30^T by using the polyphasic approaches and focus on the methylotrophy and diazotrophy of strain SM30^T based on the complete genome information and on the response of MDHs to Ln^{3+}.

1.2 Materials and methods

1.2.1 Phenotypic characterization of strain SM30^T

Cell morphology and Gram-staining (Gerhardt et al., 1994) of SM30^T were evaluated by light microscopy using an Olympus BX43 microscope with ×1000 magnification from 72 h-grown colonies. The motility of SM30^T was tested by stabbing the cells with a needle into a semisolid (0.3% agar) NMS+La with 0.5% (v/v) methanol medium and observation of the cell spread. NaCl tolerance, pH, and temperature optimum determinations were conducted according to the methods of Smibert and Krieg (1994) in triplicates. Salt tolerance was checked in R2A liquid medium containing 0.1, 0.2, 0.5, 1, 2, 5, and 10% (w/v) NaCl at 28°C for one week. Optimum pH condition was 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), Na_2HPO_4 / NaH_2PO_4 (pH 6.0–8.0), and Na_2CO_3/$NaHCO_3$ (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% (w/v) KNO_3. Urease activity was checked by urea agar medium (1 g tryptone, 5 g NaCl, 1 g glucose, 1.2 g Na_2HPO_4, 0.8 g KH_2PO_4, 0.03 g neutral red, 15 g agar, 50 ml 40% [w/v] urea solution, 1 L sterile water). Nitrogen fixation was confirmed by modified NFB medium with methanol as the carbon source instead of D, L-malic acid (Eckert et al., 2001). 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 Inc., Japan) following the manufacturer's instructions and the OD_{595} was read using a microplate reader (Powerscan HT, DS Pharma) every day for 8 days.

Quinone and fatty acid analyses were carried out by TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). Five grams and 0.1 g of SM30^T cells (wet weight) collected from seven days' cultures of mineral medium (MM) (Alamgir et al., 2015) supplemented 0.5% (v/v) methanol were used for the major quinone and cellular fatty acid methyl esters analysis, respectively.

Ubiquinones were extracted from the cells (Bligh and Dyer, 1959) and were analyzed by HPLC (ACQUITY UPLC system) according to Tamaoka et al. (1983).

MDH activity was assayed with a dye-linked MDH assay method, using phenazine ethosulfate (PES) as the electron acceptor (Ghosh and Quayle, 1979). SM30^T cells were grown in liquid 100 ml MM and NMS medium containing 0.5% methanol (0.02% yeast extract was supplied for better growth) with different metal conditions (without Ca^{2+} and La^{3+}, only Ca^{2+}, only La^{3+}, and both Ca^{2+} and La^{3+}, all added at 30 μM). After cultivation at 28°C for 5 days, the cells were harvested by centrifugation (20,600 g × 5 min, 4°C). The cells were disrupted with MINI-BEADBEATER^{TM} (BioSpec 3110BX, Ieda Trading Corporation), and the samples were centrifuged at 15,000 rpm, 4°C for 10 min. The supernatant was used as cell-free extract, and subjected to MDH activity assay.

Cells of strain SM30^T, *Pleomorphomonas diazotrophica* DSM 25022^T, *Pleomorphomonas koreensis* NBRC 100803^T, *Pleomorphomonas oryzae* DSM 16300^T and *Hartmannibacter diazotrophicus* LMG 27460^T grown on R2A plate medium were subjected to whole-cell protein profile analysis by matrix-assisted, laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Tani et al., 2012).

1.2.2 Genotypic characterization of strain SM30^T

1.2.2.1 Genome sequencing, assembly, and annotation

O. diazotrophicus strain SM30^T was cultivated in 100 ml liquid R2A medium at 28°C for 3 days. The cells were then collected by centrifugation (20,600 × g, 5 min, 4°C). Genomic DNA was extracted with the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed using a PacBio sequencer (Macrogen). The reads (97,654 reads, mean subread length 8100, N50=11,405, and 791 million bases in total) were assembled with Hierarchical Genome Assembly Process (HGAP3; Chin et al., 2013). Prokka (Seemann, 2014) was applied to identify and annotate protein-coding sequences (CDSs).

1.2.2.2 General analysis of strain SM30^T genome

Genomic islands were identified with IslandViewer (Bertelli et al., 2017). CRISPRfinder (Grissa et al., 2007) was applied to identify Clustered regularly interspaced short palindromic

repeats (CRISPR) candidates. The digital DNA-DNA hybridization (dDDH) values of strain SM30^T and close reference strains were calculated on the Genome-to-Genome Distance Calculator (GGDC) web server version 2.1 (http://ggdc.dsmz.de/) (Auch et al., 2010). The genomes of the closest relatives and species recently described were selected based on the phylogenetic tree of the 16S rRNA gene (Lv et al., 2017). The dDDH value was calculated using GGDC's formula 2 (dDDH = identities / high-scoring segment pair [HSP] length), as recommended by GGDC. Average nucleotide identity (ANI) values based on entire genomic sequences of strain SM30^T and the reference strains were analyzed with the ANIb algorithm (Goris et al., 2007) via the JSpeciesWS web service (Richter and Rosselló-Móra, 2009).

1.2.3 Phylogenetic analysis of strain SM30^T

The 16S rRNA gene sequence was screened for chimeras through DECIPHER (Wright et al., 2012). 16S rRNA gene similarity of SM30^T was calculated in the EzTaxon-e web server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). Related sequences were aligned using MEGA5 software (Tamura et al., 2011). 16S rRNA based phylogenetic trees were constructed using neighbor joining (Saitou and Nei, 1987) (Fig. 1-3 and Fig. 1-4). The multilocus sequence analysis (MLSA) phylogenetic tree (Fig. 1-5) was based on concatenated amino acid sequences of RpoB (DNA-directed RNA polymerase subunit beta), GyrB (DNA gyrase subunit B), InfB (translation initiation factor IF-2), and AtpD (ATP synthase subunit beta) (Table 1-1) (Glaeser and Kämpfer, 2015) and constructed using the maximum likelihood method with the JTT algorithm (Jones et al., 1992) via MEGA5 software (Tamura et al., 2011). The amino acid sequences of MDH homologue proteins in strain SM30^T were compared with related sequences (Keltjens et al., 2014), aligned by ClustalW, and analyzed with MEGA5 (Tamura et al., 2011). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with the JTT algorithm (Jones et al., 1992) via MEGA5 software (Fig. 1-6).

1.2.4 SDS-PAGE and LC-MS analysis

Strain SM30^T was grown in 500 ml of liquid MM (Alamgir et al., 2015) with 0.5% methanol in the absence or presence of 30 μM La^{3+} at 28°C for 3 days. The cells were harvested by centrifugation (20,600 × g, 5 min, 4°C), and broken with MINI-BEADBEATER^{TM} (BioSpec 3110BX, Ieda Trading Corporation). The supernatant obtained by centrifugation (20,600 × g, 4°C for 5 min) was subjected to SDS-PAGE analysis (Laemmli, 1970). The major bands around

60 kDa under both growth conditions were excised, trypsin-digested, and sent to the Advanced Science Research Center at Okayama University for LC-MS analysis (HPLC-Chip/QTOF-MS, Agilent Technologies). The results were analyzed with Mascot software and strain SM30^T genome data.

1.2.5 The relative expression levels of MDH homologue genes by qPCR

Strain SM30^T was grown in 100 ml of 0.5% (v/v) methanol, 0.5% (v/v) ethanol and 0.5% (v/v) propanol-containing MM without or with 30 μM La^{3+} at 28°C for 3 days, respectively (in triplicate for both conditions). The cells were collected by centrifugation (20,600 × g × 5 min, 4°C) and used to extract total RNA with TRI reagent (Sigma). Reverse transcription was performed in 20 μl of reaction mixture containing 3.0 μg RNA as template, 25 pmol random hexamer, 4 μl 5 × buffer, 8 μl 2.5 mM dNTP, and 50 U ReverTra Ace (Toyobo Co., Ltd.) under these thermal steps: 30°C for 10 min, 42°C for 60 min, and 99°C for 5 min. The mixture without the reverse transcriptase was used as a negative control. Quantitative PCR was performed with the THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd.) and the CFX ConnectTM Real-Time System (Bio-Rad). The PCR reaction mixture (20 μl) consisted of 5 μl cDNA, 10 μl THUNDERBIRD SYBR qPCR Mix, and 6 pmol of each primer. Thermal conditions were 95°C for 1 min, and 45 cycles of 15 s each at 95°C and 30 s at 60°C. *gyrA* (OHA_1_00856) was selected as a reference gene (Rocha et al., 2015) to normalize the expression levels. The primer sets for *gyrA*, *mxaF*, and *xoxF1-4* were designed using an online software tool (Table 1-2; https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool). The PCR products amplified with the strain SM30^T genomic DNA were used as calibration standards. Bio-Rad CFX Manager 3.1 was used to analyze the results. Significant differences between samples with or without La^{3+} were analyzed using Two-way RM ANOVA and Tukey's multiple comparisons test.

1.2.6 Construction of gene deletion mutants

mxaF and *xoxF1-4* genes were subjected to deletion mutagenesis. Each kilobase of the upstream and downstream regions of *mxaF* and *xoxF1-4* was amplified with the primer sets listed in Table 1-3. The fragments were tandemly cloned into the Sma I site in pK18mobSacB (Schäfer et al., 1994) with an In-Fusion cloning kit (Takara Bio Co.) to produce pK18mob-mxaF, pK18mob-xoxF1, pK18mob-xoxF2, pK18mob-xoxF3, and pK18mob-xoxF4. The plasmids were introduced into strain SM30^T via conjugation using *E. coli* S17-1. The kanamycin-resistant,

single-crossover mutant was streaked on R2A medium containing 10% sucrose to obtain double-crossover mutants, which were confirmed by diagnostic PCR. Besides, pK18mob-xoxF1 was also introduced into the *ΔmxaF* strain to construct the *ΔmxaFxoxF1* strain. Finally, *ΔmxaF*, *ΔxoxF2*, *ΔxoxF3,* and *ΔmxaFxoxF1* were obtained. The *ΔxoxF1* and *ΔxoxF4* mutants could not be generated.

The wild type SM30^T and four mutants were grown in 200 µl of MM containing 0.5% methanol, ethanol, propanol, and or with or without 30 µM La^{3+} prepared in 96 well plates at 28°C. The wild-type, *ΔmxaF*, and *ΔmxaFxoxF1* of strain SM30^T were also grown in MM containing 0.5% methanol in the presence of 1 mM Ca^{2+} with varied concentrations of La^{3+} (0 to 50 µM), and with 30 µM of each of the rare earth elements except Pm^{3+}. The OD_{600} was read using a microplate reader (Powerscan HT, DS Pharma) every day for 8 days. Final OD_{600} values were converted into that of a 1-cm light path. All growth experiments were done in triplicates.

1.2.7 The effect of strain SM30^T on rice plant

The presence of *nif* gene suggested that strain SM30^T may fix nitrogen of the air. To check the effect of strain SM30^T inoculation on rice, growth of rice plants in the absence of nitrogen supplemented with strain SM30^T cells were measured. About 100 rice seeds were washed with tap water. Then seeds were treated in 70% ethanol for 3 min, and then treated with 40 ml of 3% sodium hypochlorite solution containing 0.1-0.5% Tween 20. Then the suspension was heated at 80°C for 30 min (once check per 15 min). Then the seeds were washed with 50 ml sterile MQ water 5 times in clean bench. Ten sterile rice seeds were line-placed onto the agar plates (20 ml of 0.8% plant agar [INA TC-6, 500 g, Funakoshi frontiers in life science]). The embryo of the seeds was faced to the air. Ten microliters of 10 µM sterile $MgCl_2$ solution was used as control groups, while 10 µl strain SM30^T cell suspension (OD_{600} 0.01 or 0.1) was regarded as experiment groups. The experiment was done in triplicates. The germination number of rice plant, the longest shoot length, root length and fresh weight were measured after 15 days cultivation. The significant differences among different samples were analyzed using one-way ANOVA and Bartlett's test.

1.2.8 Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers are as follows: SM30^T 16S rRNA (LC153750), *mxaF* (LC154793), *xoxF* (LC154794), *cpn60* (LC160005), and *nifH* (LC154795); *Hartmannibacter diazotrophicus* LMG27460^T *xoxF* (LC163943), and *cpn60* (LC171325); and *Pleomorphomonas diazotrophica* DSM25022^T *cpn60* (LC171326). The complete genome data of strain SM30^T has been submitted to DDBJ under accession numbers AP017626 to AP017628.

1.3 Results and discussion

1.3.1 Phenotypic characterization of strain SM30^T

Morphological and physiological characteristics of strain SM30^T were compared with the most related type strains of the genera *Pleomorphomonas*, *Hartmannibacter*, *Methylobrevis and Labrenzia* (Table 1-4). The detailed information of strain SM30^T is given in the species description. Both NMS and MM medium supplemented with 0.5% methanol or 0.1% methylamine in the presence and absence of La^{3+} were adopted for testing the utilization of methanol or methylamine as the sole carbon source. The growth of strain SM30^T and related type strains on different media is shown in Fig. 1-1. Although strain SM30^T could grow even in the absence of La^{3+}, the presence of La^{3+} promoted its growth in both media (Fig. 1-1 A and B). Only *H. diazotrophicus* LMG 27460^T exhibited La^{3+}-dependent growth on methanol in NMS medium, which is likely due to the existence of *xoxF* (I sequenced the PCR-amplified fragment, Table 1-4), however, its methylotrophy has been unknown (Suarez et al., 2014). *mxaF* and *xoxF* were not found in the genomes of *P. koreensis* NBRC 100803^T (NZ_AULH00000000) and *P. oryzae* DSM 16300^T (NZ_AUHB00000000). I attempted to amplify *mxaF* and *xoxF* from *P. diazotrophica* DSM 25022^T but no products were obtained (Table 1-4). These results indicated that these three *Pleomorphomonas* species are not methylotrophic, and that they are critically different to SM30^T. The growth of SM30^T in MM containing 0.1% methylamine was La^{3+}-independent whereas La^{3+} could promote its growth in NMS (Fig. 1-1 C and D). *H. diazotrophicus* LMG 27460^T was able to utilize methylamine in NMS medium only in the presence of La^{3+} (Fig. 1-1 D). No *Pleomorphomonas* strains could utilize methylamine as a carbon source in either media regardless of La^{3+} (Fig. 1-1 C and D). Methylamine could be converted to formaldehyde by methylamine dehydrogenase, and XoxF-MDH was reported to be capable of formaldehyde oxidation (Pol et al., 2014; Bogart et al., 2015), which might explain

the better growth of SM30^T and *H. diazotrophicus* LMG 27460^T on methylamine in the presence of La^{3+}.

The quinone components were ubiquinone-10 (98.7%) and ubiquinone-9 (1.9%), which was similar to those of the 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 18:1 ω7c (4.23%), cyclo 19:0 ω8c (12.94%), 18:0 3OH (1.93%), 20:1 ω7c (1.54%), summed feature 2 (comprising 14:0 3OH and 16:1 isoI) (2.03%), and summed feature 8 (containing 18:1 ω7c and 18:1 ω6c) (68.62%), the pattern of which could be totally distinguished from related type strains (Table 1-5).

The MDH activity of cells grown in MM containing 0.5% methanol (+ Ca + La) was 0.09 ± 0.012 U mg $^{-1}$ (average and standard deviation, n=3) in the presence of ammonium, which was much higher than that in the absence of ammonium (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. 1-1 A and B). I 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 MALDI-TOF/MS (Tani et al., 2012) showed that strain SM30^T could be distinguished from the related species (Fig. 1-2).

1.3.2 Genome analysis of strain SM30^T

1.3.2.1 General features

The complete genome sequence of *O. diazotrophicus* strain SM30^T was obtained with a PacBio sequencer. The genome contains one single circular chromosome of 4.8 Mbp with 71.6 mol% G+C content, and two plasmids pSM30-1 (141 kbp) and pSM30-2 (21 kbp), of 72.5% and 60.7% G+C content, respectively. The putative origin of replication was located upstream of the *dnaA* (OHA_1_03267, chromosomal replication initiator protein). The chromosome encodes 4360 CDSs, including 3315 CDSs (76.03%) with assigned biological functions (Table 1-6). There are a total of 66 tRNAs (one more in pSM30-1) and nine ribosomal RNA operons in the chromosome. The chromosome also contains genes encoding RNA polymerase and DNA

polymerase I, III, and IV. The genome contains four genes encoding sigma factors: one *rpoE* family, two *rpoH*, and one *rpoN*. A total of 116 genes encode transcriptional regulators, among which 68 belong to helix-turn-helix (HTH)-type transcriptional regulators.

The genome contains 13 genomic islands (GIs) in a total of 124 kbp comprising 2.48% of the genome; GIs 1-12 are located in the chromosome, and GI 13 is in pSM30-1 (Table 1-7). Among the 103 CDSs located in the genomic islands, 59 genes encode known proteins, and 44 genes encode hypothetical proteins. Urease-related proteins were encoded in GI 13. CRISPR elements widely exist in bacterial and archaeal genomes as defense mechanisms against foreign plasmids and phages (Barrangou et al., 2007). The chromosome contains two CRISPRs (Crispr_1 and Crispr_2) and three possible CRISPRs (Crispr_3, Crispr_4, and Crispr_5), with 27, 8, 1, 1, and 1 spacers, respectively.

1.3.2.2 Phylogenetic analysis of strain SM30^T

Phylogenetic analysis of the 16S rRNA gene sequence (Fig. 1-3) showed that strain SM30^T 16S rRNA gene sequence formed a monophyletic group with a bootstrap value of >90% that of an uncultured bacterium SNR59 from rice paddy soil (AB608674). However, strain SM30^T was separately branched and distantly related to members of the genera *Pleomorphomonas* (Xie and Yokota, 2005; Im et al., 2006; Madhaiyan et al., 2013), with 93.5% to 94.2% similarity, *H. diazotrophicus* LMG 27460^T (Suarez et al., 2014) with 93.8% similarity, *M. pamukkalensis* PK2^T with 93.6% similarity and the genus *Labrenzia* with 92.1% to 92.7% similarity. In general, the minimum 16S rRNA gene sequence identity value of lower than 94.9 ± 0.4% may lead to a new genus circumscription (Yarza et al., 2008). Therefore, strain SM30^T represents a novel genus within the *Alphaproteobacteria* and the name *Oharaeibacter diazotrophicus* gen. nov. sp. nov. is proposed (Lv et al., 2017).

Recently, two new genera, *Chthonobacter albigriseus* and *Mongoliimonas terrestris* were described (Kim et al., 2017; Xi et al., 2017). According to Fig. 1-4, strain SM30^T is most closely related to *Mongoliimonas terrestris* MIMtkB18^T with 96.3% similarity and *Chthonobacter albigriseus* ED7^T with 96.28% similarity. The dDDH (Auch et al., 2010) and ANI values between genomes of strain SM30^T and closely related species were lower than the thresholds for species delineation (70% for dDDH and 95%-96% for ANI, Richter and Rosselló-Móra, 2009)

(Table 1-8). MLSA phylogenetic tree based on concatenated amino acid sequences of RpoB, GyrB, InfB, and AtpD showed that strain SM30^T is distantly related to these strains (Fig. 1-5). These results support the novel phylogenetic location of strain SM30^T.

The amino acid sequences of the MDH homologues shared 36 to 61% identity with each other. XoxFs have been grouped into five different families based on homology (XoxF1-XoxF5 families) (Keltjens et al., 2014). The phylogenetic tree of the amino acid sequences showed that the strain contains MxaF, two members of the XoxF5 family (XoxF1 and XoxF2), and one of the XoxF1 family (XoxF3). XoxF4 is located in a monophyletic branch with the XoxF from *Derxia lacustris* HL-12 (Fig. 1-6).

1.3.3 Genetic characterization of methylotrophy

Strain SM30^T can utilize methanol and methylamine as the sole carbon source (Lv et al., 2017). All genes related to methylotrophy are listed in Table 1-9. Although the genome contains *mmoXY* encoding methane monooxygenase component A alpha and beta chain, the strain could not grow in liquid NMS medium containing methane as the sole carbon source, which indicates that SM30^T is not a methanotroph.

1.3.3.1 Methanol oxidation

Similar to that of *M. extorquens* AM1 (Chistoserdova et al., 2003), strain SM30^T owns a whole gene set of *mxa* cluster encoding classic calcium-dependent MDH, while it harbors four other *xox* clusters encoding the putative Ln^{3+}-dependent MDH homologues (Table 1-9). All of these clusters were located in the chromosome. The *mxa* operon includes *mxaFJGIRSACKLDE*, while *mxaH* and *mxaB* together with *liaS* encoding sensor histidine kinase, are encoded upstream of the *mxaFI* cluster in the opposite direction. *xoxF1* and *xoxF4* are associated with *xoxJ* and *xoxG*, and *xoxF3* is associated with *xoxG* and *mxcQE*, while *xoxF2* is an orphan (Fig. 1-7). The exact function of MxaJ/XoxJ is not clear; however, they were categorized as a member of the three families of extracellular solute-binging proteins, indicating their involvement in the binding of methanol or the release of the toxic reaction product formaldehyde (Wu et al., 2015). MxaG/XoxG with the typical CXXCH motif for heme c binding would function as an electron acceptor for methanol oxidation, cytochrome c_L (Wu et al., 2015). MxcQ and MxcE consist of a two-component system regulating the expression of the *mxa* genes, yet it is still unknown

whether this regulation is direct or indirect (Vu et al., 2016). In addition, *mxbDM* encoding for another two-component regulatory system for the expression of *mxa* genes, and *pqqABCDE* and *pqqFG* in different loci for PQQ synthesis proteins were also detected in the genome.

To determine the dominant MDH in strain SM30^T, the cell-free extracts of methanol-grown cells in the absence or presence of La^{3+} were subjected to SDS-PAGE analysis. Major bands of about 60 kDa were recognized in both conditions (Fig. 1-8). The LC-MS analysis showed that the band in the absence of La^{3+} contained MxaF-MDH as well as XoxF1-MDH (with a lower score than that for MxaF-MDH), and that in the presence of La^{3+} was identified as XoxF1-MDH (Table 1-10). This result agreed with a previous report that *mxaF* responded to the absence of La^{3+}, while *xoxF* was induced in the presence of La^{3+} (Hibi et al., 2011; Vu et al., 2016). The detection of XoxF1 in the absence of La^{3+} might imply that XoxF1 is required for the expression of *mxaF* in strain SM30^T, which was also reported in *M. extorquens* AM1 (Skovran et al., 2011).

The expression level of MDH homologue genes in strain SM30^T grown on methanol, ethanol and propanol was quantified by qPCR, respectively (Fig. 1-9). When grown on methanol, *mxaF* was highly expressed in the absence of La^{3+}, and instead, *xoxF1* was highly expressed in the presence of La^{3+}, which was in line with other methylotrophs containing both types of MDHs (Keltjens et al., 2014; Farhan UI Haque et al., 2015; Vu et al., 2016). These results were consistent with the results of the LC-MS analysis, and they were regarded as main MDHs with a different metal requirement. In addition, *mxaF* was also induced in the absence of La^{3+} when grown on ethanol, which indicated that *mxaF* was involved in ethanol oxidation without La^{3+}. The expression level of *mxaF* in propanol was relatively low, suggesting that *mxaF* may be responsible for propanol oxidation but not the sole gene for propanol oxidation. *xoxF1* was induced in both ethanol and propanol-grown cells independent of La^{3+}, revealing its importance for the oxidation of ethanol and propanol. Although the expression level of *xoxF2* induced by La^{3+} was a bit low in methanol-grown cells, which might show that *xoxF2* was also associated with methanol oxidation. Additionally, the expression of *xoxF2* in ethanol and propanol was low as well. Interestingly, *xoxF3* was highly induced in the absence of La^{3+} at a level comparable to that of *mxaF* in methanol-grown cells, though XoxF3 was not detected in the LC-MS analysis. XoxF3 has a molecular weight of 65 kDa and harbors the amino acid residues of Asp317,

Tyr318, and Asp319 (its putative signal peptide was not taken into account for amino acid positions), which are identical to those in *Methylacidiphilum fumariolicum* SoIV XoxF and the distinctive feature for Ln^{3+}-binding XoxF-type MDH (Keltjens et al., 2014; Pol et al., 2014). The substrate specificity, metal requirement, and its role in the methylotrophy of XoxF3 are currently unknown. Moreover, *xoxF3* was also highly expressed in cells grown on ethanol without La^{3+}. The expression of *xoxF4* was low in methanol, ethanol and propanol-grown cells with or without La^{3+} conditions, suggesting that it may not be so important for methanol, ethanol and propanol oxidation.

Deletion mutants for MDH homologue genes were successfully created for *mxaF*, *xoxF2*, and *xoxF3*, and a double gene mutant for *mxaF* and *xoxF1*. For unknown reasons, I could not create mutants for *xoxF1* and *xoxF4*. Generally, *xoxF1* mutants have been created for many methylotrophs of *Paracoccus denitrificans* (Harms et al., 1996), *M. extorquens* AM1 (Chistoserdova and Lidstrom, 1997), *Methyloversatilis universalis* FAM5 (Kalyuzhnaya et al., 2008a), *Methylibium petroleiphilum* PM1 (Kalyuzhnaya et al., 2008a), unclassified *Burkholderiales* strains RZ18-153 and strain FAM1 (Kalyuzhnaya et al., 2008a), and *Rhodobacter sphaeroides* (Wilson et al., 2008). There has been no report for the inability of *xoxF1* mutant construction so far, however, since *xoxF1* is known to be necessary for *mxaF* expression in *M. extorquens* AM1 (Skovran et al., 2011), *xoxF1* mutant can not be created if the mutant generation steps contain growth on methanol, which I did not use. I finally could not obtain any mutant after diagnosis of ~1000 possible double-crossover mutants from ca. ~20 single-crossover mutants. Thus, the inability was not due to a simple probability but to unknown biological reasons. At the moment, it is unknown whether *xoxF1* is also necessary for facultative growth (on R2A) or the gene deletion vector construction was not appropriate (though it did create the ***Δ****mxaF****Δ****xoxF1* double mutant).

The growth of wild type and the mutants on methanol, ethanol, and propanol in the absence or presence of La^{3+} is shown in Fig. 1-10. La^{3+} could increase the growth of the wild type on methanol, while it did not affect the growth on ethanol or propanol so much. The ***Δ****mxaF* mutant did not grow on methanol in the absence of La^{3+}, indicating the necessity of *mxaF* for the growth on methanol in the absence of La^{3+}. Compared with those of wild type strain $SM30^{T}$, the growth of ***Δ****mxaF* mutant on ethanol and propanol implied that *mxaF* was partly involved in the

oxidation of ethanol and propanol in the absence of La^{3+} as well. A mutant for *xoxF1* was not obtained, but by comparing the growth of *ΔmxaF* and *ΔmxaFxoxF1*, it could be concluded that *xoxF1* is necessary for the growth on methanol in the presence of La^{3+} and also involved in the oxidation of ethanol and propanol. The slower growth of *Δ xoxF2* on methanol in the presence of La^{3+} compared to the wild type suggested that *xoxF2* participates in methanol oxidation in the presence of La^{3+}, however, *xoxF2* had little effect on ethanol and propanol oxidation. In comparison with wild type, *ΔxoxF3* showed lower growth on methanol and ethanol in the absence of La^{3+}, indicating that *xoxF3* was responsible for methanol and ethanol oxidation in the absence of La^{3+}. There was one report about the purification of XoxF1 from "*Candidatus* Methylomirabilis oxyfera" in the absence of any Ln^{3+} (Wu et al., 2015). XoxF3 shared 70% amino acid identity with XoxF1 of "*Ca*. Methylomirabilis oxyfera", and they were classified as the XoxF1 family (Fig. 1-6). Therefore, XoxF3 may be Ca^{2+}-binding XoxF-MDH. All these mutants were not defective when grown on ethanol and propanol, which suggested that there are other enzymes, possibly including XoxF4, that participate in the oxidation of these substrates.

To determine the concentration of La^{3+} that allows the La^{3+}-dependent growth on methanol, wild type, *ΔmxaF*, and *ΔmxaFxoxF1* mutants were grown on methanol in the presence of 1 mM Ca^{2+} and varied La^{3+} concentrations from 0 to 50 μM (Fig. 1-11). Ten micromolar La^{3+} could effectively increase the growth of the wild type on methanol, compared with La^{3+} absence condition, while 10 nM La^{3+} supported the growth of *ΔmxaF* on methanol. Thirty micromolar La^{3+} resulted in maximal growth of wild type and *ΔmxaF*. Compared with that of a *ΔmxaF* strain of *M. extorquens* AM1 that shows the maximal growth with 1 μM La^{3+} (Vu et al., 2016), a much higher concentration of La^{3+} was necessary to achieve maximal growth of $SM30^T$ *ΔmxaF* under our experimental conditions. The *ΔmxaFxoxF1* mutant did not grow at all in these conditions, which showed that *xoxF1* was necessary for strain $SM30^T$ to utilize methanol.

To explore the metal requirement specificity, wild type, *ΔmxaF,* and *ΔmxaFxoxF1* mutants were grown on methanol in the presence of different rare earth elements (30 μM) (Fig. 1-12). La^{3+} and Nd^{3+} could enhance the growth of wild type, while the other rare earth elements such as Y^{3+}, Gd^{3+}, Tb^{3+}, Dy^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+} inhibited its growth, which was not reported for strain AM1 in previous reports (Vu et al., 2016), but the reason is unknown. Only La^{3+}, Ce^{3+}, Pr^{3+}, and Nd^{3+} allowed the growth of *ΔmxaF*. Sm^{3+} was not effective on a *ΔmxaF*

mutant of strain SM30^T, whereas a *mxaF xoxF1 xoxF2* triple mutant of *M. extorquens* AM1 grows on methanol in the presence of Sm^{3+} (Vu et al., 2016).

1.3.3.2 Methylamine utilization

SM30^T can utilize methylamine as the sole carbon and energy source (Lv et al., 2017). There are three different systems for methylamine utilization: methylamine oxidase in gram-positive methylotrophs, eukaryotes, and certain members of the *Enterobacteriaceae* family; methylamine dehydrogenase (MADH) in some gram-negative methylotrophs and facultative autotrophs; and N-methylglutamate-mediated methylamine utilization pathway in the remaining gram-negative methylotrophs (Chistoserdov et al., 1994). Different from *M. extorquens* AM1, which possesses an entire set of *mauFBEDACJGIMN* encoding methylamine dehydrogenase (Chistoserdov et al., 1994), SM30^T harbors incomplete *mau* gene set, such as *cycA12* encoding for cytochrome c-552, *mauD* and *mauE* encoding methylamine utilization proteins, and *mauAB* encoding methylamine dehydrogenase light and heavy chain. However, strain SM30^T contains all enzyme genes responsible for the N-methylglutamate pathway, which indicates that SM30^T may utilize methylamine via the N-methylglutamate pathway, similarly to *M. extorquens* DM4 (Gruffaz et al., 2014).

1.3.3.3 Assimilation and dissimilation of methanol

Strain SM30^T contains all of the necessary genes for H_4MPT-linked formaldehyde oxidation. 3-Hexulose-6-phosphate synthase as a key enzyme for the ribulose monophosphate pathway (Chistoserdova et al., 2007b) is missing in strain SM30^T, which indicates that SM30^T does not utilize this pathway for formaldehyde fixation. A gene for S-hydroxymethyl glutathione dehydrogenase was found, whereas a gene for S-formyl glutathione hydrolase was absent in the genome, suggesting that SM30^T may oxidize formaldehyde only via the H_4MPT pathway.

Three non-homologous formate dehydrogenases gene clusters (*fdh1AB, fdh2ABCD,* and *fdh3ABC*) were detected in the *M. extorquens* AM1 genome (Chistoserdova et al., 2003), while the *fdh2* group was found in the genome of SM30^T. FDH2 is a common formate dehydrogenase in methylotrophs (Vuilleumier et al., 2009). Besides, *fdh3B* was also found. Mutation of the fourth FDH enzyme system in the triple *fdh* mutant of *M. extorquens* AM1 showed methanol-

negative phenotype, indicating that formate oxidation is an essential step in the methylotrophic growth (Chistoserdova et al, 2007a).

The existence of an entire gene set for the serine cycle in the SM30^T genome, together with the methylotrophy genes described above, support the idea that SM30^T possesses the typical alpha-proteobacterial methylotrophic carbon assimilation pathway (Table 1-9). In *Methylobacterium* species, one carbon transfer pathway is associated with the tetrahydrofolate (H_4F) pathway. *M. extorquens* strains AM1 and DM4 contain *mtdA*, *fch,* and *ftfL* for the H_4F pathway, while SM30^T showed a different gene organization and contains *folD* encoding for bifunctional methylene-H_4F cyclohydrolase instead of *mtdA* and *fch* in *M. extorquens*. A similar gene structure also exists in other α-proteobacteria such as *Ruegeria pomeroyi* DSS-3 (NC_003911). In addition, all genes involved in the ethylmalonyl-CoA pathway for glyoxylate regeneration were found in the strain SM30^T genome.

Besides one-carbon compounds, SM30^T can also utilize multi-carbon substances such as glucose. All genes for the tricarboxylic acid (TCA) cycle were found in the SM30^T genome. The TCA cycle plays a classical role in the metabolism of multi-carbon compounds. Aside from sharing malate dehydrogenase with the serine cycle, the TCA cycle is also associated with glyoxylate regeneration as well. Metabolisms of both one- and multi-carbon compounds supported the facultative methylotrophic lifestyle of strain SM30^T.

1.3.4 Nitrogen fixation and the effect of strain SM30^T on rice plant

Strain SM30^T grows also on a nitrogen-free medium and possesses a *nif* gene cluster similar to those of its close relatives, the *Pleomorphomonas* species (Fig. 1-13). Different from *Pleomorphomonas* sp., *nifHDK* are separated from *nifENX* by other genes, and there is an extra *nifU* in the SM30^T *nif* gene cluster. The cluster in SM30^T was analogous to the *nif* gene cluster in *Paenibacillus riograndensis* SBR5^T (Fernandes Gde et al., 2014), while *hesA* is absent in SM30^T. Despite minor modification, SM30^T has developed its own gene set suitable for nitrogen fixation. It was reported that type II methanotrophs (α-proteobacteria) can fix atmospheric nitrogen (Hanson and Hanson, 1996). The metaproteomic analysis of root associated bacteria from rice roots disclosed that dinitrogenase reductase (NifH), the alpha (NifD) and beta subunit (NifK) of dinitrogenase were mainly derived from type II methanotrophs of the family *Methylocystaceae*

(Bao et al., 2014). As a new member of *Methylocystaceae* family, strain SM30^T harbors *nifHDK* clusters, which is in accordance with the previous report.

The analysis of catalyzed reporter deposition–fluorescence in situ hybridization (CARD-FISH) showed that type II methanotrophs were localized around the epidermal cells and vascular cylinders in the root tissues of rice plants, indicating that they may have endophytic and epiphytic lifestyles in rice roots (Bao et al., 2014). The effect of strain SM30^T on the growth of rice plants was shown in Fig. 1-14. Compared with those of control groups, strain SM30^T cells with OD_{600} 0.01 decreased the root length and root number of rice plants significantly; strain SM30^T cells with OD_{600} 0.1 had no effect on rice plants. Based on these result obtained *in vitro*, I could not conclude the importance of diazotrophy of SM30 for plant-growth promotion.

1.3.5 Urea metabolism

Strain SM30^T is positive for urea reduction, and there were two *ure* gene clusters in the SM30^T genome. One cluster is in the chromosome, containing structural genes (*ureA1*, *ureB1*, and *ureC1*) and accessory genes (*ureD1*, *ureE1*, *ureF1*, and *ureG1*), while the other was in pSM30-1. The difference between them was that *ureB* was absent in the plasmid; however, *ureA2* had the same function of *ureA1* and *ureB1*, encoding urease subunit beta and gamma at the same time. The *ure* genes structure in strain SM30^T was almost the same as that of *Corynebacterium glutamicurn* ATCC13869 (Puskás et al., 2000).

1.4 Conclusion

The growth of strain SM30^T on one-carbon compounds depends on specific metabolic pathways, for which the genes were confirmed in this study. Similar to those of *M. extorquens* AM1, the first step is the oxidation of methanol to formate via MDH and the H_4MPT pathway. Among the complicated MDH systems, MxaF and XoxF1 are the dominant MDHs for strain SM30^T methylotrophy in the absence and presence of La^{3+}, respectively, as shown by protein identification and qPCR results. Only light Ln^{3+} (La^{3+}, Ce^{3+}, Pr^{3+}, and Nd^{3+}) could support the growth of the *ΔmxaF* strain, which may be because weaker Lewis acids could not polarize C_5 of PQQ; or that heavier Ln^{3+} interrupt the exact orientation of methanol or PQQ binding in the active site; or that cells only recognize and transport the specific Ln^{3+} (Martinez-Gomez et al., 2016). Different from that of *xoxF1* and *xoxF2*, the expression of *xoxF3* was highly induced in

the absence of La^{3+}. The physiological role of *xoxF3* is unclear due to the limited information on different XoxF isozymes. Although the XoxF3 amino acid sequence conserves "Asp317, Tyr318, and Asp319," which is considered to be important for Ln^{3+} coordination, the induction of the gene in the absence of La^{3+} in this study may blur the boundary of metal requirement specificities of MxaF and XoxF-type MDHs. A similar result was also obtained with XoxF-type MDH purified from "*Candidatus* Methylomirabilis oxyfera"; the purified active MDH belongs to the XoxF-type but has no Ln^{3+} (Wu et al., 2015). Formate is the branch point for carbon dissimilation and assimilation. The dissimilation is due to the oxidation of formate to CO_2, while the assimilation requires the conversion of formate to methylene-H_4F (Peyraud et al., 2011). In the serine cycle, methylene-H_4F and glycine from glyoxylate are converted to C3 compounds, such as phosphoenol pyruvate (PEP), and CO_2 is added to PEP to be further converted to oxaloacetate, which links the serine and TCA cycles together. The continuous function of the serine cycle requires the ethylmalonyl-CoA pathway, which offers glyoxylate regeneration. These pathways function tightly with one another and indicate the facultative methylotrophy position of strain SM30^T. As facultative methylotrophic bacteria, strain SM30^T harbors an entire gene set for the TCA cycle, which may offer strain SM30^T the advantage of adapting to various environments.

The novel phylogenetic position of strain SM30^T within the family *Methylocystaceae* (Webb et al., 2014) was confirmed based on the genome data. The most characteristic difference is that strain SM30^T is both a methylotroph and diazotroph, which is supported by the presence of C1-metabolism genes and nitrogen fixation genes. According to Fig. 1-4, strain SM30^T is most closely related to two new genera described recently: *M. terrestris* MIMtkB18 and *C. albigriseus* ED7. Although the genome of *C. albigriseus* ED7 is unavailable, it is still distinguished from strain SM30^T because *C. albigriseus* ED7 did not contain *nifH* gene (Kim et al., 2017), while strain SM30^T possesses *nif* gene clusters encoding nitrogenase. *M. terrestris* MIMtkB18 is non-motile and oxidase-negative (Xi et al., 2017), while strain SM30^T is motile and oxidase-positive, indicating that they are different species. Compared with the close reference strains, strain SM30^T contains *mxaF*, whereas *Methylobrevis pamukkalensis* PK2, *Hartmannibacter diazotrophicus* E19, and *Pleomorphomonas* spp. do not harbor *mxaF*. Strain SM30^T contains many more *xoxF* copies among all strains, and strain SM30^T can fix nitrogen,

whereas *Methylobrevis pamukkalensis* PK2 cannot (Table 1-11). Strain SM30^T harbors a *mxaF* as well as four *xoxF*-type genes, which may be important for utilizing not only methanol but also other alcohols available in the natural environment.

Plants harbor diverse bacteria that may promote their growth directly or indirectly, through such activities as secreting growth-promoting substances, supplying fixed nitrogen, and solubilizing phosphorus (Rosenblueth and Martı´nez-Romero, 2006). The association of nitrogen-fixing bacteria with rice plants has long been investigated (Fujii et al., 1987; Barraquio et al., 1997; Elbeltagy et al., 2001). Many nitrogen-fixing bacteria have been isolated from rice plants, such as *Azospirillum doebereinerae*, *P. oryzae*, and *H. diazotrophicus* (Eckert et al., 2001; Xie and Yokota, 2005; Suarez et al., 2014). The strain SM30^T genome encodes nitrogenase, which may suggest an effective plant-growth promoting ability of the strain. The frequent detection of sequences derived from the related bacterial group (including *Pleomorphomonas*) from the rice rhizosphere also suggests their importance for root colonization in rice (Edwards et al., 2015). Urea, accounting for more than 50% of the N-fertilizer used worldwide today (Myrach et al., 2017), is fully degraded to ammonium in the soil by the urease of microbes. Urease encoded by two sets of *ure* gene clusters in strain SM30^T may release ammonium to benefit the rice plant, which identifies strain SM30^T as a candidate for improving rice plant growth.

Description of *Oharaeibacter* gen. nov.

Oharaeibacter (O.ha.ra.e.i.bac'ter. N.L. masc. n. bacter [from Gr. n. bakterion] a rod-shaped bacterium; N.L. masc. n. Oharaeibacter, referring to Magosaburō Ōhara [1880-1943], 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-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 are positive. Major fatty acids are summed feature 8 ($C_{18:1}$ ω7c/$C_{18:1}$ ω6c) and cyclo $C_{19:0}$ ω8c. The major respiratory quinone component is Q-10. The DNA G+C content is 71.6 mol%. Phylogenetically, the genus is a member of the class *Alphaproteobacteria*, order *Rhizobiales*. The type species is *Oharaeibacter diazotrophicus*.

Description of *Oharaeibacter diazotrophicus* sp. nov.

Oharaeibacter diazotrophicus (di.a.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).

Cell size is 1.5-2.1 μm × 1.1-1.4 μm and appears singly. Colonies are 1 to 2 mm, smooth, raised, translucent, viscous, white colonies on MM containing 0.5% (v/v) methanol and 30 μM La^{3+}. It is able to utilize methanol and methylamine as a sole carbon and energy source. Lanthanum can promote the growth on methanol. In the BIOLOG GN2 system, the following substrates are oxidized: Tween40, α-D-glucose, D-mannitol, methyl pyruvate, acetic acid, citric acid, D-glucosaminic acid, beta-OH-butyric acid, alpha-ketoglutaric acid, D, L-lactic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-histidine, L-threonine, urocanic acid, inosine, and putrescine. The following substrates are *weakly* oxidized: dextrin, glycogen, gentiobiose, beta-methyl-D-glucose, D-raffinose, turanose, mono-methyl succinate, cis-aconitic acid, formic acid, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D-serine, D, and L-carnitine. According to API 20NE tests, positive results are obtained for hydrolysis of gelatin, assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, gluconate, and malate; negative for reduction of NO_3^-, indole production, glucose fermentation, arginine dihydrolase, urease, hydrolysis of esculin, β-galactosidase, assimilation of capric acid, adipic acid, citrate, and phenylacetic acid. Grows well on R2A, R2A containing 0.1% methylamine, NMS and MM medium containing 0.5% (v/v) methanol and 30 μM La^{3+}. In addition, it can grow weakly on LB and nutrient broth medium. The optimal medium for strain $SM30^T$ is MM medium containing 0.5% (v/v) methanol and 30 μM La^{3+}. Grows at temperature range 5 to 40°C and a pH range 3 to 8, with optimal growth at 28°C and pH 7. Strain $SM30^T$ cannot tolerate higher than 2% (w/v) NaCl in R2A medium. The growth on the modified NFB medium and the PCR-amplification product of the *nifH* gene suggests that $SM30^T$ is diazotrophic. The Christensen urease test is positive, which is contradictory to the API 20NE test. Nitrate reduction to nitrite is negative. Major fatty acids are summed feature 8 ($C_{18:1}$ ω7c and $C_{18:1}$ ω6c) and cyclo $C_{19:0}$ ω8c. The major respiratory quinone is Q-10.

The type strain SM30^{T} (=NBRC 111955^{T} =DSM 102969^{T}) was isolated from rhizospheres of rice cultivar Norin 18 from an experimental field of the Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan.

Chapter 2 *Novimethylophilus kurashikiensis* gen. nov. sp. nov., a new lanthanide-dependent methylotrophic species of *Methylophilaceae*

2.1 Introduction

Dr. Sachiko Masuda tried to obtain several novel La^{3+}-dependent methanotrophs and methylotrophs from rice rhizosphere in IPSR with methane as the sole carbon and energy source, in the presence of nitrogen and La^{3+} as growth factors, which demonstrated that La^{3+} can be key factors for methylotrophic bacteria (Masuda et al., unpublished). In this chapter, I broadened the screening sources for methanotrophic bacteria, including plants not only rice. Light Ln^{3+}-related methylotrophy has been reported by several papers (Pol et al., 2014; Vu et al., 2016), however, reports on heavy Ln^{3+}-associated methylotrophy are still scarce. In this research, I chose both light (La^{3+}) and heavy (Ho^{3+}) Ln^{3+} as growth factors with methane as the only carbon and energy source to aim at discovering novel Ln^{3+}-dependent methanotrophs or methylotrophs.

In this chapter, I isolated and characterized *Novimethylophilus kurashikiensis* strain La2-4^T, a novel Ln^{3+}-dependent methylotroph, by polyphasic approach and investigated its Ln^{3+}-dependent methylotrophy based on the draft genome information. In addition, several known species, such as *Burkholderia ambifaria*, *Cupriavidus necator*, and *Dyadobacter endophyticus*, with unexpected methylotrophic ability only in the presence of Ln^{3+} are also discussed.

2.2 Material and methods

2.2.1 Isolation of Ln^{3+}-dependent methanotrophs and methylotrophs

Samples that included various plants and plant root soil (approximately 50 mg; Table 2-1) collected at IPSR, were put in a NMS+La or NMS+Ho prepared in 70 ml vials capped with rubber seals. Twenty percent (v/v, final concentration) methane was added to the gas phase with a syringe. After three rounds of enrichment cultivation (3 weeks total), the culture was spread onto NMS+La or NMS+Ho agar medium, and the plates were incubated at 28°C for 1 week under 20% methane in an acryl chamber. Colonies showing unique morphology were purified several times. The pure isolates were subjected to a growth test on 20% (v/v) methane or 0.5% (v/v) methanol in the absence or presence of 30 μM La^{3+} or Ho^{3+}, respectively. Methylotrophic bacteria were chosen for 16S rRNA gene sequence analysis. The 16S rRNA gene sequences were screened for chimeras through DECIPHER (Wright et al., 2012). The PCR amplification of *xoxF*

gene from Ln^{3+}-dependent methylotrophic bacteria was performed with *xoxF* genes primer sets (Taubert et al., 2015) and KOD FX Neo DNA polymerase (Toyobo Co., Ltd.) under the following conditions: an initial step at 95°C for 5 min, followed by 30 cycles of 30 seconds each at 95°C, 56°C, and 72°C, and a final extension for 5 min at 72°C.

2.2.2 Phenotypic characterization of strain La2-4^T

The purity of strain La2-4^T was confirmed through light microscopy (Olympus BX43 microscope, ×1000 magnification) from 72-h grown colonies. The motility of La2-4^T was observed using a semisolid (0.3% agar) NMS medium containing 0.5% (v/v) methanol and 30 μM La^{3+} (NMS+MeLa); strain La2-4^T was stabbed into the medium with an inoculation loop, and 1 week later the spread of the cells was checked. Catalase and oxidase activities were determined as described previously (Doronina et al., 2014). Optimum pH and temperature for growth, along with NaCl tolerance, were tested in triplicate as follows: salt tolerance was checked with a NMS+MeLa liquid medium containing 0.1, 0.2, 0.5, 1, 2, 5, and 10% (w/v) NaCl; the optimum pH condition was determined with a NMS+MeLa liquid medium adjusted to pH values of 3, 4, 5, 6, 7, 8, 9, and 10; optimum temperature was also checked in a NMS+MeLa liquid medium at temperatures of 5, 10, 15, 20, 25, 28, 35, and 40°C. Urease activity was checked by the method described by Christensen (Christensen, 1946). Nitrogen fixation was confirmed using modified NFB medium with 0.5% (v/v) methanol as the carbon source instead of D, L-malic acid (Eckert et al., 2001). The enzyme activity pattern was determined with API ZYM (bioMérieux). Utilization of different carbon sources was tested with API 20 NE (bioMérieux) and Biolog GN2 Microplates (CSC Inc., Japan) following the manufacturer's instructions, and the results were read after 7 days' incubation.

NMS methanol medium containing additional calcium chloride (100 μM) or lanthanum chloride (10 nM to 30 μM) or varied lanthanides (30 μM) was used for testing the effect of different La^{3+} concentrations and various Ln^{3+} on the growth of strain La2-4^T. NMS methanol medium without additional calcium or La was regarded as a negative control. NMS medium supplemented with 0.1% methylamine in the absence (NMS+MA) or presence of 30 μM La^{3+} (NMS+MALa) was also used to test the utilization of methylamine as the sole carbon source.

Methanol dehydrogenase was assayed with a dye-linked MDH assay method with 100 mM Tris-HCl (pH 9.0) buffer containing 15 mM NH_4Cl and PES as the electron acceptor (Ghosh

and Quayle, 1979). Strain La2-4^T was harvested by centrifugation (20,600 × g, 5 min, 4°C) after growing in 500 ml of NMS+MeLa and 500 ml liquid NMS+MA or NMS+MALa at 28°C for 3 days. Cells were washed with 25 mM MES buffter (pH 5.5) twice, then mixed with zirconia / silica beads (Biospec Product Inc.) and disrupted with the Mini-Beadbeater^{TM} (BioSpec 3110BX, Ieda Trading Corporation; 4600 rpm for 30 sec, three times). The samples were centrifuged at 20,600 g, 4°C for 5 min, and the supernatant was designated as cell-free extract. The methanol-grown La2-4^T cell-free extract in the presence of La^{3+} was used for SDS-PAGE analysis (Laemmli, 1970). The protein bands of 63 kDa and 40 kDa were excised from the gel, in-gel digested with trypsin, and then sent to the Advanced Science Research Center at Okayama University for LC-MS analysis (HPLC-Chip/QTOF-MS Agilent Technologies). The data were analyzed with Mascot software and La2-4^T draft genome data.

Fatty acid analysis was carried out by TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). A wet cell paste of strain La2-4^T (0.1 g) collected from 7-d cultures of NMS+MeLa was used for cellular fatty acid methyl esters analysis. Fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The respiratory quinones and polar lipids were analyzed by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany, using 1 g of strain La2-4^T cells (dry weight) collected from 10 L of 1-week cultures of NMS+MeLa medium.

Cells of strain La2-4^T (grown on NMS+MeLa), *Methylophilus methylotrophus* DSM 46235^T (NMS+MeLa), *Methylotenera mobilis* DSM 17540^T (R2A containing 0.1% methylamine), and *Methylotenera versatilis* JCM 17579^T (R2A containing 0.1% methylamine) were subjected to whole-cell protein profile analysis by MALDI-TOF/MS (Tani et al., 2012).

2.2.3 Genomic characterization of strain La2-4^T

The genomic DNA of strain La2-4^T was extracted with the DNeasy® Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. The genome sequencing was performed with a MiSeq sequencer. The reads were assembled with CLCbio-a (QIAGEN). The annotation of protein CDS was conducted via the online BlastKOALA tool (Kanehisa et al., 2016). The dDDH value between La2-4^T and close reference species was calculated on the GGDC web server version 2.1 (http://ggdc.dsmz.de/) (Auch et al., 2010). The genome sequences of *Methylophilus methylotrophus* DSM 46235^T (NZ_KB905141), *Methylotenera mobilis* JLW8^T

(CP001672), *Methylobacillus glycogenes* JCM 2850^T (NZ_BAMT01000001), and *Methylovorus glucosetrophus* SIP3-4 (NC_012969) were used in this analysis. The dDDH value was calculated using GGDC's formula 2 (dDDH = identities / HSP length), as recommended by GGDC. ANI values between La2-4^T and these close reference strains based on their whole genome sequences were analyzed with the ANIb algorithm (Goris et al., 2007) via the JSpeciesWS web service (Richter and Rosselló-Móra, 2009).

2.2.4 Quantification of *xoxF* expression

La2-4^T cells grown in 100 ml of NMS+MeLa, NMS+MA, and NMS+MALa for 2 days (triplicate in each set of conditions) were subjected to total RNA extraction with TRI reagent (Sigma). The reverse transcription was done in 20 µl of reaction mixture containing 3.0 µg RNA as template, 25 pmol random hexamer, 4 µl 5 × buffer, 8 µl 2.5 mM dNTP, and 50 U ReverTra Ace (Toyobo Co., Ltd.) under these thermal steps: 30°C for 10 min, 42°C for 60 min, and 99°C for 5 min. The mixture without the reverse transcriptase was used as a negative control. Quantitative PCR was performed with the THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd.) and the CFX Connect^{TM} Real-Time System (Bio-Rad). The PCR reaction mixture (20 µl) consisted of 5 µl cDNA, 10 µl THUNDERBIRD SYBR qPCR Mix, and 6 pmol of each primer. Thermal conditions were 95°C for 1 min, and 45 cycles of 15 s each at 95°C and 30 s at 60°C. The *gyrB* gene (NMK_1321) was selected as the reference gene (Rocha et al., 2015). The primers for *gyrB* gene and *xoxF1-5* were designed using an online software tool (Table 2-2; https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool). The PCR products amplified with La2-4^T genomic DNA were used as calibration standards. Bio-Rad CFX Manager 3.1 was used to analyze the results.

2.2.5 Phylogenetic analysis of La2-4^T

The phylogenetic analysis of 16S rRNA gene (Fig. 2-2), MLSA (Fig. 2-8; the accession number of amino acid sequences used for MLSA phylogenetic tree are listed in Table 2-3) and amino acid sequences of MDH homologue proteins (Fig. 2-9) were done as described previously. Additionally, the 16S rRNA gene phylogenetic tree was also constructed by maximum parsimony (Fig. 2-3) (Nei and Kumar, 2000) and PhyML (Fig. 2-4) (Jukes and Cantor, 1969) algorithms.

2.2.6 Nucleotide sequence accession numbers

The accession number of strain La2-4^T 16S rRNA gene is LC199478. The draft genome sequences of strain La2-4^T have been deposited in DDBJ, and their accession numbers were BDOQ01000001 to BDOQ01000032.

2.3 Results and discussion

2.3.1 Isolation of Ln^{3+}-dependent methanotrophs and methylotrophs

A total of 35 samples including various plants and plant root soil (Table 2-1) were used as sources of bacteria. After three rounds of enrichment culture in liquid NMS medium (Whittenbury et al., 1970) with 20% (v/v) methane (NMS+M liquid) as a sole carbon source and 30 μM La^{3+} or Ho^{3+} as supplements, the bacterial isolates were purified on a solid agar plate under 20% (v/v) methane in the presence of La^{3+} or Ho^{3+} (NMS+MLa / NMS+MHo agar). Finally, ca. 300 strains were isolated and then subjected to growth tests on 20% (v/v) methane or 0.5% (v/v) methanol in the absence or presence of La^{3+} or Ho^{3+}.

I found that two isolates (Ho311 and Ho312) were capable of growing on methane, and they were identified as *Methylomonas koyamae* (with 99.34% and 99.33% 16S rRNA gene sequence identity, respectively). Their growth on methane in the presence and absence of La^{3+} or Ho^{3+} was comparable; therefore, they are not Ln^{3+}-dependent methanotrophs (data not shown). The genus *Methylomonas* belongs to type I methanotroph in the *Gammaproteobacteria* group (Ogiso et al., 2012). The genomes of *M. koyamae* strains contain the *mxaFI* gene, which can support their growth on methanol without Ln^{3+} (Heylen et al., 2016). I was not able to isolate other methanotrophs. It is known that certain methanotrophs such as *Methylocella* prefer low ionic strength (Dedysh et al., 2000), and the ionic strength of NMS medium is relatively high. It has also been reported that a methanotroph *Methylobacter tundripaludum* 31/32 could not utilize its XoxF in the co-culture with a methylotroph *Methylotenera mobilis* JLW8 but could utilize its XoxF in pure cultures in the presence of Ln^{3+}; this was considered to be due to the sequestration of Ln^{3+} by the methylotroph (Krause et al., 2017). These findings might explain the limited numbers of methanotrophs isolated in our study.

On the other hand, 101 isolates could grow on NMS+Me in either the presence or absence of Ln^{3+}. These isolates contained 11 strains belonging to the *Methylobacterium* species

(*M. aminovorans, M. longum, M. oryzae, M. populi, M. pseudosasae,* and *M. thiocyanatum* with 98.52–100% 16S rRNA gene identities). They are members of facultative methylotrophs ubiquitously colonizing plant surfaces. They usually contain both *mxaF* and *xoxF*, and our isolates also showed growth on methanol irrespective of the presence of Ln^{3+} (data not shown).

I found five La^{3+}-dependent methylotrophs (Fig. 2-1). Strain La20-1 was identified as *Burkholderia ambifaria* (99.39% 16S rRNA gene identity with *B. ambifaria* AMMD^{T}). The information on its methylotrophy was unavailable (Coenye et al., 2001). Two *xoxF* genes (BAMB_RS29555 and BAMB_RS30720) were detected in the genome of another strain of the species (*B. ambifaria* RZ2MS16, LKPJ00000000; Batista et al., 2016); therefore, strain La20-1 may also contain *xoxF*.

Strain Ho1-7 was identified as *Cupriavidus necator* (100% 16S rRNA gene identity with *C. necator* N-1^{T}), for which its methylotrophy was unknown (Makkar and Casida, 1987). Wu et al. reported that *C. necator* N-1 possessed a NAD-dependent MDH to oxidize methanol, but it does not utilize methanol as a carbon source in the absence of La^{3+} (Wu et al., 2016). However, the genome of *C. necator* N-1 contains a putative *xoxF* gene (CNE_RS23020; CP002878; Poehlein, et al., 2011), which indicates that *C. necator* N-1 may be a La^{3+}-dependent methylotroph.

Strain Ho17-2 was identified as *Dyadobacter endophyticus* (99.76% 16S rRNA gene identity with *D. endophyticus* 65^{T}). There was no data on its methylotrophy in the first paper on the species (Gao et al., 2016). The genome of the species is unavailable, but those of close species contain *xoxF* genes (DFER_RS07945 in *D. fermentans* DSM 18053 and H144_RS0114235 in *D. beijingensis* DSM 21582; Lang et al., 2009).

These three isolates were known species, but their Ln^{3+}-dependent methylotrophy has not been described. I could not amplify *xoxF* genes from these isolates with *xoxF1-xoxF5* primer sets (Taubert et al., 2015). Although strains Ho1-7 and Ho17-2 were isolated from enrichment cultures containing Ho^{3+}, they could not grow on either methane or methanol in the presence of Ho^{3+}. I assume that they might grow on some other carbon sources provided by methylotrophs. In addition, I was able to isolate several La^{3+}-dependent methylotrophic isolates but not Ho^{3+}-dependent methylotrophs; they differ in terms of their ionic radius, which may hamper the

insertion of the metal into the XoxF active site, its transport into the cells, or the activation of the genes (Vu et al., 2016).

Strain La3113 was identified as *Methylotenera mobilis* with 98.32% 16S rRNA gene identity to that of *M. mobilis* JLW8^T, suggesting that the strain may belong to a novel species among the genus *Methylotenera*. *M. mobilis* JLW8^T was initially reported as an obligate methylamine utilizer (Kalyuzhnaya et al., 2006), but it was later found that the strain showed nitrate-dependent growth on methanol (Kalyuhznaya et al., 2009). *M. versatilis* 301, another member of the genus *Methylotenera*, showed weak growth on solid agar medium supplemented with methanol despite lacking the *mxaFI* gene (Kalyuzhnaya et al., 2012). Beck et al. later revealed that these strains could grow on methanol plates including La^{3+} due to *xoxF* genes in their genome (Beck et al., 2014).

The growth of strain La2-4^T on methanol was also La^{3+}-dependent. The 16S rRNA gene sequence of strain La2-4^T was most closely related (99.1% identity) to that of an uncultured methylotroph 10-3Ba28 (AY360532) from rice field soil (Lueders et al., 2004), and it formed a monophyletic group with 10-3Ba28 in phylogenetic trees (Fig. 2-2, Fig. 2-3 and Fig. 2-4). The phylogenetic trees showed that strain La2-4^T is distantly related to known methylotrophic bacteria of the family *Methylophilaceae*. Strain La2-4^T was most closely related to *Methylophilus luteus* Mim^T, with 93.4% of the 16S rRNA gene sequence identity. The sequence shares 92.58 to 93.43% identity with *Methylophilus* species, 91.14 to 92.59% identity with *Methylotenera* species, 92.01 to 93.31% identity with *Methylobacillus* species, and 92.68 to 93.02% identity with *Methylovorus* species. According to Yarza et al. (2008), a threshold 16S rRNA gene sequence identity value of lower than 94.9 ± 0.4% may lead to a new genus circumscription. Therefore, I characterized the strain La2-4^T and proposed its name as *Novimethylophilus kurashikiensis*.

2.3.2 Phenotypic characterization of *N. kurashikiensis* strain La2-4^T

Cells of the isolate La2-4^T were straight or slightly curved rods, 1.3–2.2 μm in length and 0.5–0.7 μm in width, occurring singly. Cells of the early exponential phase (3 days) were motile, and spore formation was not observed. Gram staining of cells was negative, and catalase and oxidase was positive. It grows at a temperature range of 15 to 40°C and a pH range of 6 to 9, with optimal growth at 28°C and pH 7 in liquid NMS+MeLa. Strain La2-4^T cannot tolerate

higher than 2% (w/v) NaCl in NMS+MeLa. The Christensen urease test is negative. The strain also cannot grow or fix nitrogen on a nitrogen-free medium under air. Differential characteristics between strain La2-4^T and related species of the genera *Methylophilus*, *Methylotenera*, *Methylobacillus*, and *Methylovorus* are listed in Table 2-4 (Urakami and Komagata, 1986; Jenkins et al., 1987; Doronina et al., 2005; Kalyuzhnaya et al., 2006; Gogleva et al., 2010). Detailed information on strain La2-4^T is given in the species description.

The major fatty acids were 16:00 and summed feature 3 (comprising $C_{16:1}$ ω7c/$C_{16:1}$ ω6c), the pattern of which could be clearly distinguished from related type strains (Table 2-5). The quinone components were ubiquinone-8 (60%) and an additional unidentified component (40%) that was different from the phylogenetically closest taxa. The polar lipids were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), six unknown phospholipids (PL), one unknown phosphoaminolipid (PNL), and one unknown lipid (L) (Fig. 2-5). The results of whole-cell MALDI-TOF/MS analysis revealed that strain La2-4^T has different spectra from these species (Fig. 2-6).

La2-4^T grew on 0.5% (v/v) methanol at higher concentrations of La^{3+} up to 30 µM, suggesting that La2-4^T prefers relatively high La^{3+} concentration (Fig. 2-7 A). In the case of an *mxaF* mutant of *M. extorquens* AM1, in which XoxF1 was the major MDH, 1 µM La^{3+} resulted in maximal growth and culture density (Vu et al., 2016). La2-4^T XoxF may have lower affinity for La^{3+}. Besides La^{3+}, other light Ln^{3+} such as Ce^{3+}, Nd^{3+}, and Pr^{3+} could also support the growth of La2-4^T on methanol (Fig. 2-7 B). All the other heavier Ln^{3+} did not support its growth on methanol (data not shown). Additionally, strain La2-4^T can also utilize methylamine, and the presence of La^{3+} slightly enhanced its growth on methylamine (Fig. 2-7 C). To date, only PQQ-dependent alcohol/methanol dehydrogenases are known to be Ln^{3+}-dependent enzymes, and there has been no report on Ln^{3+}-related enzymes involved in methylamine metabolism. It is possible that La2-4^T may contain an enzyme whose activity can be enhanced by Ln^{3+} engaged in the utilization of methylamine.

The MDH activity of La2-4^T grown on 0.5% (v/v) methanol in the presence of 30 µM La^{3+} was 0.039 ± 0.004 U mg^{-1} (average ± standard deviation, n=3). The cell-free extract from methylamine-grown cells with or without La^{3+} did not show any MDH activity.

2.3.3 Genome analysis of strain La2-4^T

The draft genome sequence of La2-4^T obtained with the MiSeq platform consists of 32 scaffolds, 3.69 Mb, with an average G+C content of 56.1 mol%. A total of 3579 putative CDS and 84 tRNAs were detected. The dDDH showed that it shares low relatedness with those of *Methylophilus methylotrophus* DSM 46235^T (16.3%; 2.86 Mb; NZ_KB905141), *Methylotenera mobilis* JLW8^T (18.7%; 2.68 Mb; CP001672), *Methylobacillus glycogenes* JCM 2850^T (17.5%; 3.25 Mb; NZ_BAMT01000001), and *Methylovorus glucosetrophus* SIP3-4 (19.3%; 3.08 Mb; NC_012969), which suggests that La2-4^T belongs to a new genus and species (dDDH values lower than 70% for different species; Auch et al., 2010). ANI values for the La2-4^T genome against these species were 67.5%, 66.8%, 69.0%, and 69.8%, respectively, which were lower than the threshold for species delineation (95%-96%; Richter and Rosselló-Móra, 2009), suggesting the novelty of La2-4^T. The MLSA phylogenetic tree (Fig. 2-8) based on concatenated amino acid sequences of RpoB (DNA-directed RNA polymerase subunit beta), GyrB (DNA gyrase subunit B), InfB (translation initiation factor IF-2), and AtpD (ATP synthase subunit beta) (Glaeser and Kämpfer, 2015) also showed that strain La2-4^T was classified as a different branch from those of species of the family *Methylophilaceae*.

2.3.4 Methylotrophy-related genes

All methylotrophy-related genes are listed in Table 2-7. The genome of La2-4^T did not contain *mxaFI* gene encoding calcium-dependent MDH, while it did harbor five *xoxF*-type genes, which explains its Ln^{3+}-dependent methanol utilization. Based on the phylogenetic tree of XoxF from closely related species, XoxF2, 3, and 4 (NMK_1556, NMK_2270 and NMK_2902) belong to XoxF4-family MDHs, while XoxF1 (NMK_0372) belongs to XoxF3-family MDHs. XoxF5 (NMK_3457) is very close to XoxF of *Methylosinus trichosportium* OB3b, and these two sequences form a monophyletic group distinct from the families (Fig. 2-9).

In the genome, *xoxF1* is clustered with the genes for cytochrome c (*xoxG1*) and cytochrome c oxidase (*coxABC*), which are also found in the XoxF cluster of *Sinorhizobium medicae* WSM419 (Keltjens et al., 2014) (Fig. 2-10). Genes for diguanylate cyclase (NMK_0359 and NMK_0362) are also found near *xoxF4*- and *xoxF5*-type clusters in some methylotrophic bacteria (Keltjens et al., 2014). The cluster of *xoxF2* contains genes encoding for ABC transporter and TonB-dependentreceptor. It was reported that TonB is highly conserved in all

organisms carrying *xoxF*, and it might participate in the acquirement/uptake of REEs from the environment (Keltjens et al., 2014). Molybdate transport system (*modABCE*) and cytochrome c biogenesis protein is encoded with *xoxF3*, yet its relevance for methylotrophy is currently unknown. *xoxF4* is clustered with genes for ABC transporter substrate-binding protein, cytochrome c (*xoxG4a* and *xoxG4b*), PhoH-like ATPase, periplasmic protein TonB, beta-lactamase (*gloB*), cytochrome b561 (*cybB*), and sulfur-oxidizing protein SoxY. Among them, *xoxF4*, *xoxG4a*, and *xoxG4b* will be important structural component proteins for XoxF4. *phoH* is related to *xoxF4* in *Methylotenera mobilis* JLW8 (Mmol_2042; CP001672) and *Methylovorus glucosetrophus* SIP3-4 (MSIP34_RS11780; NC_012969). Furthermore, the *gloB* gene is related to the *xoxF* cluster of *Methylobacterium aquaticum* 22A (VP06_RS30975; AP014704), which may indicate that these genes are involved in methylotrophy. *xoxF5* and *xoxG5* are clustered with formate dehydrogenase genes (*fdhE* and *fdoG2*, *fdoG3*, *fdoH2*, and *fdoI2*).

The relevance of these five *xoxF* genes to methylotrophy was evaluated with real-time PCR analysis. La2-4^T was grown on 0.5% (v/v) methanol with 30 μM La^{3+}, and on 0.1% (v/v) methylamine with or without 30 μM La^{3+}. The results showed that *xoxF4* (NMK_2902) was most highly and constantly expressed in these conditions, which suggests that *xoxF4* expression is constitutive in La2-4^T (Fig. 2-11). In addition, SDS-PAGE analysis was also performed for La2-4^T cell-free extract grown in these conditions (Fig. 2-12). The major protein bands of 63 kDa and 40 kDa under methanol with La^{3+} condition were subjected to LC-MS analysis. The former contained chaperonin GroEL (NMK_1763) and XoxF4 (NMK_2902), and the latter contained an uncharacterized protein (NMK_0924) and transaldolase (NMK_0161) (Table 2-8). These results indicated that XoxF4 is the dominant MDH in La2-4^T.

MDH requires PQQ as a cofactor (Anthony, 2004). PQQ synthesis genes (*pqqABCDE*) were found with extra *pqqD*2 and *pqqE2*, and *pqqFG* genes were also present in a different locus.

Methylamine is oxidized by MADH or methylamine oxidase, or is utilized in the N-methylglutamate pathway (Chistoserdova et al., 2009; Latypova et al., 2010). The La2-4^T genome does not contain genes for methylamine dehydrogenase or oxidase. Essential enzymes such as glutamine amidotransferase, glutamate synthase, sarcosine oxidase (*soxABCD*), and glutamine synthetase (*glnA*) involved in N-methylglutamate pathway are found in the La2-4^T genome.

The genes for H_4MPT-linked formaldehyde oxidation pathway were also present in La2-4^T: formaldehyde activating enzyme (*fae*), methylene-tetrahydromethanopterin dehydrogenase (*mtdB*), methenyltetrahydromethanopterin cyclohydrolase (*mch*), and formylmethanofuran dehydrogenase (*fwdABC*). Sulfide/quinone reductase (SQR) purified from *Hyphomicrobium zavarzinii* (Klein et al., 1994), *Methylosinus trichosporium* OB3b (Patel et al., 1980), and *Methylococcus capsulatus* (Zahn et al., 2001) was reported to be involved in formaldehyde oxidation. Two genes encoding for sulfide/quinone reductase were also found in the La2-4^T genome; however, it was unknown whether they were actually involved in formaldehyde oxidation.

Two gene clusters for formate dehydrogenase were found in La2-4^T: the *fdsD*, *fdhD*, and *fdoG1H1I1* gene cluster (NMK_2922-NMK_2926) and the *fdhE*, *fdoG2G3H2I2* gene cluster (NMK_3459-NMK_3463). The former was located downstream of the *xoxF4* cluster, while the latter was located upstream of the *xoxF5* cluster.

All genes involved in the ribulose monophosphate (RuMP) cycle were found in the La2-4^T genome: 3-hexulose-6-phosphate synthase (*hxlA*, three copies), 6-phospho-3-hexuloisomerase (*hxlB*), glucose-6-phosphate isomerase (*gpi*), glucose-6-phosphate 1-dehydrogenase (*g6pd*), and 6-phosphogluconate dehydrogenase (*pgd*).

Thus, I could locate all of the important genes for the growth of strain La2-4^T on C1-compounds including methanol and methylamine. At the time of writing, the family *Methylophilaceae* included five genera: *Candidatus* Methylopumilus, *Methylobacillus*, *Methylophilus*, *Methylotenera*, and *Methylovorus*. Strain La2-4^T, most closely related with *Methylophilus luteus* Mim^T, is identified here as a new member of the *Methylophilaceae* family. With several available genomes of *Methylophilaceae*, its genetic information on methylotrophy has been increasing. The comparison of metabolic modules of C1 metabolism between La2-4^T and related species is summarized in Table 2-9. MxaF-MDH is lacking in La2-4^T, *Methylotenera versatilis* 301^T, and *Methylotenera mobilis* JLW8^T, and the MADH gene cluster is absent in La2-4^T, *Methylotenera versatilis* 301^T, *Methylovorus glucosetrophus* SIP3-4, and *Methylovorus* sp. MP688. Additionally, the glutamate-mediated methylamine utilization pathway is incomplete in *Methylotenera mobilis* JLW8^T. All the bacteria possess XoxF-MDH for methanol oxidation, assimilate formaldehyde to biomass through the RuMP pathway, or dissimilate formaldehyde via

the H_4MPT pathway. The members in the family *Methylophilaceae* may utilize methanol or methylamine with different MDHs or a different methylamine utilization pathway, while they may use the H_4MPT pathway and RuMP pathway for the metabolism of formaldehyde.

2.3.5 Multi-carbon substrate metabolism, nitrogen metabolism, transport, and secretion systems

The La2-4^T genome lacks a gene for α-ketoglutarate dehydrogenase in the TCA cycle, suggesting its preference for a methylotrophic lifestyle (Lapidus et al., 2011; Beck et al., 2014). Different from that of *Methylophilus* sp. TWE2, the La2-4^T genome encodes the complete glycolysis process (Xia et al., 2015). Similar to that of *Methylotenera mobilis*, the La2-4^T genome contains the genes involved in the methylcitric acid cycle (Kalyuzhnaya et al., 2008b), which functions in the utilization of propionate derived from demethylation of dimethylsulfoniopropionate, a typical compound found in aquatic environments.

The genome encodes urea carboxylase and urease accessory protein (*UreFG*), nitrate reductase/nitrite oxidoreductase (*narGHI*), and nitrate reductase molybdenum cofactor assembly chaperone NarJ/NarW (*narJ*). In addition, genes for nitric oxide reductase (*norBCDEQ1Q2*) were also found. Additionally, genes for nitrite reductase (NADH), nitrogen fixation protein, nitrogen regulatory protein, and nitrous oxide reductase were all found. However, *nifH* gene encoding for nitrogenase iron protein was absent in the genome, and La2-4^T is not a diazotroph.

The La2-4^T genome contains 223 genes encoding transporter-related proteins. They include 29 ABC transporters, 13 biopolymer transport proteins, 17 MFS (major facilitator superfamily) transporters, 15 multidrug transporters, 22 RND (resistance-nodulation-division) transporters, and many transporters for zinc, potassium, molybdate, magnesium, and cobalt. Additionally, 11 genes encoding porins, including two for phosphate-selective porins, were found. The genes for type I, II, and III secretion systems were also found.

2.4 Conclusions

By adding Ln^{3+} to the culture medium, I obtained several Ln^{3+}-dependent methylotrophs, including *Burkholderia ambifaria* strain La20-1, *Cupriavidus necator* strain Ho1-7, and *Dyadobacter endophyticus* strain Ho17-2. Our study is the first to describe them as Ln^{3+}-dependent methylotrophs. In addition, I isolated a novel Ln^{3+}-dependent methylotroph, strain

La2-4^T. The remarkable characteristic of the strain La2-4^T genome from those of closely related species is the lack of the *mxaF* gene. Based on the genetic and phenotypic characterization supported by genome information, I propose establishing a novel genus within the family *Methylophilaceae*.

Description of *Novimethylophilus* gen. nov.

Novimethylophilus, L. adj. *novus*, new; N.L. n. *methylum* (from French *méthyle*, back-formation from French *méthylène*, coined from Gr. n. *methu*, wine and Gr. n. *hulê*, wood), the methyl radical; N.L. pref. *methylo-*, pertaining to the methyl radical; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*), friend, loving; N.L. masc. n. *Novimethylophilus*, a new methyl-radical-loving.

They are aerobic, gram-stain-negative, non-spore-forming, motile, rod-shaped bacteria. Catalase and oxidase are positive. They are able to grow on methylamine but not methane. They require light Ln^{3+} (La^{3+} – Nd^{3+}) for methanol-utilization. The major fatty acids are summed feature 3 ($C_{16:1}$ ω7c and $C_{16:1}$ ω6c) and $C_{16:0}$. The major phospholipid is phosphatidylethanolamine, and the major respiratory quinone component is Q-8. Phylogenetically, the genus is a member of the class *Betaproteobacteria*, order *Methylophilales*. The type species is *Novimethylophilus kurashikiensis*.

Description of *Novimethylophilus kurashikiensis* sp. nov.

Novimethylophilus kurashikiensis (kura.shi.ki.en'sis N.L. masc. adj. *kurashikiensis* pertaining to Kurashiki, the city in Okayama prefecture, Japan, where the type strain was isolated).

Cell size is 0.5–0.7 μm × 1.3–2.2 μm and appears singly. Colonies are around 1 mm, smooth, raised, and translucent on NMS+MeLa agar plates (light orange in NMS+MeLa liquid medium). Its utilization of methanol occurs only in the presence of light Ln^{3+}. It also grows on methylamine as a sole carbon and energy source. According to API ZYM tests, positive results are obtained for esterase lipase (C8), esterase (C4), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase, and α-glucosidase; negative results are obtained for alkaline phosphatase, lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin,

α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. In the BIOLOG GN2 system, the following substrates are weakly oxidized: alpha-D-glucose, methyl pyruvate, p-OH-phenylacetic acid, alpha-ketobutyric acid, sebacic acid, alaninamide, L-leucine, L-pyroglutamic acid, D, L-carnitine, gamma-amino butyric acid, and glycerol. Based on API 20NE tests, positive results are obtained for nitrate reduction, hydrolysis of gelatin, and assimilation of glucose, N-acetyl-glucosamine, maltose, gluconate, and malate; negative results are obtained for indole production, glucose fermentation, arginine dihydrolase, urease, hydrolysis of esculin, β-galactosidase, and assimilation of arabinose, mannose, mannitol, capric acid, adipic acid, citrate, and phenylacetic acid. It grows well on NMS+MeLa agar, NMS+MA agar, and R2A supplemented with 0.1% methylamine. There is no growth on LB and nutrient broth medium. It grows at a temperature range of 15 to 40°C and a pH range of 6 to 9, with optimal growth at 28°C and pH 7. Strain La2-4^T cannot tolerate higher than 2% (w/v) NaCl in NMS+MeLa. The Christensen urease test is negative. The strain cannot grow or fix nitrogen on a nitrogen-free medium under air. The nitrogenase gene is absent. Major fatty acids are summed feature 3 ($C_{16:1}$ ω7c and $C_{16:1}$ ω6c) and $C_{16:0}$. The major phospholipid was phosphatidylethanolamine, and the quinone system predominantly comprises Q-8. The DNA G+C content is 56.1 mol%.

The type strain La2-4^T was isolated from the rhizosphere soil of purple rice in the experimental field of IPSR, Okayama University, Kurashiki, Japan. Strain La2-4^T was deposited as NBRC 112378^T and KCTC 62100^T.

Appendix

1. Methanol exudation by rice plant roots

Plants can emit methanol into the atmosphere due to their structure and metabolic properties, the amount of methanol produced by the leaf tissue has been quantified (MacDonald and Fall, 1993; Nemecek-Marshall et al., 1995; Kirstine et al., 1998; Fukui and Doskey, 1998). As an important habitat for methylotrophs, rice root also can offer methanol to methylotrophs, however, the concentration of methanol from rice root has not been investigated, although other many metabolites have been reported to be exudated from rice roots (Tawaraya et al., 2009). For quantification of methanol, gas chromatography or proton-transfer reaction mass spectrometry (PTR-MS) are the most suited means, however, PTR-MS is for gaseous compounds and not readily available. Methanol is too small molecule to detect with advanced mass spectrometry devices (like liquid chromatography-MS, GC-MS, and capillary electrophoresis MS). Thus, GC analysis is the most practical method that I can use. In addition, it was likely that root methanol emission in soil can not be readily quantified. So, I took advantage of hydroponic culture system for rice, and quantified methanol in the culture medium.

Twenty sterile rice seeds (prepared as described above) were grown on 20 ml agar plates (0.8%) prepared in petri dishes. One week later, rice plants with similar size were chosen to be transferred to 50 ml sterile plastic tube containing ca. 35 ml glass beads (diameter, 6 mm) supplemented with 18 ml sterile one-half strength of Kimura B nutrient solution (Table A-1; Ma et al., 2001). The same setup without rice plant was regarded as control group. All samples were put into plant cultivation chamber (CLE-303, TOMY) at 26°C, with light/dark cycle of 14/10 hours, 10 klux fluorescent light (135 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}). Triplicates were prepared for both control and experiment groups. The experimental setup was shown in Fig. A-1. A hundred-microliter culture medium was sampled from each sample at appropriate timing of 0 to 5 d and kept frozen in a 200-μl tube. One microliter sample was injected to a gas chromatograph (GC-2014, SHIMADZU) equipped with a InterCap WAX column (diameter, 0.53 mm; length, 30 m; GL Sciences Inc.) and a flame ionization detector, with the following condition: injection temperature 230°C, detector temperature 250°C, column temperature 110-152°C (initial temperature 110°C, increasing temperature at the rate of 6°C/minute for 7 min, holding 152°C

for 3 min), flow rate of the carrier gas (N_2) 10 ml/min. The following concentrations of methanol were used as standard solution for methanol concentration analysis and calculation: 100 μM, 200 μM, 500 μM, 1 mM, 2 mM, and 5 mM. Methanol was detected at retention time 0.8-0.9 min, and the peak area was linear against the methanol concentration within the used range.

The size of the plants used in the experiment was 0.07 ± 0.01 g (fresh weight), 11.28 ± 1.05 cm (shoot length), and 8.58 ± 1.71 cm (root length), in average, at the end of the experiment (5 d). As shown in Fig. A-2, rice plants can accumulate 3.5 mM (~ 0.01%) methanol in the medium in 8 h, then the amount of methanol gradually decreased. This decrease may be due to the consumption by microorganisms inside rice root (though the plants had been sterilized) or the evaporation of methanol into outside because of the open experimental setup. Even though, such amount of methanol could be available for the microorganisms surrounded in the rhizosphere. Additionally, rice root can also produce other carbon sources such as glucose and acetic acid, supporting the growth of facultative methylotrophs (Aulakh et al., 2001; Kerdchoechuen, 2005).

2. Soluble Ln^{3+} concentration measurement in rice field (IPSR)

Even though Ln^{3+} belong to the REE, they are relatively rich in the Earth's crust; their content is similar to that of copper and zinc (La, 39 ppm; Cu, 60 ppm; Zn, 70 ppm) (Krishnamurthy and Gupta, 2004). REE ions are reported to form complexes with soil minerals that display a low solubility and only at a less extent to constitute a water-soluble fraction, which represents the potentially bioactive fraction (Pang et al., 2001). Therefore, the quantification of soluble Ln^{3+} in soil samples was necessary. Here I report Ln^{3+} content of the rice field soil in the instiutute. The concentration of other common 23 metal ions was also analyzed to compare with those of Ln^{3+}.

Soil samples (rice field in IPSR) were air-dried, sieved through 2 mm sifter, homogenized and stored in plastic bags prior to laboratory analysis (Parraga-Aguado et al., 2014). Exact 1.0 g soil samples measured by an analytical balance (CP224S, Sartorius stedim biotech) were dried in a drying oven (DX600, Yamato Scientific Co., Ltd.) at 80°C for 24 h, and soil samples were weighed to measure water content of the samples. For Ln^{3+} and common 23 metal concentration measurement, about 1 g soil sample was mixed with 5, 25, 125, and 625-ml sterile MQ water, respectively, and the mixed samples were stirred for 2 h. After shaking, the soil

suspension samples were centrifuged (MX-305, TOMY) at 9100 × g for 10 min at room temperature. The filtered supernatant sample with 0.45 μm fiter (Minisart, Sartorius stedim biotech) was used for further analysis. Soil pH was determined by a edge^{pH} meter (HI 2002-01, HANNA) with 2 ml filtered supernatant sample. One-mililiter filtered supernatant was combined with 1-ml 60% HNO_3 solution and 10 ml sterile MQ water, and the mixture was used as water-extractable metal samples and was subjected to analyze Ln^{3+} concentration by ICP-MS (Agilent7500cx, Agilent) (Ernst, 1996). Triplicate samples were measured for all analyses.

The soil samples contained 1.57% combined water and the soil supernatant had pH 6.45. When 1 g soil was mixed with less volume of sterile MQ water, the soluble concentration of Ln^{3+} was higher. In short, the concentration of soluble Ln^{3+} in soil was at the similar level with that of Co, Mo, Cd, Sb and Pb (Table A-2). Such nanomolar soluble Ln^{3+} may be available for microorganisms, for example, 25 nM La^{3+} could support the growth of strain La2-4 on methanol and 10 nM La^{3+} was able to activate the XoxF1 of strain SM30, indicating that Ln^{3+}-dependent methylotrophy could take part in methanol or methane metabolism in the rice rhizosphere.

Summary

Ln^{3+} have been shown to be essential cofactors for the activity and expression of XoxF type MDH. Although XoxF proteins are widely encoded in various microbes, the robust activity of XoxF can be evident only in the presence of Ln^{3+}. Therefore, methylotrophs that have never been isolated in Ln^{3+}-free laboratory conditions may exist, and the discovery of such microorganisms would contribute to both the understanding the importance of Ln^{3+} in (micro)organisms and the activity of as-yet un-isolated microorganisms.

In chapter 1, a novel facultative methylotroph and diazotroph, strain $SM30^T$ isolated from rice rhizosphere was characterized. Strain $SM30^T$ did not grow on methane but grew on methanol. In addition, La^{3+} enhanced its growth on methanol. Due to its low 16S rRNA gene identity to known species *Pleomorphomonas oryzae* DSM 16300^T (94.17%) and its distinct phenotypic characteristics, the name *Oharaeibacter diazotrophicus* gen. nov., sp. nov. was proposed. The phylogenetic ananlysis based on 16S rRNA gene and MLSA combined with the dDDH and ANI values confimed the novel phylogenetic location of strain $SM30^T$. The complete genomic information revealed that strain $SM30^T$ harbors one chromosome and two plasmids with a total 71.6 mol% GC content. Typical alpha-proteobacterial methylotrophy genes were encoded in strain $SM30^T$ genome: PQQ dependent MDH (*mxaF* and *xoxF1-4*), methylotrophy regulatory proteins (*mxbDM* and *mxcQE*), PQQ synthesis, H_4F pathway, H_4MPT pathway, formate oxidation, serine cycle, and ethylmalonyl-CoA pathway. Among five MDH genes (*mxaF* and *xoxF1-4*), *mxaF* and *xoxF1* were involved in the oxidation of methanol, ethanol, and propanol, and the expression of *mxaF* and *xoxF1* was switched by La^{3+}; *xoxF2* was partly related with methanol oxidation in the presence of La^{3+}; *xoxF3* was engaged in the growth on methanol and ethanol in the absence of La^{3+}. In addition, four kinds of light Ln^{3+} such as La^{3+}, Ce^{3+}, Pr^{3+}, and Nd^{3+} served as cofactors for XoxF1. Two related new genera and species, *Mongoliimonas terrestris* from desert soil and *Chthonobacter albigriseus* from grass-field soil showing 96.3% and 96.28% 16S rRNA gene identity to that of strain $SM30^T$, respectively, have been published after the publication of strain $SM30^T$.

In chapter 2, I also explored Ln^{3+}-dependent methylotrophs using NMS medium with methane as the sole carbon source supplemented with La^{3+} and Ho^{3+} as growth cofactors. I obtained two methanotrophs of *Methylomonas koyamae*, but their growth was not dependent on

Ln^{3+}. Among the isolates, the growth of strain La2-4^T on methanol, isolated from rice rhizosphere soil, was strictly Ln^{3+} dependent. Due to the low 16S rRNA gene identity to known species *Methylophilus luteus* Mim^T (93.4%), strain La2-4^T was named as *Novimethylophilus kurashikiensis* gen. nov. sp. nov. Its draft genome encoded 3579 putative CDSs and 84 tRNAs with total 56.1 mol% G+C content. Beta-proteobacterial methylotrophy genes were found in its genome: PQQ dependent MDH (*xoxF1-5*), PQQ synthesis, H_4MPT pathway, formate oxidation, and RuMP cycle. The genome harbors five *xoxF*s but no *mxaFI*. Among five XoxF proteins, XoxF4 was the major MDH in the cells grown on methanol and methylamine. Genes for the glutamate-mediated methylamine utilization pathway were detected. Additionally, the Ln^{3+}-dependent methylotroph of known species, such as *Burkholderia ambifaria*, *Cupriavidus necator*, and *Dyadobacter endophyticus* were disclosed.

This research succeeded in isolating novel methylotrophs with light Ln^{3+} (La^{3+}) as growth cofactors, implying the important role of Ln^{3+} in the biochemistry of life inhabiting this planet. However, Ln^{3+}-dependent methanotrophs had not been isolated in this study. The traditional enrichment cultivation method used in this study might cause the competition among methanotrophs and methylotrophs, resulting in the loss of Ln^{3+}-dependent methanotrophs. Therefore, other direct isolation methods such as direct spreading of bacteria without enrichment cultivation, or cell sorting may be adopted to isolate Ln^{3+}-dependent methanotrophs. *Methylacidiphilum fumariolicum* SolV isolated from an Italian volcanic mudpot is strictly dependent on the presence of Ln^{3+}, implying the presence of Ln^{3+}-dependent methanotrophs in extreme environment; hence, isolating Ln^{3+}-dependent methanotrophs from extreme environments should be a worthy of trial. Both strain SM30^T and strain La2-4^T contain more than one copy of *xoxF* gene, but their functional roles and interactions await further characterization. Additionally, rice plants can accumulate as much as 3.5 mM methanol, which offers carbon source for methylotrophs. The rice field soil in IPSR contains soluble Ln^{3+} at the level of nM which is available to Ln^{3+}-utilizing microorganisms. Research on the effect of Ln^{3+} on the diversity and abundance of microorganisms in natural condition of rice field should be necessary and interesting to assess the importance of Ln^{3+}-dependent methylotrophy.

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Table 1-1. Accession numbers of amino acid sequences used in MLSA maximum likelihood phylogenetic tree.

Strains	RpoB	GyrB	InfB	AtpD
SM30^T	OHA_1_01289	OHA_1_01474	OHA_1_03353	OHA_1_03098
Mongoliimonas terrestris MIMtkB18^T	WP_075218193	WP_075216478	WP_075216405	WP_075214121
Methylobrevis pamukkalensis PK2^T	ODN68938	WP_069307704	ODN72328	ODN72530
Pleomorphomonas oryzae DSM16300^T	WP_026789298	WP_026791533	WP_026789891	WP_026790640
Hartmannibacter diazotrophicus E19^T	SON55049	SON53545	SON58214	SON57522
Pleomorphomonas koreensis DSM23070^T	WP_026782430	WP_026785062	WP_026781854	WP_026783262
Prosthecomicrobium hirschii 16^T	WP_054357651	WP_054358743	WP_054358827	WP_054358697

Table 1-2. Primer sets for real time PCR used in this paper.

Targets	Locus	Forward primer	Reverse primer	Product length (bp)	TmF (°C)	TmR (°C)
gyrA	OHA_1_00856	GCCGACGAGATCAAGGACTA	GGTCTTGAGGTCGGTGAGTT	90	56	56
mxaF	OHA_1_04073	ACATCTGCATGGACTGGGAA	GTTGTACGCCTTCACCTGAC	140	56	56
xoxF1	OHA_1_00064	GAACGGTGAAGACGTGAACA	TAGTCCATGCAGACGTGGTT	129	56	56
xoxF2	OHA_1_04184	AACGTGATGACCTACGCCTT	TCGTGGTGTACTTGGACAGG	157	56	56
xoxF3	OHA_1_03539	TACGTCTTCGCCAACGTCTA	TGCCAGACCAGTTCGGATAC	127	56	57
xoxF4	OHA_1_01165	AAGTGGGTCTACCAGCTGAC	GAAGTGGACGATCACCTTGC	114	56	56

Table 1-3. Primer sets for generation of MDH gene deletion mutant strains used in this paper.

Targets	Forward primer	Reverse primer	Product length (bp)
mxaF upstream	GTCGACTCTAGAGGATCCCCTGAGGACGCCGAACCATCCG	CGAGAACGGTCTGCAGTTCC	790
mxaF downstream	GGAACTGCAGACCGTTCTCGCAAGAGCTGATCCGAGACGG	CGAATTCGAGCTCGGTACCCCTCCCACTTGCCCGTGTACT	640
xoxF1 upstream	CTCTAGAGGATCCCCCTCCAGCGCGACGTTCAGGT	CCTAGACCACACCTCTCGAG	855
xoxF1 downstream	GAGGTGTGGTCTAGGCCGTGTTCTCGCTGCCCTGA	TCGAGCTCGGTACCCGACGTTGAGGTTCGTGCCGA	826
xoxF2 upstream	GTCGACTCTAGAGGATCCCCTCGCGGGTCGACGAGTTCAT	ACATCGGCGGGATCCGTTCG	564
xoxF2 downstream	CGAACGGATCCCGCCGATGTCCGTGTTCTCCCTGCCATGA	CGAATTCGAGCTCGGTACCCAACTGGTCGGCCGTGACTCG	704
xoxF3 upstream	GTCGACTCTAGAGGATCCCCACCACGTGATCGTCCGCGAA	GCCGTCACCGATCGCGTGTA	850
xoxF3 downstream	TACACGCGATCGGTGACGGCTGCTCTACACCTTCCGCGTC	CGAATTCGAGCTCGGTACCCAGGATGCGCTCGTAGCCGTA	560
xoxF4 upstream	GTCGACTCTAGAGGATCCCCGCCGAAGACGGTGAAGTCCT	GATGCCCTCCCGTCATGTCC	659
xoxF4 downstream	GGACATGACGGGAGGGCATCACGTCTTCCGCCTGCCGTGA	CGAATTCGAGCTCGGTACCCCGGCGATCTCGTAGCTGAAG	737

The sequences that were used as extensions for recombination with the vector and inter-fragments are underlined.

Table 1-4. Differential characteristics of strain SM30^T from species of the *Pleomorphomonas*, *Hartmannibacter*, *Methylobrevis*, and *Labrenzia*.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cell shape	Rods	Pleomorphic rods	Rods	Pleomorphic rods	Rods	short rods	Large Rods, joined
Motility	+	—	—	—	+	+	+
Catalase	+	+	+	+	—	+	+
Nitrate reduction to nitrite (NO_2^-)	—	—	+*	+	+	—	+
Temperature range for growth (°C)	5-40*	15-40*	5-40*	5-40*	15-40*	4-40	15-37
NaCl tolerance (%)	≤2	≤2	≤2	≤2	≤3	≤5.8	≤10
Assimilation of (API 20NE):							
Gluconate	+	+	—	—	—	—	+ ¶
Malate	+	+	+	—	—	—	+ ¶
N-Acetylglucosamine	+	(+)	(+)	+	—	—	(+)¶
Glucose fermentation	—	+	—	—	—	—	— ¶
Utilization of:							
Methanol	+*	—*	—*	—*	+ *, §	+	nd
Methylamine	+*	—*	—*	—*	+ *, §	(+)	nd
PCR amplification of:							
mxaF	+	—*	—**	—**	—*	nd	nd
xoxF	+	—*	—**	—**	+*	nd	nd
Major fatty acids (>10%)	SF 8, $C_{19:0}$ cyclo ω8c	SF 8, $C_{16:0}$	SF 8, $C_{19:0}$ cyclo ω8c, $C_{16:0}$	SF 8, $C_{19:0}$ cyclo ω8c	SF 8	SF 3, SF 8	SF 2, SF8

Continued Table 1-4

Characteristic	1	2	3	4	5	6	7
Hydroxy fatty acids	$C_{14:0}$ 3-OH, $C_{18:0}$ 3-OH	$C_{14:0}$ 3-OH, $C_{18:0}$ 3-OH	$C_{14:0}$ 3-OH, $C_{18:0}$ 3-OH	$C_{14:0}$ 3-OH, $C_{18:0}$ 3-OH	$C_{14:0}$ 3-OH, $C_{16:0}$ 3-OH	$C_{14:0}$ 3-OH, $C_{16:0}$ 3-OH, $C_{18:0}$ 3-OH	$C_{14:0}$ 3-OH, $C_{18:0}$ 3-OH
Quinone type	Q-10 (98.7%), Q-9 (1.3%)	Q-10 (86%), Q-8 (14%)	Q-10	Q-10	Q-10	Q-10	Q-10
Isolation source	Rhizospheric soil of rice (cv Norin 18)	Root tissue of *Jatropha curcas*	Contaminated culture of *Rhodopseudomonas palustris*	Roots of rice plants	Rhizospheric soil of *Plantago winteri*	Saline hot spring	Marine sediment
DNA G+C content (mol %)	71.6**	63.2 (HPLC)	67.2**	63**	63.99**	68.9**	59.1**

1, strain SM30^T; 2, *Pleomorphomonas diazotrophica* DSM 25022^T (Madhaiyan et al., 2013); 3, *Pleomorphomonas koreensis* NBRC 100803^T (Im et al., 2006); 4, *Pleomorphomonas oryzae* DSM 16300^T (Xie and Yokota, 2005); 5, *Hartmannibacter diazotrophicus* LMG 27460^T (Suarez et al., 2014); 6, *Methylobrevis pamukkalensis* PK2^T (Poroshina et al., 2015); 7, *Labrenzia aggregata* CCUG 61128^T (Biebl et al., 2007; Bibi et al., 2014). *, this study; **, genome data; +, positive reaction; —, negative reaction; (+), weakly positive reaction; §, La-depended; ¶, Data obtained from under the respective strain entry at www.ccug.se. SF, Summed feature; nd, not determined.

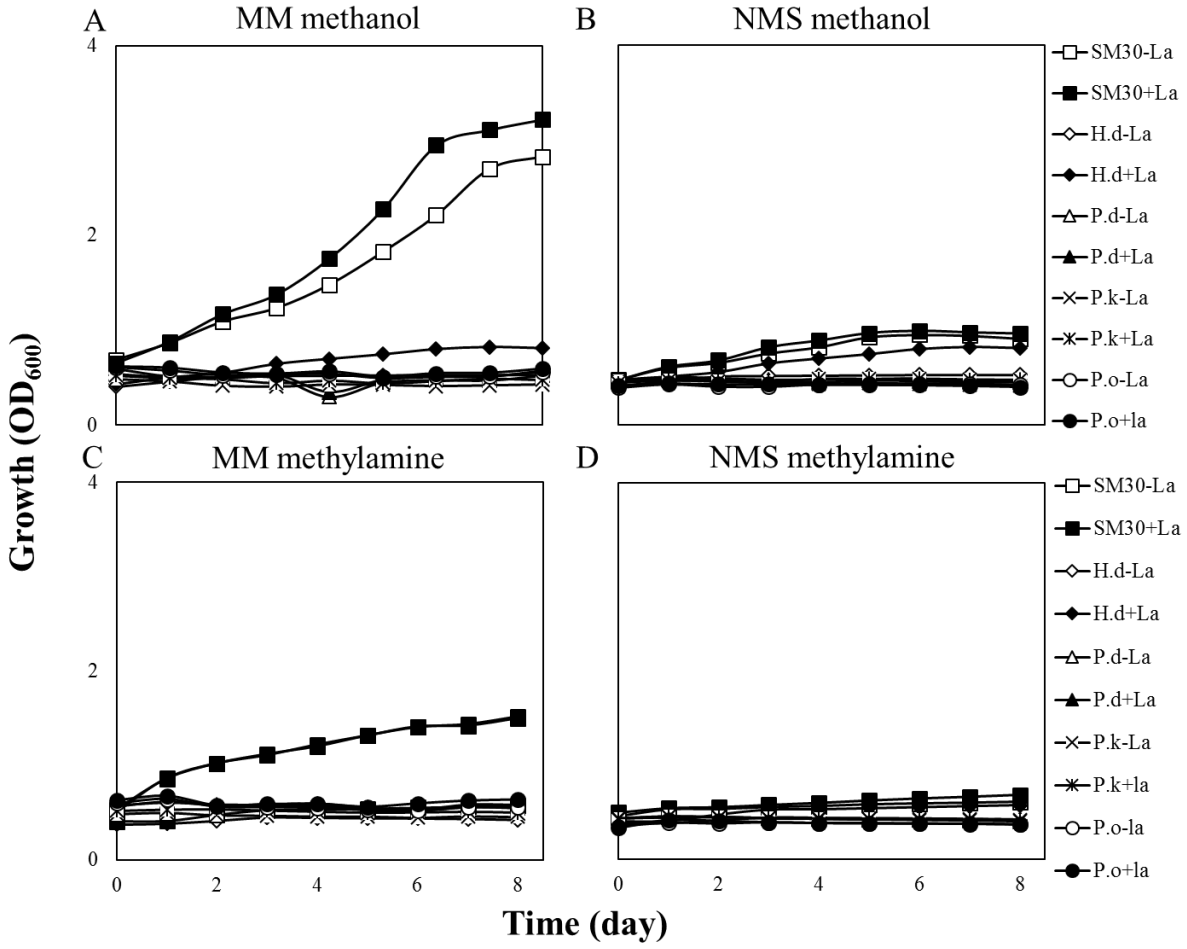

Fig. 1-1. Growth of strain SM30^T and reference type strains on methanol and methylamine, in different media. Five strains were grown on methanol (A, B) and methylamine (C, D) in MM (A, C) and NMS medium (B, D). - La, without lanthanum; + La, with lanthanum. H.d, *Hartmannibacter diazotrophicus* LMG 27460^T; P.d, *Pleomorphomonas diazotrophica* DSM 25022^T; P.k, *Pleomorphomonas koreensis* NBRC 100803^T; P.o, *Pleomorphomonas oryzae* DSM 16300^T. Data are presented as average of technical triplicate preparations. Standard deviation is not shown due to complexity but relative standard deviation is less than 15%.

Table 1-5. Cellular fatty acid profiles of strain SM30^T and phylogenetically related taxons.

Fatty acids	**1**	**2**[a]	**3**[a]	**4**[a]	**5**[b]	**6**[c]	**7**[d]
$C_{8:0}$ 3OH	—	—	—	—	tr	—	—
$C_{10:0}$ 3OH	—	—	—	—	tr	—	—
$C_{14:00}$	—	tr	—	tr	—	0.5	—
$C_{15:00}$	—	—	—	—	—	1	—
$C_{16:1}$ ω11c	—	—	—	—	0.8	—	—
$C_{16:00}$	0.7	11.7	8.3	18.4	6.1	8	—
$C_{17:0}$ CYCLO	—	0.5	tr	1.3	—	2.4	—
$C_{17:00}$	1.3	—	1.4	0.7	—	7	—
$C_{17:1}$ ω8c	—	—	—	—	—	5.6	—
$C_{16:0}$ 3OH	—	—	—	—	1.8	1	—
$C_{18:00}$	6.7	3.9	7.8	3.3	—	4.4	3.2
11 methyl $C_{18:1}$ ω7c	4.2	—	—	—	—	1	4.4
$C_{19:0}$ CYCLO ω8c	12.9	8.2	13.6	40.3	1.5	7	—
$C_{19:1}$	—	—	—	—	—	0.7	—
$C_{18:0}$ 3OH	1.9	4	2.8	2.1	—	tr	2
$C_{20:1}$ ω7c	1.5	—	—	—	—	—	3.7
$C_{20:00}$	—	—	—	—	7.9	—	—
Summed feature 2	2	5.6	2.8	3.6	8.3	1	13.7
Summed feature 3	—	1.7	0.7	tr	1.1	13	1.6
Summed feature 8	68.6	62.6	61.8	28.3	71.4	47	68.4

Strains: 1, SM30^T; 2, *Pleomorphomonas diazotrophica* R5-392^T; 3, *Pleomorphomonas oryzae* KCTC 12245^T; 4, *Pleomorphomonas koreensis* KCTC 12246^T; 5, *Hartmannibacter diazotrophicus* E19^T; 6, *Methylobrevis pamukkalensis* PK2^T and 7, *Labrenzia aggregata* KACC 15203^T. tr; Fatty acids less than 0.5%. *a*, Data from Madhaiyan et al., 2013; *b*, Data from Suarez et al., 2014; *c*, Data from Poroshina et al., 2015; *d*, Data from Bibi et al., 2014. Summed features (SF) are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. SF 2: $C_{14:0}$ 3-OH/iso-$C_{16:1}$ I; SF 3: $C_{16:1}$ ω7c/$C_{16:1}$ ω6c; SF 8: $C_{18:1}$ ω7c/$C_{18:1}$ ω6c.

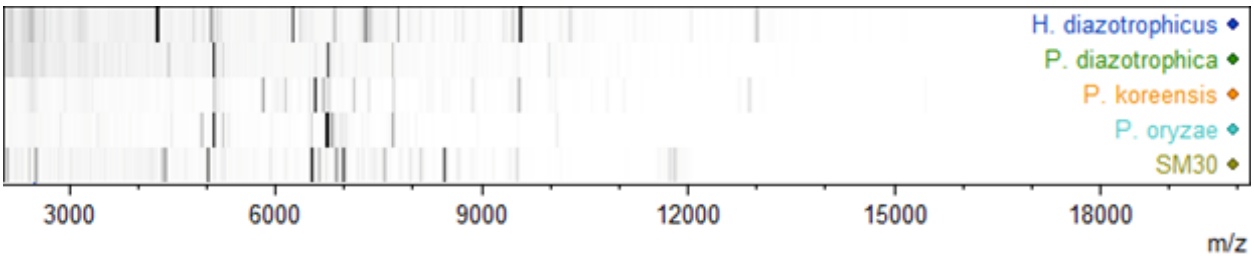

Fig. 1-2. Comparison of whole cell protein profiles of SM30^T and related type strains by MALDI-TOF/MS.

Table 1-6. General features of *O. diazotrophicus* SM30^T genome.

	Chromosome	pSM30-1	pSM30-2
Size (bp)	4,841,392	141,326	21,379
G+C content (mol%)	71.6	72.5	60.70
Number of CDSs	4360	113	24
CDS summary:			
Protein of assigned function	3315	97	6
Protein of unassigned function	1045	16	18
Not in KEGG orthology	2141	45	22
Percentage coding	87.92	90.16	89.36
Average gene length (bp)	976	1128	796
tRNA	66	1	0
rRNA operon	9	0	0
GenBank accession number	AP017626	AP017627	AP017628

Table 1-7. Genomic islands in *O. diazotrophicus* SM30^T genome.

GI	Start	End	Locus (OHA_)	Major products
GI 1	24146	31793	1_00022-1_00026	Major facilitator superfamily protein; excalibur calcium-binding domain protein
GI 2	1703943	1710146	1_01580-1_01586	HPr kinase/phosphorylase; sulfotransferase domain protein; coenzyme PQQ synthesis protein D (PqqD); asparagine synthetase [glutamine-hydrolyzing] 3; PAN domain protein
GI 3	2293140	2297513	1_02115	Hypothetical protein
GI 4	2517238	2522524	1_02321-1_02329	Trehalose transport system permease protein SugB; trehalose import ATP-binding protein SugC; PTS-dependent dihydroxyacetone kinase, ADP-binding subunit DhaL; PTS-dependent dihydroxyacetone kinase, phosphotransferase subunit DhaM; phosphocarrier protein NPr; phosphoenolpyruvate-protein phosphotransferase
GI 5	2788200	2855209	1_02558-1_02605	TVP38/TMEM64 family inner membrane protein YdjZ; mercuric reductase; nitrogen regulation protein NR(II); alpha/beta hydrolase family protein; tRNA(Glu)-specific nuclease WapA precursor; tyrosine recombinase XerC; putative deoxyribonuclease RhsA; DNA-invertase hin; IS2 repressor TnpA; IS2 transposase TnpB; excalibur calcium-binding domain protein; succinoglycan biosynthesis protein ExoI; putative serine protease HtrA; putative peptidoglycan binding domain protein; radical SAM superfamily protein; molybdenum cofactor biosynthesis protein A; von Willebrand factor type A domain protein; transposase; integrase core domain protein; tyrosine recombinase XerD; putative zinc metalloprotease Rip3; alkaline phosphatase D precursor
GI 6	2791975	2804845	1_02562-1_02570	Alpha/beta hydrolase family protein; tRNA(Glu)-specific nuclease WapA precursor; tyrosine recombinase XerC
GI 7	2805825	2850878	1_02571-1_02602	Putative deoxyribonuclease RhsA; DNA-invertase hin; IS2 repressor TnpA; IS2 transposase TnpB; excalibur calcium-binding domain protein; succinoglycan biosynthesis protein ExoI; putative serine protease HtrA; putative peptidoglycan binding domain protein; radical SAM superfamily protein; molybdenum cofactor biosynthesis protein A; von Willebrand factor type A domain protein; transposase; integrase core domain protein; tyrosine recombinase XerD

Continued Table 1-7

GI	Start	End	Locus (OHA_)	Major products
GI 8	3145809	3151207	1_02877-1_02883	Fatty acid desaturase; transglutaminase-like superfamily protein; Na(+)/H(+) antiporter NhaA; esterase PHB depolymerase
GI 9	4211837	4224000	1_03850-1_03863	Maf-like protein YhdE; zinc-binding protein; prophage CP4-57 integrase; transposase; coenzyme PQQ synthesis protein D; asparagine synthetase [glutamine-hydrolyzing] 3; transposase; GTP pyrophosphokinase rsh; lipopolysaccharide export system ATP-binding protein LptB
GI 10	4213816	4220675	1_03853-1_03860	Prophage CP4-57 integrase; transposase; coenzyme PQQ synthesis protein D; asparagine synthetase [glutamine-hydrolyzing] 3; transposase
GI 11	4239279	4244015	1_03879-1_03863	Hypothetical protein
GI 12	4576204	4580906	1_04191-1_04196	Helix-turn-helix domain protein; phage Terminase
GI 13	97676	104203	2_00076-2_00083	Dihydrolipoyllysine-residue acetyltransferase component of acetoin cleaving system; deoxyribonucleoside regulator; urease accessory protein UreE, UreG, UreD; urease subunit alpha

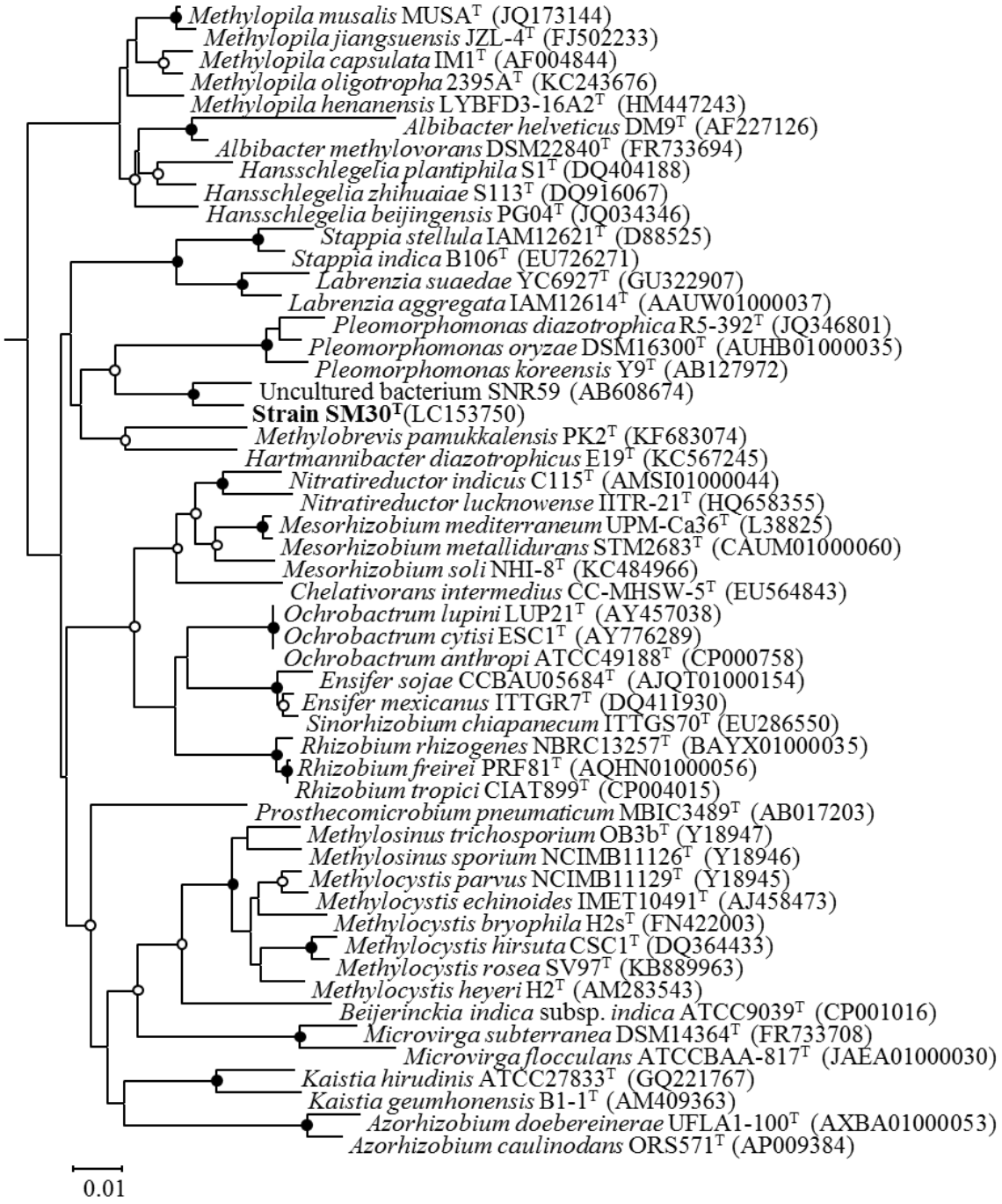

Fig. 1-3. Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship of the new genus within the *Rhizobiales*, class *Alphaproteobacteria*. The evolutionary distances were computed using the Kimura 2-parameter method. Circles indicate consensus bootstrap values from neighbor-joining, maximum-likelihood, and parsimony analyses. Nodes supported at ≥90% in the majority of analyses are indicated by filled circles. Nodes supported at 70 to 90% in most analyses are indicated by open circles. Unsupported nodes (≤70%) have no circle. There was a total of 1261 positions in the final dataset. Bar, 0.01 substitutions per site.

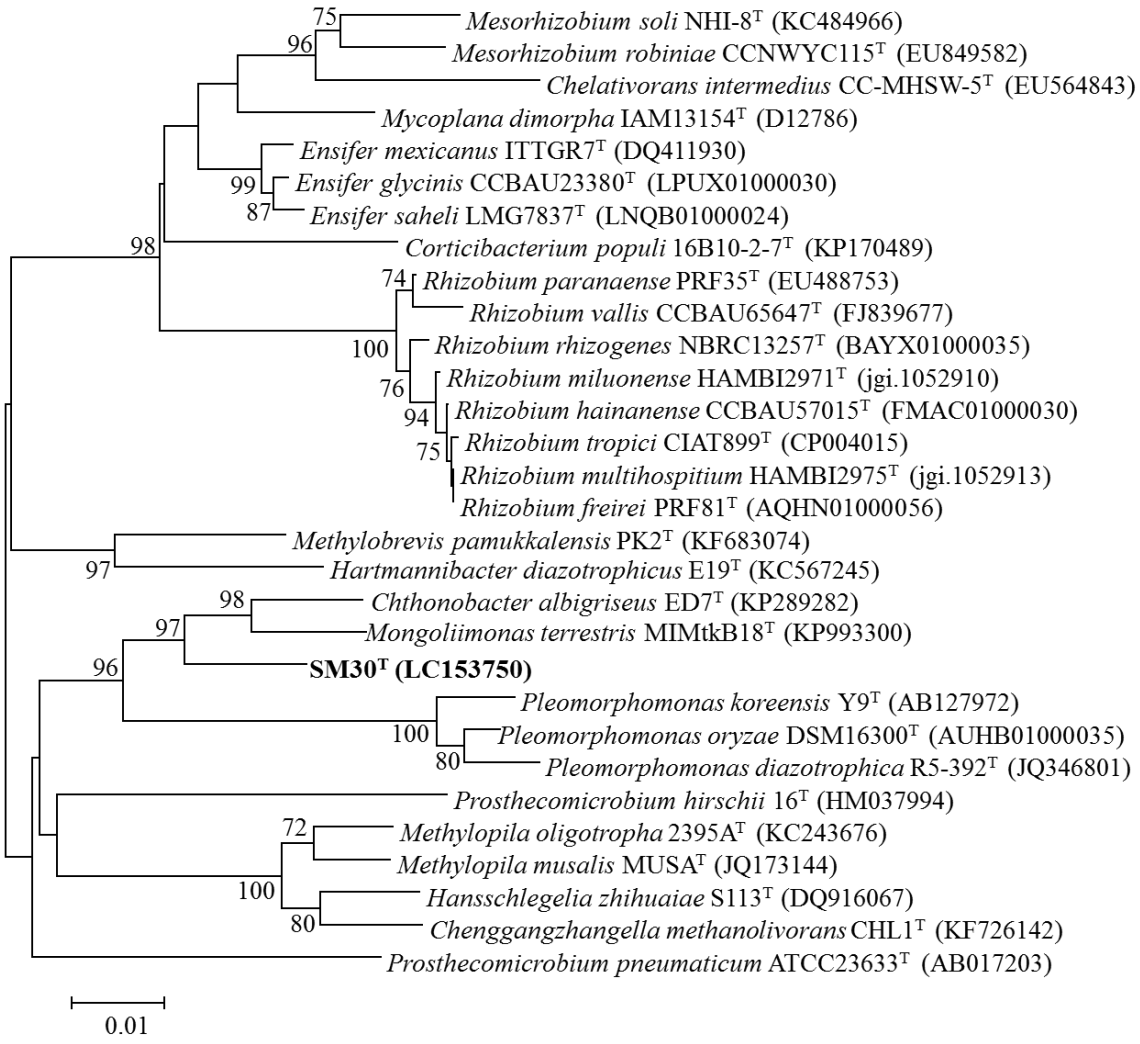

Fig. 1-4. Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship of the new genus within the *Rhizobiales*, class *Alphaproteobacteria*. The evolutionary distances were computed using the Kimura 2-parameter method. Bootstrap values greater than 70% are listed as percentages at the branching points. Bar, 0.01 substitutions per site.

Table 1-8. dDDH and ANI values between genomes of strain SM30^T and closely related species.

Reference strains	Accession number	dDDH (Formula: 2)	ANI
Mongoliimonas terrestris MIMtkB18^T	NZ_MSIG01000001	20.60%	79.35%
Methylobrevis pamukkalensis PK2^T	NZ_MCRJ01000001	21.00%	80.71%
Pleomorphomonas oryzae DSM16300^T	NZ_AUHB01000001	20.40%	79.17%
Hartmannibacter diazotrophicus E19^T	LT960614	20.60%	78.48%
Pleomorphomonas koreensis DSM23070^T	NZ_AULH01000001	20.10%	77.65%
Prosthecomicrobium hirschii 16^T	NZ_LJYW01000001	20.10%	77.75%

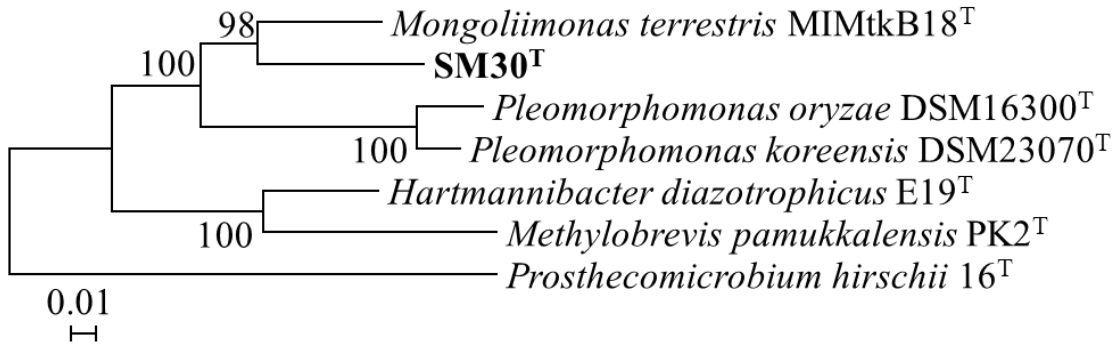

Fig. 1-5. MLSA-based maximum likelihood phylogeny of concatenated partial amino acid sequences of RpoB, GyrB, InfB, and AtpD of strain SM30^T and other related type strains using the JTT algorithm. (Accession numbers are listed in Table 1-1.) Bootstrap values greater than 70% are listed as percentages at the branching points. Bar, 0.01 substitutions per site.

Fig. 1-6. Neighbor-joining phylogeny of MDH homologues amino acid sequences of strain SM30^{T} and other related methylotrophic bacteria using the JTT algorithm. Bootstrap values greater than 70% are listed as percentages at the branching points. Bar, 0.1 substitutions per site.

Table 1-9. Methylotrophy islands of *O. diazotrophicus* SM30^T genome.

Cluster	Start	End	Locus_tag (OHA_1_)	Gene	Product
MxaF	4447204	4448085	4069		TraB family protein
	4448101	4448745	4070	*mxaB*	DNA-binding response regulator
	4448742	4450184	4071	*liaS*	Sensor histidine kinase LiaS
	4450181	4450687	4072	*mxaH*	MxaH protein
	4451093	4452982	4073	*mxaF*	Methanol dehydrogenase large subunit protein, MxaF
	4453145	4453981	4074	*mxaJ*	Methanol oxidation protein MaxJ
	4454020	4454556	4075	*mxaG*	Periplasmic cytochrome c class I
	4454710	4455003	4076	*mxaI*	Methanol dehydrogenase small subunit
	4455214	4456239	4077	*mxaR*	Methanol dehydrogenase regulatory protein MxaR
	4456236	4457102	4078	*mxaS*	MxaS protein
	4457106	4458002	4079	*mxaA*	MxaA protein
	4457999	4459030	4080	*mxaC*	MxaC protein
	4459027	4459629	4081	*mxaK*	MxaK protein
	4459626	4460678	4082	*mxaL*	MxaL protein
	4460675	4461208	4083	*mxaD*	MxaD gene product
	4461211	4462158	4084	*mxaE*	MxaE protein
XoxF1	62909	63304	59		RlpA-like protein precursor
	63713	64063	61		Hypothetical protein
	64146	64991	62	*soxY*	Sulfur oxidation protein SoxY
	65107	65931	63	*gloB*	Beta-lactamase
	66512	68317	64	*xoxF1*	Methanol dehydrogenase XoxF1
	68746	69264	65	*xoxG1*	Cytochrome c
	69463	70305	66	*xoxJ1*	Extracellular solute-binding protein
	70426	71004	67	*pspE*	Rhodanese
	71102	72031	68	*fabG*	3-oxoacyl-ACP reductase
	72281	73000	69		ABC transporter ATP-binding protein
	72984	73745	70		ABC transporter permease
XoxF2	4567971	4568948	4179		Magnesium transport protein CorA
	4568945	4569154	4180		Hypothetical protein
	4569426	4570250	4181	*thyA*	Thymidylate synthase
	4570366	4570818	4182		Hypothetical protein
	4570854	4571294	4183		Hypothetical protein
	4571327	4573141	4184	*xoxF2*	Methanol dehydrogenase [cytochrome c] subunit 1 precursor
	4573165	4573644	4185	*greA*	Transcription elongation factor GreA
	4573654	4573875	4186		Protein SlyX
	4573980	4574483	4187		Hypothetical protein

Continued Table 1-9

Cluster	Start	End	Locus_tag (OHA_1_)	Gene	Product
XoxF2	4574897	4575958	4189		Phage integrase family protein
XoxF3	3874609	3875517	3534		EamA-like transporter family protein
	3875646	3876545	3535		N-formylglutamate amidohydrolase
	3876717	3876971	3536		Hypothetical protein
	3877311	3878696	3537	*mxcQ*	Signal transduction histidine kinase, MxcQ
	3878693	3879352	3538	*mxcE*	Response regulator receiver protein, MxcE
	3879705	3881570	3539	*xoxF3*	Methanol dehydrogenase [cytochrome c] subunit 1 precursor
	3881719	3883506	3540		Ankyrin repeat protein with 3 copies
	3883553	3884377	3541		Cytochrome c
	3884385	3885632	3542		Hypothetical protein
	3885656	3886630	3543	*vgb*	Virginiamycin B lyase
	3886761	3887171	3544		EF hand
XoxF4	1255039	1255749	1162	*dnaJ*	Chaperone protein DnaJ
	1255884	1256624	1163		Hypothetical protein
	1256775	1258307	1164	*accD*	Methylmalonyl-CoA carboxyltransferase
	1258676	1260457	1165	*xoxF4*	Methanol dehydrogenase [cytochrome c] subunit 1 precursor
	1260472	1261314	1166	*xoxJ4*	Bacterial extracellular solute-binding proteins, family 3
	1261386	1261754	1167	*xoxG 4*	Cytochrome c
	1262053	1262487	1168		Hypothetical protein
	1262711	1263580	1169	*yedZ*	Sulfoxide reductase heme-binding subunit YedZ
	1692054	1692737	1573	*mxbM*	Response regulator receiver protein, MxbM
	1692734	1695412	1574	*mxbD*	Sensor histidine kinase
PQQ synthesis	2024333	2025709	1882	*pqqF*	Coenzyme PQQ biosynthesis protein F
	2025751	2027097	1883	*pqqG*	Coenzyme PQQ biosynthesis protein G
	3907630	3907908	3563	*pqqD 2*	Coenzyme PQQ synthesis protein D
	4216726	4217031	3857	*pqqD 3*	Coenzyme PQQ synthesis protein D
	4707064	4708188	4315	*pqqE*	Coenzyme PQQ synthesis protein E
	4708185	4708487	4316	*pqqD 1*	Coenzyme PQQ synthesis protein D
	4709074	4709829	4317	*pqqC*	Coenzyme PQQ synthesis protein C
	4709826	4710770	4318	*pqqB*	Coenzyme PQQ synthesis protein B
	4710835	4710912	4437	*pqqA*	Coenzyme PQQ biosynthesis protein A
Methylamine dehydrogenase	2155152	2155685	2005	*mauA*	Methylamine dehydrogenase light chain
	2155691	2156308	2006	*mauD*	Methylamine utilization protein MauD
	2156375	2156908	2007	*mauE*	Methylamine utilization protein MauE

Continued Table 1-9

Cluster	Start	End	Locus_tag (OHA_1_)	Gene	Product
Methylamine dehydrogenase	2156905	2158041	2008	*mauB*	Methylamine dehydrogenase heavy chain
N-glutamate-mediated methylamine utilization pathway	3437831	3438385	3144	*mgdD*	N-methyl glutamate dehydrogenase/oxidoreductase, subunit D
	3438378	3441338	3145	*mgdC*	N-methyl glutamate dehydrogenase/oxidoreductase, subunit C
	3441335	3441607	3146	*mgdB*	N-methyl glutamate dehydrogenase/oxidoreductase, subunit B
	3441619	3442872	3147	*mgdA*	N-methyl glutamate dehydrogenase /oxidoreductase, subunit A
	3444759	3445667	3150	*mgsA*	N-methyl glutamate synthase, subunit A
	3445686	3446372	3151	*mgsB*	N-methyl glutamate synthase, subunit B
	3446384	3447712	3152	*mgsC*	N-methyl glutamate synthase, large subunit C
	3447817	3449124	3153	*gms*	Gamma-glutamylmethylamide synthetase
H_4MPT-linked formaldehyde oxidation	532970	533779	508	*fhcC*	Formylmethanofuran dehydrogenase
	533779	534684	509	*fhcD*	Formylmethanofuran-tetrahydromethanopterin formyltransferase
	534672	536309	510	*fhcA*	Formyltransferase/hydrolase complex Fhc subunit A
	536306	537391	511	*fhcB*	Formyltransferase/hydrolase complex Fhc subunit B
	1124949	1126001	1030	*orf9*	H_4MPT-linked C1 transfer pathway protein
	1126176	1126877	1031	*orf17*	H_4MPT-linked C1 transfer pathway protein
	1127753	1128595	1033	*orf7*	H_4MPT-linked C1 transfer pathway protein, Orf7
	1128592	1129527	1034		Alpha-aminoadipate--LysW ligase LysX
	1129511	1130488	1035	*mch*	Methenyltetrahydromethanopterin cyclohydrolase
	1129511	1130488	1036		Tetrahydromethanopterin C1 transfer protein
	1131837	1132739	1037	*mtdA*	Methylenetetrahydromethanopterin dehydrogenase
	1132819	1133814	1038		NAD(P)-dependent methylenetetrahydromethanopterin dehydrogenase
	2192292	2192831	2036	*fae*	Aldehyde-activating protein
formate oxidation	2581834	2582055	2384	*fdh2D*	NAD-dependent formate dehydrogenase delta subunit
	2582042	2582941	2385	*fdhD*	Formate dehydrogenase accessory protein FdhD
	2582947	2585820	2386	*fdh2A*	NAD-dependent formate dehydrogenase alpha subunit
	2585831	2587396	2387	*fdh2B*	NAD-dependent formate dehydrogenase beta subunit
	2587393	2587866	2388	*fdh2C*	NAD-dependent formate dehydrogenase gamma subunit
	2907765	2908103	2657	*fdh3B*	Formate dehydrogenase
H_4F pathway	680096	681523	635	*trmFO*	Methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase TrmFO
	1843755	1845428	1713	*ftfL*	Formate-tetrahydrofolate ligase
	2194488	2195357	2038	*folD*	Bifunctional methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase
	2307571	2308479	2126	*metF*	Methylenetetrahydrofolate reductase
	3125796	3126680	2865	*purU*	Formyltetrahydrofolate deformylase
	4056137	4056742	3702		5-formyltetrahydrofolate cyclo-ligase familyprotein

Continued Table 1-9

Cluster	Start	End	Locus_tag (OHA_1_)	Gene	Product
H_4F pathway	4732727	4733491	4340		5-formyltetrahydrofolate cyclo-ligase familyprotein
	552746	554050	522	*glyA1*	Serine hydroxymethyltransferase 1
	601983	603257	567	*eno*	Enolase
	725973	726875	679	*sucD_1*	Succinyl-CoA ligase [ADP-forming] subunit alpha
	726892	728073	680	*sucC_1*	Succinyl-CoA ligase [ADP-forming] subunit beta
	728321	729280	681	*mcl1_1*	Malyl-CoA lyase/malate thiokinase
	729496	730683	682	*sga*	Serine--glyoxylate aminotransferase
	835047	836096	777	*mcl1_2*	Malyl-CoA lyase/malate thiokinase
serine cycle	2003713	2005389	1864	*pckA*	Phosphoenolpyruvate carboxykinase [ATP]
	2082882	2085617	1936	*sucD_2*	Succinyl-CoA ligase [ADP-forming] subunit alpha
	2508768	2509709	2315	*ghrA*	Glyoxylate/hydroxypyruvate reductase A
	3529107	3530006	3223	*sucD_3*	Succinyl-CoA ligase [ADP-forming] subunit alpha
	3530218	3531414	3225	*sucC_2*	Succinyl-CoA ligase [ADP-forming] subunit beta
	3531522	3532484	3226	*mdh_2*	Malate dehydrogenase
	3816587	3817552	3483	*ghrB*	Glyoxylate/hydroxypyruvate reductase B
	4714342	4715619	4323	*ttuD*	Glycerate kinase
	140460	141752	13	*gltA_1*	Citrate synthase
	16641	17852	14	*icd*	Isocitrate dehydrogenase
	32560	33888	27	*fumC*	Fumarate hydratase, class II
	140460	141752	134	*gltA*	Type II citrate synthase
	1540977	1542599	1422	*ttdA*	Fumarate hydratase
	3306094	3308769	3013	*acnA*	Aconitate hydratase 1
TCA cycle	3485700	3486479	3188	*sdhB*	Succinate dehydrogenase
	3488354	3488746	3190	*sdhD*	Succinate dehydrogenase
	3488758	3489156	3191	*sdhC*	Succinate dehydrogenase
	3525964	3528948	3222	*sucA*	2-oxoglutarate dehydrogenase E1 component
	3529107	3530006	3223	*sucD_3*	Succinyl-CoA ligase [ADP-forming] subunit alpha
	3530218	3531414	3225	*sucC_2*	Succinyl-CoA ligase [ADP-forming] subunit beta
	3531522	3532484	3226	*mdh_2*	Malate dehydrogenase
	32560	33888	27	*fumC*	Fumarate hydratase, class II
	342833	343237	318	*gloA*	Methylmalonyl-CoA epimerase
EMP pathway	725973	726875	679	*sucD_1*	Succinyl-CoA ligase [ADP-forming] subunit alpha
	726892	728073	680	*sucC_1*	Succinyl-CoA ligase [ADP-forming] subunit beta
	728321	729280	681	*mcl1_1*	Malyl-CoA lyase

Continued Table 1-9

Cluster	Start	End	Locus_tag (OHA_1_)	Gene	Product
	1256775	1258307	1164	*pccB*	Propionyl-CoA carboxylase beta chain
	1350196	1352208	1241	*accA1*	Acetyl/propionyl-CoA carboxylase subunit alpha
	1540977	1542599	1422	*ttdA*	Fumarate hydratase
	1814614	1816785	1688	*mcmA*	Methylmalonyl-CoA mutase large subunit
	1816796	1818643	1689	*mcmB*	Methylmalonyl-CoA mutase small subunit
	3019851	3021023	2766	*phaA*	Acetyl-CoA acetyltransferase
	3021108	3021836	2767	*phaB*	Acetoacetyl-CoA reductase
	3087059	3089068	2830	*meaA*	Ethylmalonyl-CoA mutase
EMP pathway	3089480	3090760	2831	*ccrA*	Crotonyl-CoA reductase
	3094032	3095708	2835	*ibd2*	Acyl-CoA dehydrogenase
	3485700	3486479	3188	*sdhB*	Succinate dehydrogenase
	3488354	3488746	3190	*sdhD*	Succinate dehydrogenase
	3488758	3489156	3191	*sdhC*	Succinate dehydrogenase
	3492055	3493107	3194	*meaC*	Mesaconyl-CoA hydratase
	3529107	3530006	3223	*sucD_3*	Succinyl-CoA ligase [ADP-forming] subunit alpha
	3530218	3531414	3225	*sucC_2*	Succinyl-CoA ligase [ADP-forming] subunit beta
	4691718	4692479	4298		3-hydroxyacyl-CoA dehydrogenase

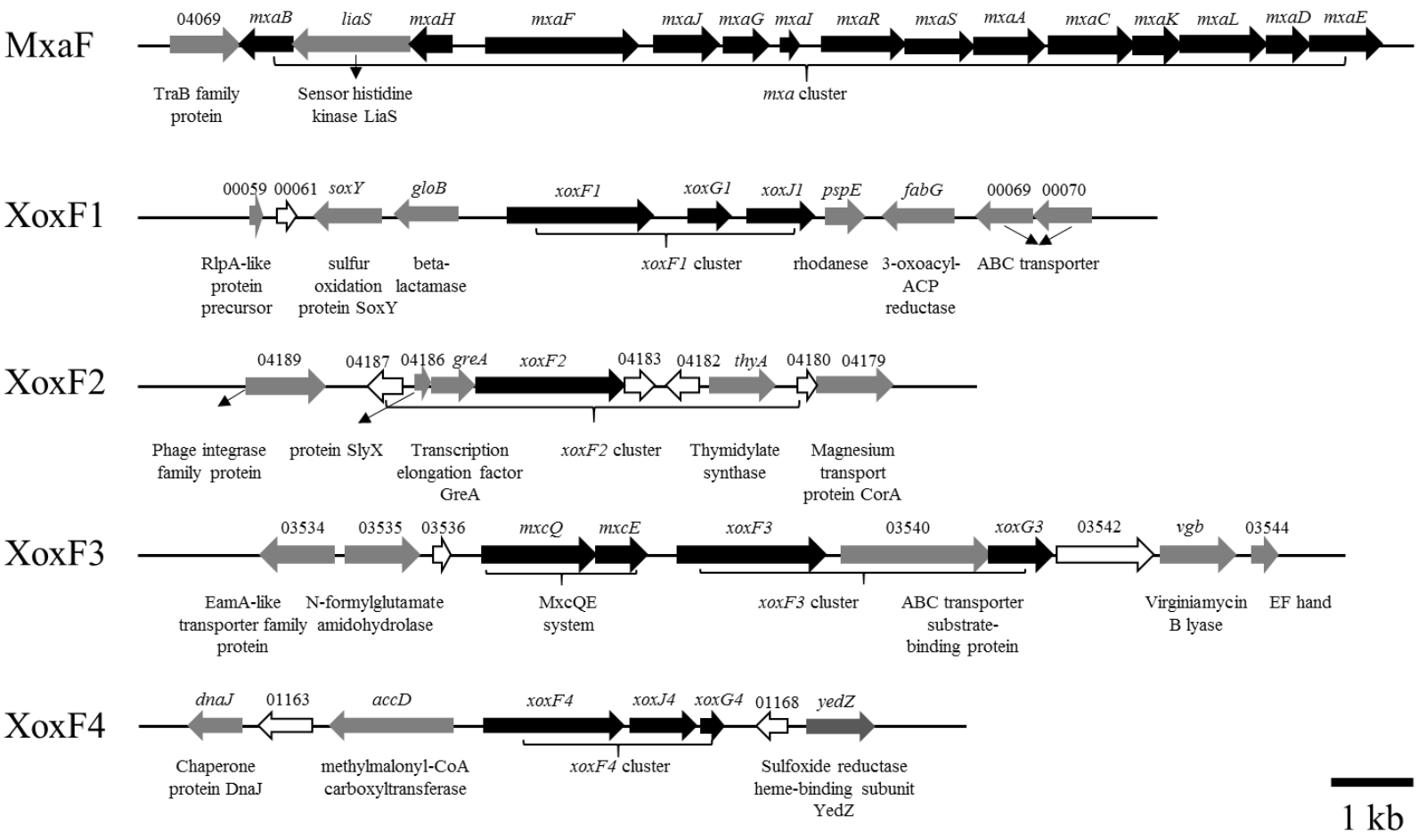

Fig. 1-7. Genomic organization of MDH homologue genes in the strain SM30^{T} genome. Black, gray, and white arrows show genes important for methylotrophy, those for proteins with known function, and those for hypothetical proteins, respectively. The accession numbers are shown without suffix (OHA_1_).

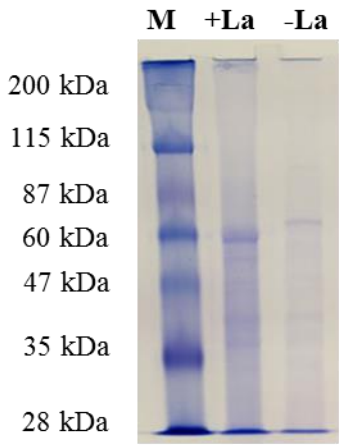

Fig. 1-8. SDS-PAGE analysis of strain SM30^{T} cell-free extracts grown on methanol in the presence (+La) or absence (-La) of La^{3+}. M, protein marker (nacalai tesque). The protein contents of the two samples were 105 μg and 23.7 μg, respectively.

Table 1-10. LC-MS analysis of major protein bands of *O. diazotrophicus* SM30^T grown on methanol in the absence or presence of La^{3+}, respectively. *a*, score ≥ 23 is statistically significant hit.

Condition	Hits	Annotation	Score [a]	Mass (Da)
-La	OHA_1_04073	Methanol dehydrogenase (MxaF)	1854	69449
	OHA_1_01892	60 kDa chaperonin 7	491	57160
	OHA_1_02483	Carbon monoxide dehydrogenase large chain	68	83149
	OHA_1_03343	30S ribosomal protein S1	45	62671
	OHA_1_03189	Succinate dehydrogenase flavoprotein subunit	42	67273
	OHA_1_00788	Transcriptional regulator PdhR	27	37033
	OHA_1_00064	Methanol dehydrogenase (XoxF1)	26	64776
	OHA_1_00700	Minor extracellular protease vpr precursor	26	103955
	OHA_1_03666	Oligo endopeptidase F, plasmid	23	66495
+La	OHA_1_00064	Methanol dehydrogenase (XoxF1)	1290	64776
	OHA_1_01892	60 kDa chaperonin 7	1070	57160
	OHA_1_01713	Formate--tetrahydrofolate ligase	660	59310
	OHA_1_02835	Acyl-CoA dehydrogenase	100	60013
	OHA_1_03189	Succinate dehydrogenase flavoprotein subunit	50	67273
	OHA_1_00682	Soluble hydrogenase 42 kDa subunit	46	43299
	OHA_1_03096	ATP synthase subunit alpha	44	54927
	OHA_1_00680	Succinyl-CoA ligase [ADP-forming] subunit beta	42	42136
	OHA_1_03013	Aconitate hydratase 1	41	96255
	OHA_1_00934	Electron transfer flavoprotein-ubiquinone oxidoreductase	40	61424
	OHA_1_04266	Putative D, D-dipeptide-binding periplasmic protein DdpA precursor	36	58106
	OHA_1_00218	Hypothetical protein	35	75858
	OHA_1_03420	Bacterial extracellular solute-binding protein	31	63897
	OHA_1_00390	Glutamine synthetase 1	26	52714
	OHA_1_02452	Hypothetical protein	23	13473

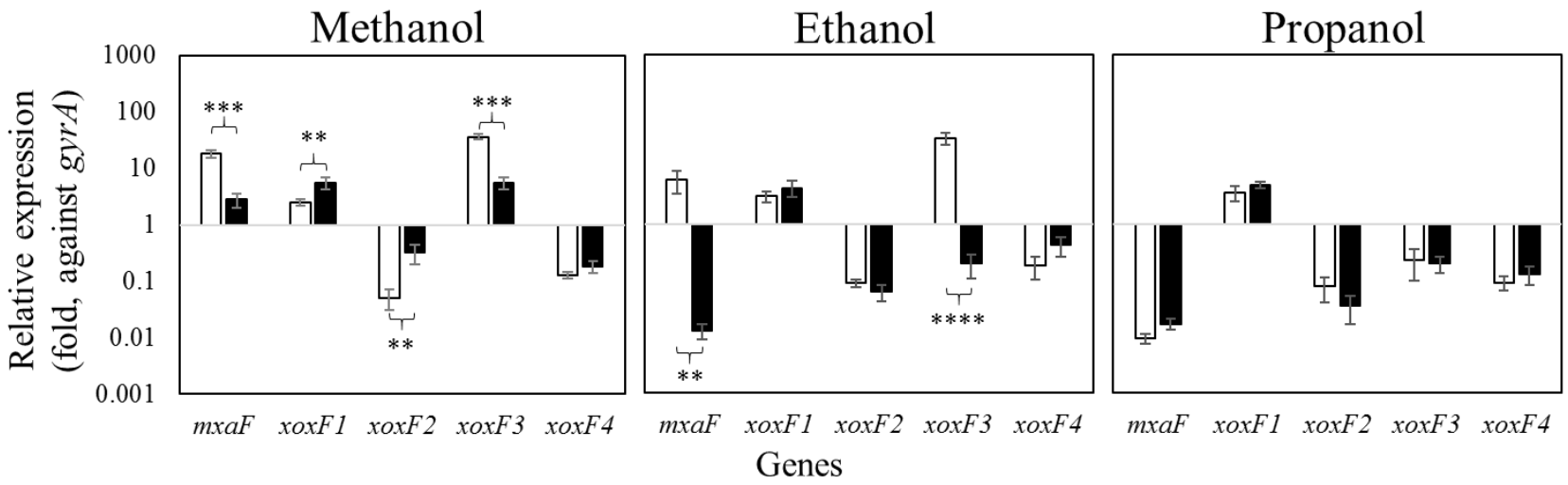

Fig. 1-9. Relative expression levels of MDH homologue genes in strain SM30^{T} grown on 0.5% (v/v) methanol, 0.5% (v/v) ethanol and 0.5% (v/v) propanol in the absence (white bar) and presence (black bar) of 30 μM La^{3+}, respectively, measured by quantitative PCR. Their expression level was normalized to that of *gyrA*. Values are average values ± standard deviation (biological replicates, n=3). Statistical significance between samples with or without La^{3+} were tested using Two-way RM ANOVA and Tukey's multiple comparisons test. ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

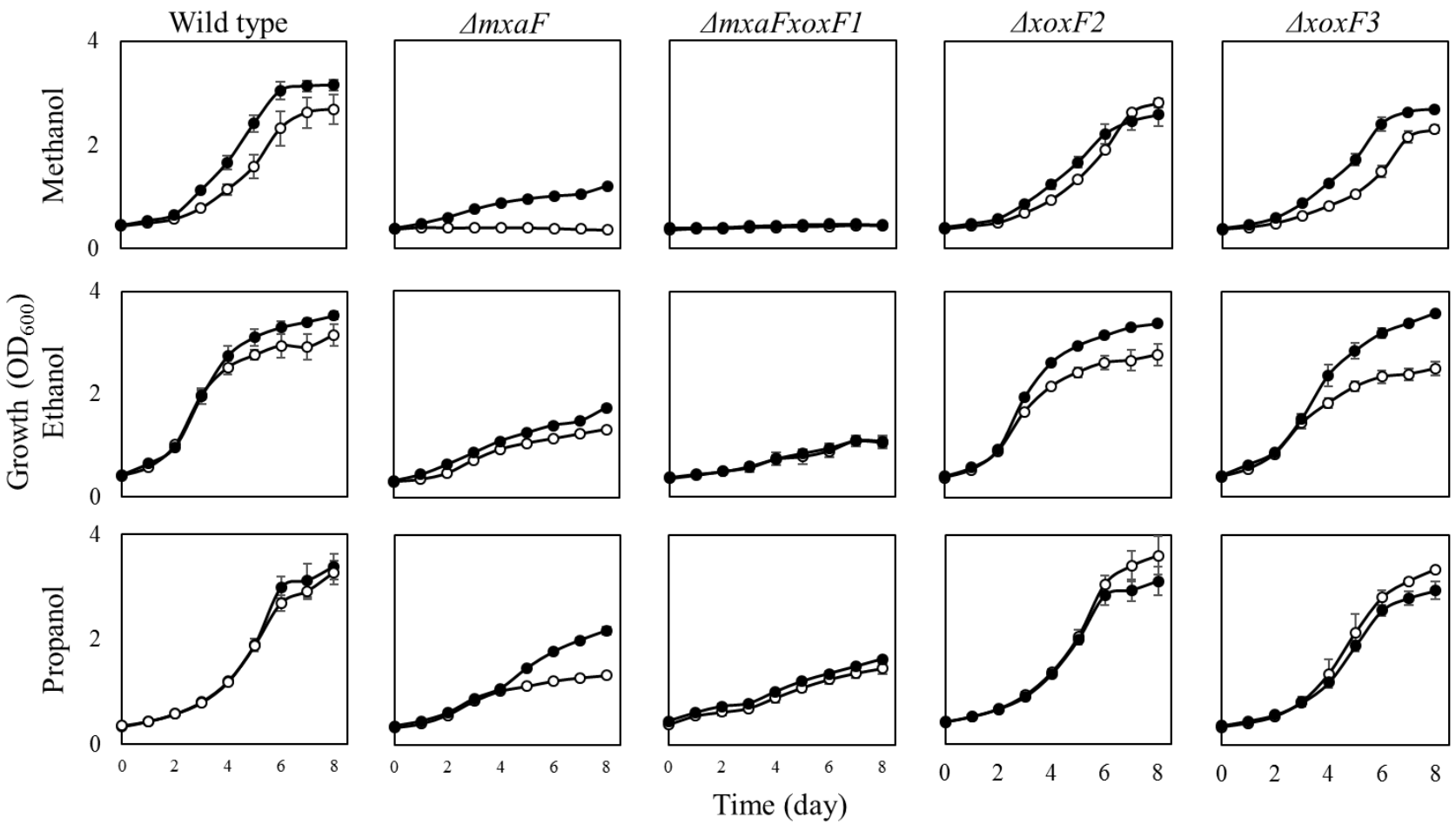

Fig. 1-10. Growth of the wild-type, *ΔmxaF*, *ΔxoxF2*, *ΔxoxF3*, and *ΔmxaFxoxF1* of strain SM30^{T} on 0.5% (v/v) methanol, 0.5% (v/v) ethanol and 0.5% (v/v) propanol in the absence (white circle) and presence (black circle) of 30 μM La^{3+}, respectively. Values are average values ± standard deviation (biological replicates, n=3).

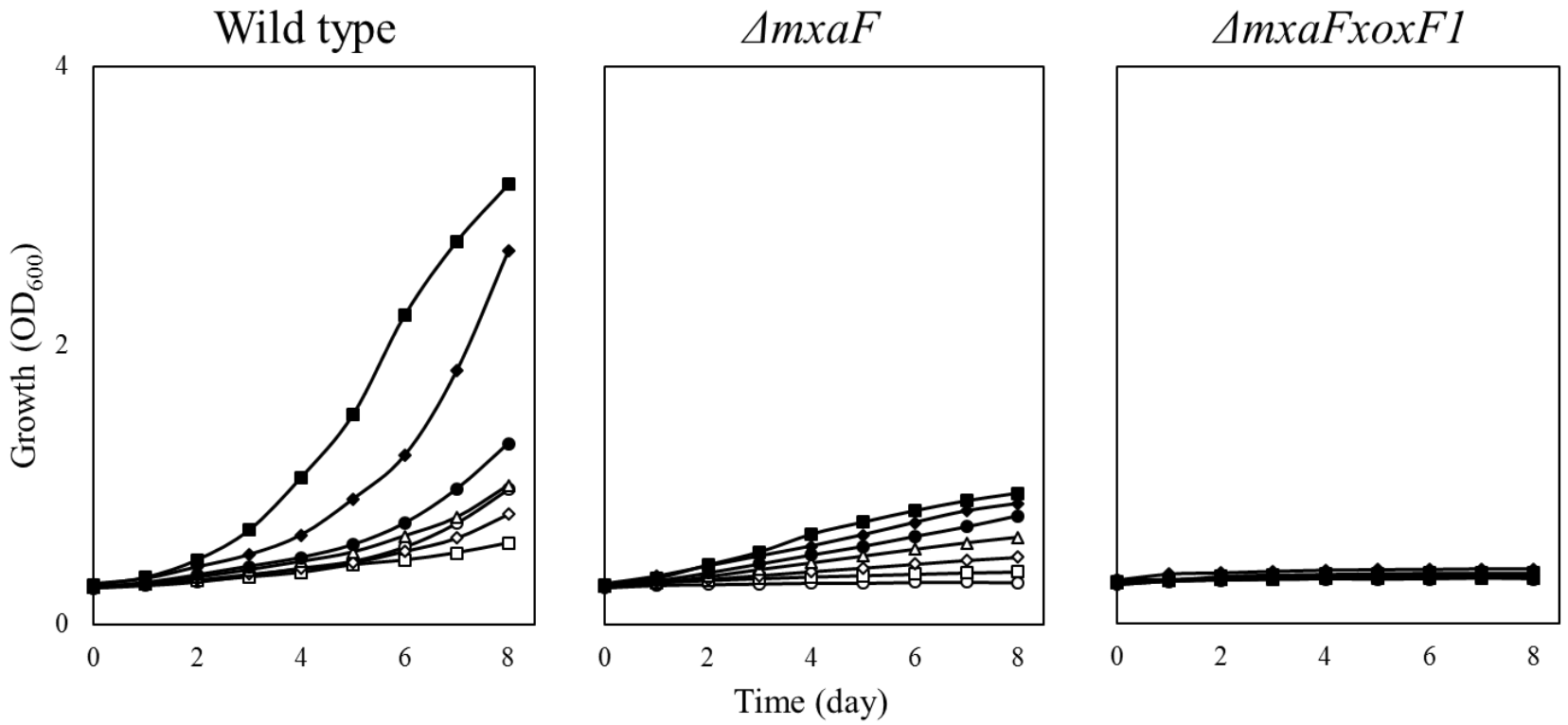

Fig. 1-11. Growth of the wild-type, *ΔmxaF*, and *ΔmxaFxoxF1* of strain SM30^{T} in MM containing 0.5% (v/v) methanol and 1 mM Ca^{2+} with varied concentrations of La^{3+}, 0 nM (○), 10 nM (□), 100 nM (◇), 1 μM (△), 10 μM (●), 30 μM (■) and 50 μM (◆). Standard deviation is not shown due to complexity but relative standard deviation is less than 29.9%.

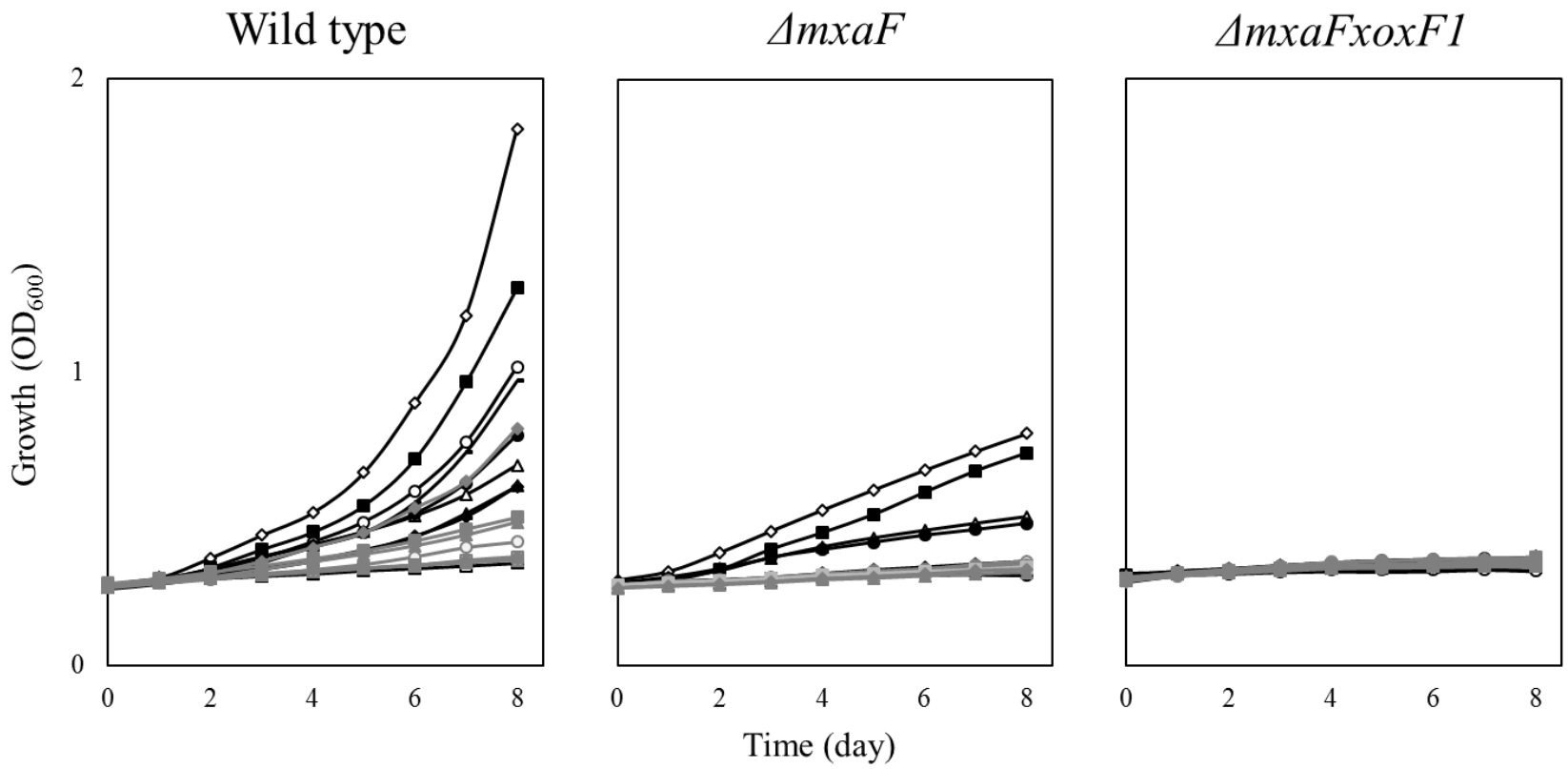

Fig. 1-12. The growth of the wild-type, *ΔmxaF*, and *ΔmxaFxoxF1* of *O. diazotrophicus* SM30^{T} in MM containing 0.5% methanol and 1 mM Ca^{2+} (-) with 30 μM different rare earth elements, Sc^{3+} (○), Y^{3+} (□), La^{3+} (◇), Ce^{3+} (△), Pr^{3+} (●), Nd^{3+} (■), Sm^{3+} (◆), Eu^{3+} (▲), Gd^{3+} (○), Tb^{3+} (□), Dy^{3+} (◇), Ho^{3+} (△), Er^{3+} (●), Tm^{3+} (■), Lu^{3+} (◆) and Yb^{3+} (▲). Standard deviation is not shown due to complexity but relative standard deviation is less than 19.1%.

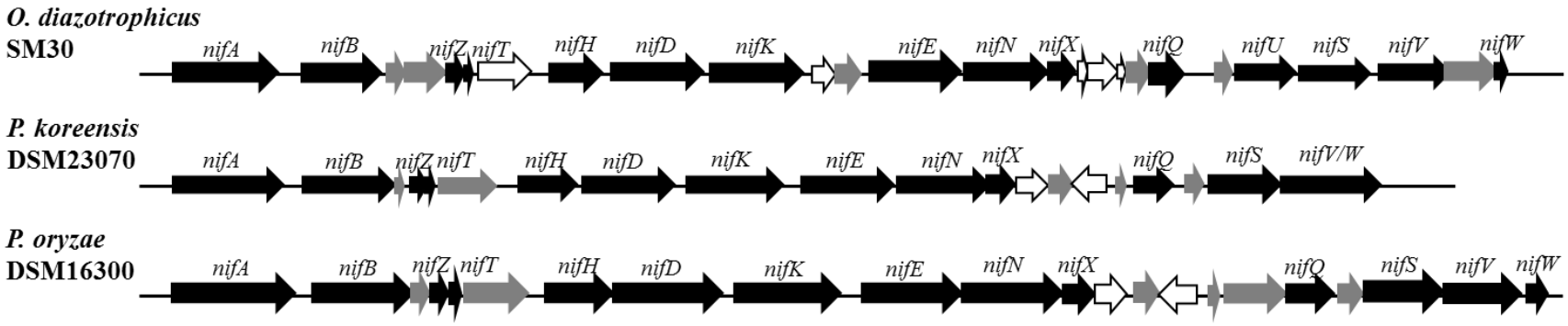

Fig. 1-13. Genomic organization of *nif* genes in strain SM30^T and *Pleomorphomonas* species. Black, gray, and white arrows show genes for structural proteins for nitrogenase, those for proteins with known function, and those for hypothetical proteins, respectively.

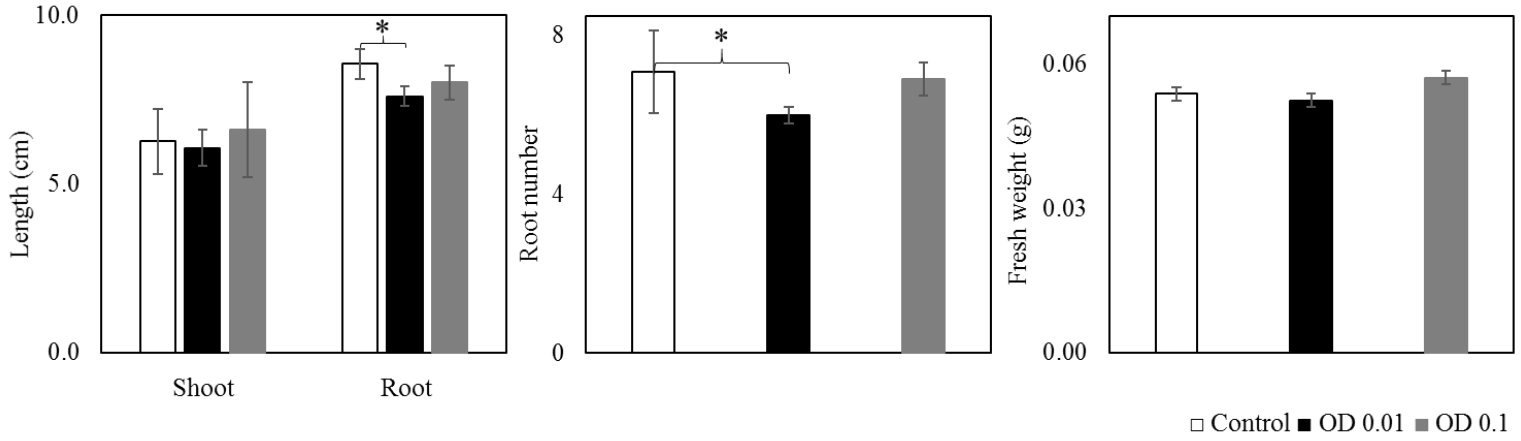

Fig. 1-14. The growth of rice plants with or without strain SM30^T in the absence of nitrogen. * $p < 0.05$ (one-way ANOVA and Bartlett's test).

Table 1-11. Metabolic modules of one-carbon metabolism between strain SM30^{T} and related species.

Strains	Accession number	16S rRNA gene identity (against SM30^{T})	Methane monoxygenase	Methanol oxidation		PQQ synthesis	H_4MPT pathway	H_4F pathway	Formate oxidation	Serine cycle	Methylamine dehydrogenase	Glutamate-mediated methylamine utilization pathway	TCA cycle	EMP pathway	nitrogenase	urease
				mxaF	*xoxF*											
SM30^{T}	AP017626-AP017628	-	-	+	+ (4)	+	+	+	+	+	-	+	+	+	+	+
Mongoliimonas terrestris MIMtkB18^{T}	NZ_MSIG01000001	96.30%	-	+	+ (1)	+	+	+	+	+	-	+	+	+	+	+
Methylobrevis pamukkalensis PK2^{T}	NZ_MCRJ01000001	94.07%	-	-	+ (2)	+	+	-	+	+	-	+	+	-	-	+
Pleomorphomonas oryzae DSM16300^{T}	NZ_AUHB01000001	94.03%	-	-	-	-	-	+	+	-	-	-	+	-	+	+
Hartmannibacter diazotrophicus E19^{T}	LT960614	93.87%	-	-	+ (2)	+	+	+	+	+	-	+	+	+	+	+
Pleomorphomonas koreensis DSM23070^{T}	NZ_AULH01000001	93.81%	-	-	-	-	-	+	+	-	-	-	+	-	+	+
Prosthecomicrobium hirschii 16^{T}	NZ_LJYW01000001	93.16%	-	+	+ (1)	+	+	+	+	+	+	+	+	+	+	+

Table 2-1. Samples used in this study.

Samples	Number of isolates	
	La^{3+}	Ho^{3+}
Oryza sativa cultivar Akitakomachi root soil	3	7 (Ho1-7)
Ammannia coccinea Rottb. root soil	3	4
Artemisia sp. root soil	4	4
Breeding wheat root soil	3	4
Triticum aestivum L. cultivar Chinese spring leaves	3	1
Triticum aestivum L. cultivar Chinese spring root soil	4	1
Cyperus difformis L. root soil 1	3	5
Cyperus difformis L. root soil 2	5	4
Digitaris (Retz.) Koeler root soil	3	5
Equisetum arvense L. root soil 1	6	4
Equisetum arvense L. root soil 2	7	4
Heliotropium arborescens L. root soil	3	5
Hordeum bulgare L. cultivar Haruna nijo soil (5 cm)	1	5
Lemna japonica landolt root soil	5	5
Monochoria vaginalis (Burm.f.) C. Presl ex Kunth var. vaginalis root soil	3	2
Hordeum bulgare L. cultivar Morex root soil 1	5	3
Hordeum bulgare L. cultivar Morex root soil 2	5	4
Triticum aestivum L. cultivar Norin No. 61 leaves	4	4
Triticum aestivum L. cultivar Norin No. 61 root soil	6	4
Paddy mud soil (5 cm)	1	4
Papaver dubium root soil 1	5	8
Papaver dubium root soil 2	5	5
Paspalum dilatatum Poir. root soil 1	4	5
Paspalum dilatatum Poir. root soil 2	5	5
Porttulaca oleracea L. root soil	3	2
Oryza sativa (Purple rice) root soil	4 (La2-4^{T})	5
Rice field soil 1	4 (La3113)	4
Rice field soil 2	4	4
Sedum sp. root soil	2	2
Sewage soil	4	2
Triadica sebifera root soil 1	2	2
Triadica sebifera root soil 2	3	5 (Ho17-2)
Trifolium repens root soil	2	4
Wild land soil	4	4
Wild mushroom root soil	2 (La20-1)	2

Table 2-2. The primer sets for real time PCR used in this paper.

Targets	NMK number	Forward primer	Reverse primer	Product length (bp)	TmF (°C)	TmR (°C)
gyrB	NMK_1321	TCCTTCGCGCCATTCTCGAT	GTATGGCTGCCGTGCAGTTG	103	61	61
xoxF1	NMK_0372	ACTGCAATTGGCCTGGACCT	TTGGGATAGGGCGTCACCAC	102	61	61
xoxF2	NMK_1556	ACGCCAACGTCAAAGGCATC	TGGTTGAGCGGCACGTAGAA	103	61	61
xoxF3	NMK_2270	TGCTGTGCTGCGATAACGTG	CCAGACTTTCTCGCCGGTCT	116	60	61
xoxF4	NMK_2902	TCGTCCTGGCGACAACAAGT	CCGTCGTAGTCCCACTCGTC	111	61	61
xoxF5	NMK_3457	TCACCTGGGCTTCGGGATTC	GCGGCGAAGGACAGATGTTG	105	61	61

Table 2-3. Accession numbers of amino acid sequences used in MLSA maximum likelihood phylogenetic tree.

Strains	RpoB	GyrB	InfB	AtpD
La2-4^T	NK_3644	NK_1321	NK_3517	NK_1304
Methylotenera versatilis 301^T	WP_013147033	WP_013146710	WP_013146825	WP_013149440
Methylotenera mobilis JLW8^T	WP_015831290	WP_012777371	WP_012777578	WP_015833266
Methylobacillus flagellatus KT^T	WP_011478640	WP_011478371	WP_011478435	WP_011480960
Methylovorus glucosetrophus SIP3-4	WP_013441123	WP_015829058	WP_015829107	WP_013443517
Methylophilus sp. TWE2	WP_049637760	WP_049637360	WP_049639543	WP_049639664
Methylovorus sp. MP688	WP_013441123	WP_041361886	WP_013440895	WP_013443517

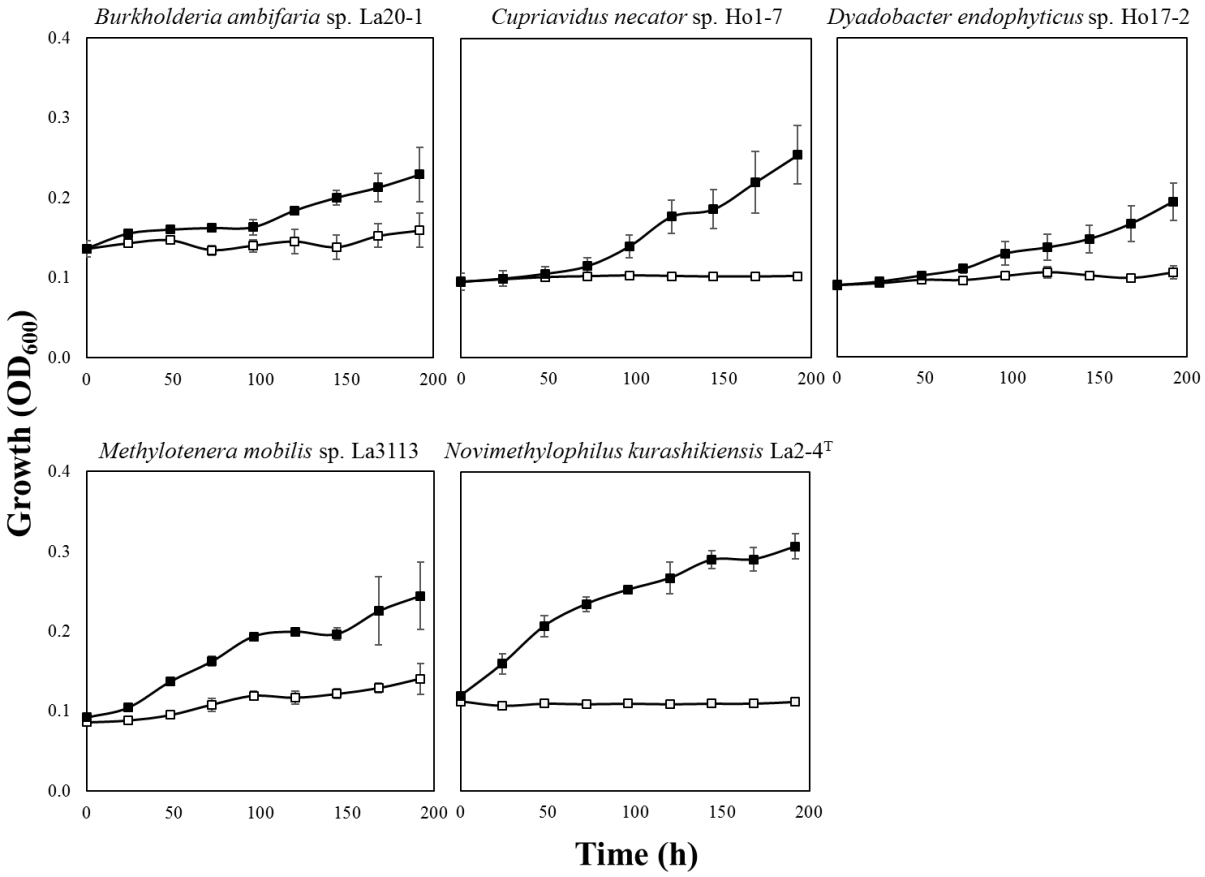

Fig. 2-1. Growth of strains La20-1, Ho1-7, Ho17-2, La3113, and La2-4^T in liquid NMS medium with 0.5% (v/v) methanol in the absence (□) or presence (■) of 30 μM La^{3+}. Data are presented as an average of technical triplicate preparations with standard deviation.

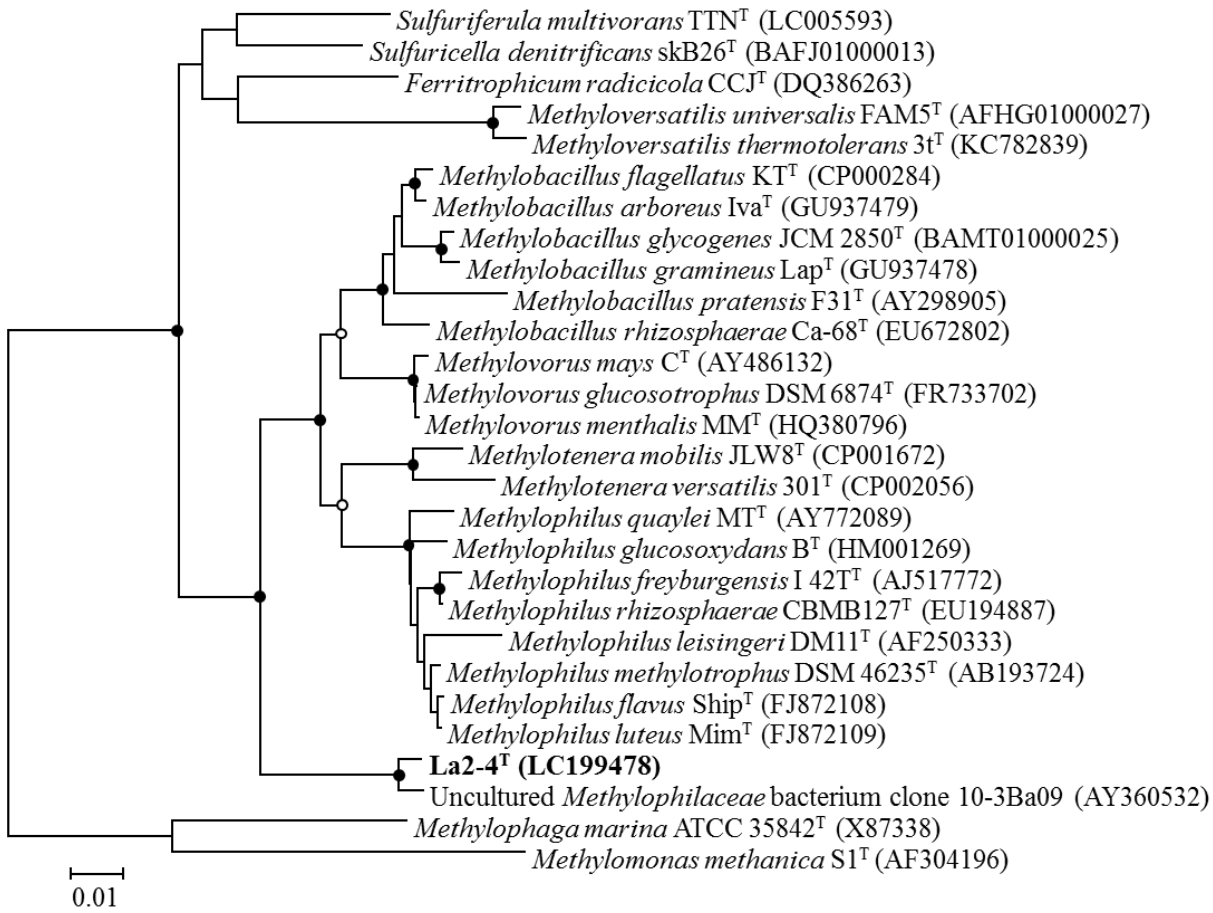

Fig. 2-2. The neighbor-joining tree based on 16S rRNA gene sequences for the relationship of the new genus within the family *Methylophilaceae*, order *Methylophilales*, class *Betaproteobacteria*. The evolutionary distances were computed using the Kimura 2-parameter method. Circles indicate consensus bootstrap values from neighbor-joining analysis. Nodes supported at ≥90% in the majority of analyses are indicated by filled circles. Nodes supported at 70 to 90% in most analyses are indicated by open circles. Unsupported nodes (≤70%) have no circles. There were a total of 1305 positions in the final dataset. Bar, 0.01 substitutions per site.

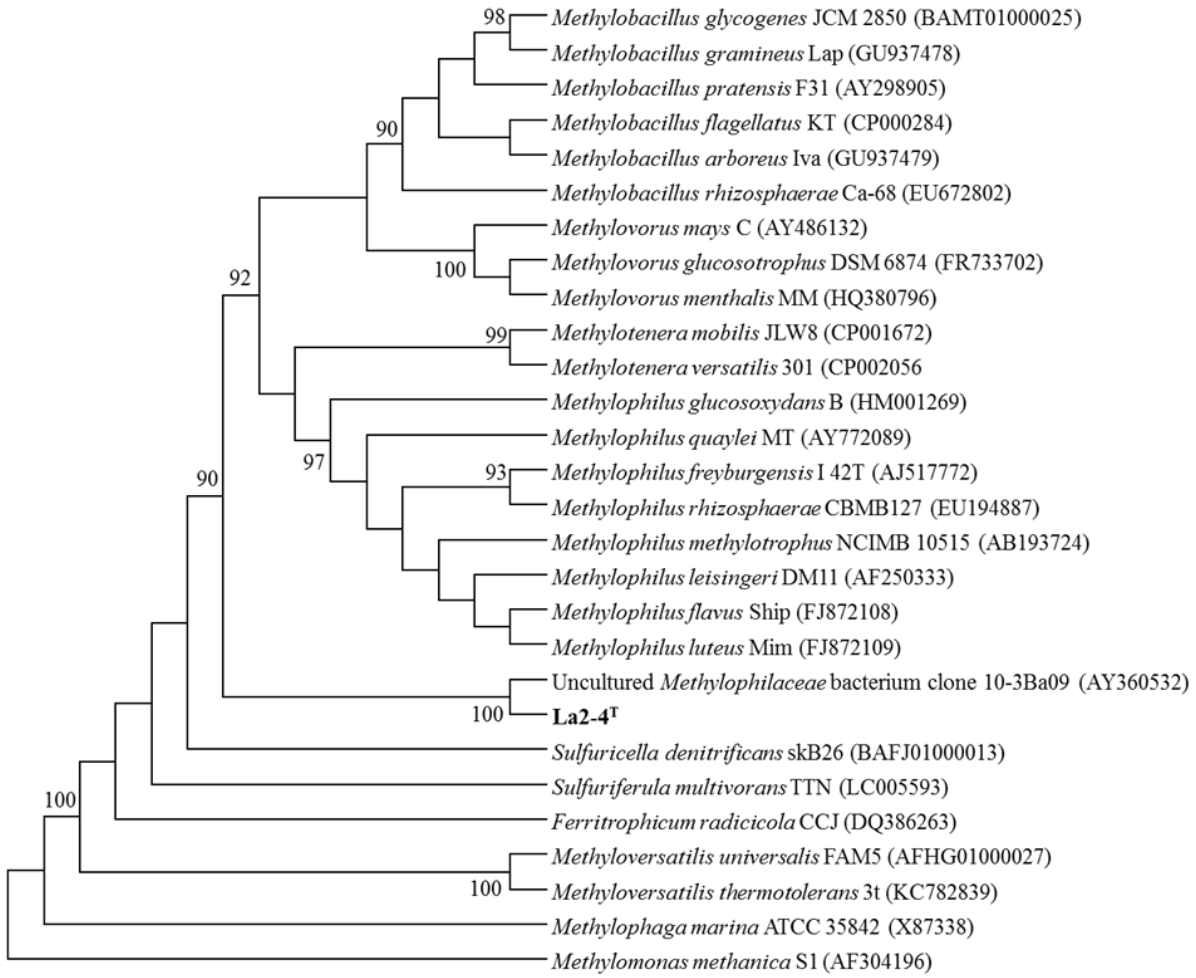

Fig. 2-3. Maximum-parsimony tree based on 16S rRNA gene sequences of strain La2-4^T and other related type strains, calculated using the close-neighbor interchange (CNI) algorithm. Bootstrap values greater than 70% are listed as percentages at the branching points.

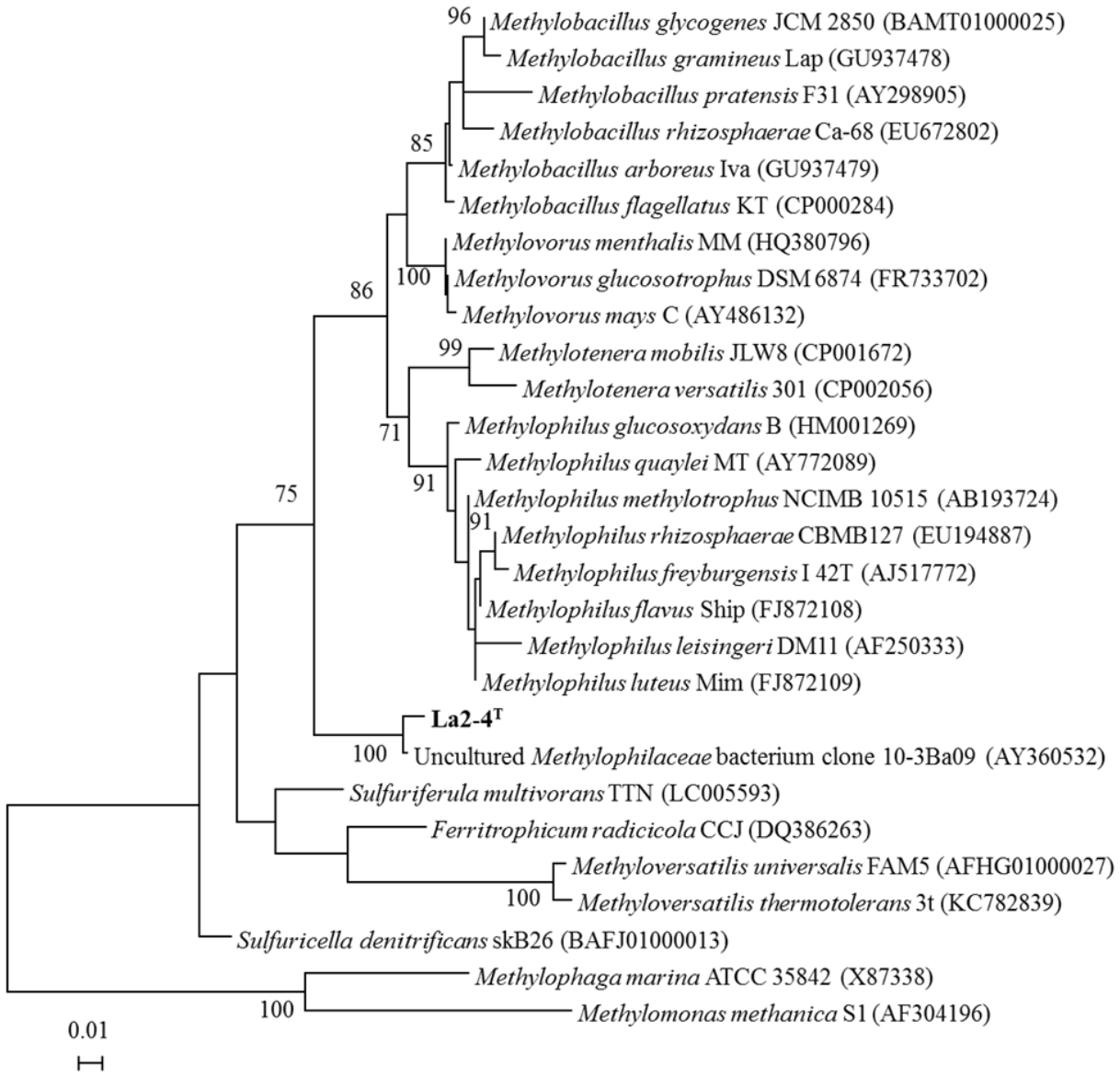

Fig. 2-4. Maximum likelihood phylogeny of 16S rRNA gene sequences of strain La2-4^T and other related type strains. Bootstrap values greater than 70% are listed as percentages at the branching points. Bar, 0.01 substitutions per site. The topology was inferred using the phyML method with the GTR model, with a gamma distribution and a proportion of invariable sites.

Table 2-4. Differential characteristics of strain La2-4[T] from species of *Methylophilus*, *Methylotenera*, *Methylobacillus*, and *Methylovorus*.

Characteristic	La2-4[T]*	*Methylophilus methylotrophus* DSM 46235[Ta]	*Methylophilus luteus* Mim[Tb]	*Methylotenera mobilis* JLW8[Tc]	*Methylobacillus glycogenes* JCM 2850[Td]	*Methylovorus glucosotrophus* DSM 6874[Te]
Isolation source	Rhizosphere soil of purple rice plant	Activated sludge	Phyllosphere of coltsfoot (*Tussilago farfara* L.)	Lake Washington sediment	Soil	Wastewater
Cell size (μm)	0.5-0.7 × 1.3-2.2	0.3-0.6 × 0.8-1.5	0.2-0.3 × 1.9-2	0.3-0.4 × 0.6-1.2	0.5-0.8 × 1.0-1.6	0.5-0.6 × 1.0-1.3
Motility	motile	motile *	non-motile	motile	non-motile	motile
Urease activity	—	+ *	—	—	+	+
Nitrate reduction	+	+ *	—	—	+	not available
Temperature range for growth (°C)	15 - 40	10 - 40 *	24 - 26	10 - 40	20 - 37	20 - 45
pH range for growth	6 - 9	6 - 8 *	7.2 - 7.8	5 - 8.5	6 - 8	6.5 - 8.5
NaCl tolerance (%)	≤ 2	≤ 1 *	≤ 0.05	≤ 0.5	≤ 3	≤ 3
Utilization of:						
Methanol	+[§]	+	+	+ *[§]	+	+
Methylamine	+	+	—	+	+	—
D-Glucose	(+)	+	+	—	—	+
D-Fructose	—	(+)	—	—	—	—
L-Arabinose	—	—	not available	—	—	—
Citrate	—	(+)	not available	—	—	—

Continued Table 2-4

Characteristic	La2-4^{T}*	*Methylophilus methylotrophus* DSM 46235Ta	*Methylophilus luteus* Mim^{Tb}	*Methylotenera mobilis* JLW8^{Tc}	*Methylobacillus glycogenes* JCM 2850^{Td}	*Methylovorus glucosotrophus* DSM 6874Te
mxaF	—	+ **	+	— **	+ **	not available
xoxF	+	+ **	not available	+ **	+ **	not available
Major fatty acids (>10%)	$C_{16:0}$ and SF 3	SF 3 and $C_{16:0}$	$C_{16:0}$ and $C_{16:1}$	$C_{16:1}\omega7c$ and $C_{16:0}$	$C_{16:0}$ and $C_{16:1}$	$C_{16:0}$ and $C_{16:1}$
Predominant polar lipids	PG, PE, and PNL	PG and PE	PG and PE	PE	not available	PG, PE, and DFG
Quinone type	Q-8	Q-8	Q-8	not available	Q-8	Q-8
DNA G+C content (mol%)	56.1 **	49.6 **	54.5	44.1 **	53.4 **	55.8

a, Data from Jenkins et al., 1987; *b*, Data from Gogleva et al., 2010; *c*, data from Kalyuzhnaya et al., 2006; *d*, data from Urakami and Komagata, 1986; and *e*, data from Doronina et al., 2005.

*, this study; **, genome data; +, positive reaction; —, negative reaction; (+), weakly positive reaction; §, La-dependent; SF, summed feature.

PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; and PNL, phosphoaminolipid.

Table 2-5. Cellular fatty acid profiles of strain La2-4^T and phylogenetically related taxons.

Fatty acids	La2-4^T	*Methylophilus methylotrophus* DSM 46235^T *a*	*Methylophilus luteus* Mim^T *a*	*Methylotenera mobilis* JLW8^T *b*	*Methylobacillus glycogenes* JCM 2850^T *c*	*Methylovorus glucosotrophus* DSM 6874^T *d*
$C_{12:0}$	-	-	-	-	-	-
$C_{14:0}$	2.77	1.5	tr	-	1.1	0.81
$C_{15:0}$	-	0.6	tr	-	0.5	0.93
$C_{16:0}$	29.2	34.3	32.4	32	43.4	49.07
$C_{16:1}$ ω5c	-	-	-	-	-	-
$C_{17:0}$	-	tr	tr	-	0.5	tr
$C_{18:0}$	0.79	0.6	-	0.3	0.8	0.56
$C_{18:1}$	-	2	0.9	-	4.6	-
$C_{20:00}$	2.24	-	-	-	-	-
$C_{20:1}$ ω7c	tr	-	-	-	-	-
cyclo $C_{17:0}$	tr	1.8	3.2	-	1.3	9.95
$C_{10:0}$ 3-OH	4.11	1.1	0.6	-	5.3	-
$C_{15:0}$ iso 3-OH	1.3	-	-	-	-	-
SF 3	58.12	57.3	52.3	66	42.5	33.6
SF 8	0.64	-	-	0.7	-	1.67

a, Data from Gogleva et al., 2010; *b*, Kalyuzhnaya et al., 2006; *c*, Gogleva et al., 2011; and *d*, Govorukhina and Trotsenko, 1991. tr; Fatty acids less than 0.5%. -, not detected. Summed features (SF) are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. SF 3: $C_{16:1}$ ω7c/$C_{16:1}$ ω6c; SF 8: $C_{18:1}$ ω7c/$C_{18:1}$ ω6c.

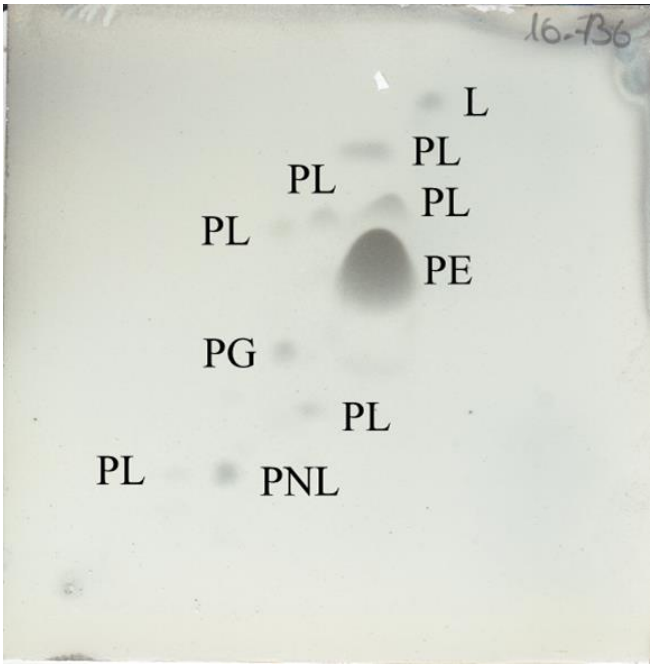

Fig. 2-5. A two-dimensional thin-layer chromatogram of polar lipids of strain La2-4^T: L, lipid; PL, phospholipid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; and PNL, phosphoaminolipid.

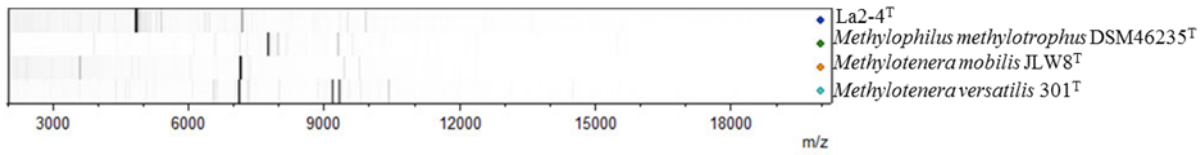

Fig. 2-6. Comparison of whole-cell protein profiles of strain La2-4^T and related type species of the facultatively methylotrophic genera (*Methylophilus methylotrophus* DSM 46235^T, *Methylotenera mobilis* JLW8^T, and *Methylotenera versatilis* 301^T) by MALDI-TOF/MS.

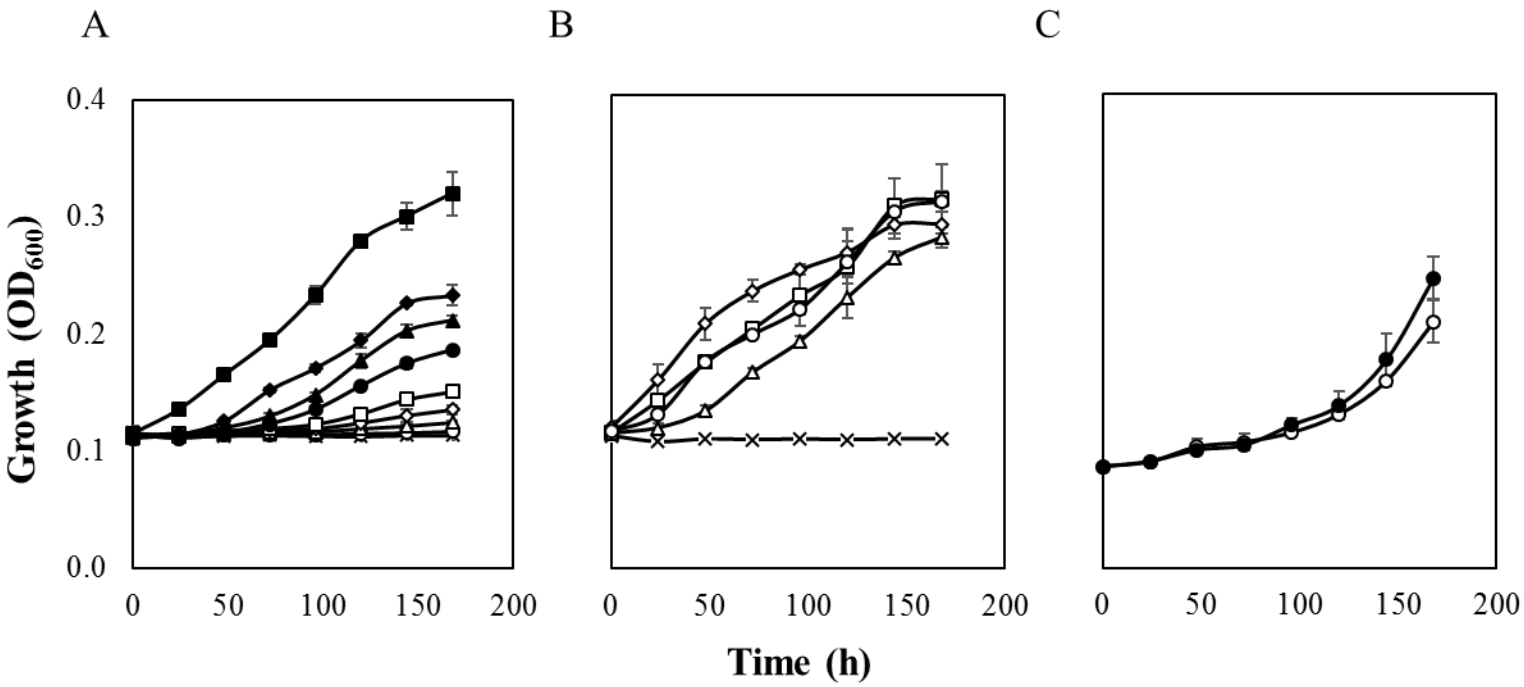

Fig. 2-7. Growth of strain La2-4^{T} in liquid NMS medium with: (A) 0.5% (v/v) methanol plus an additional 100 µM Ca^{2+} (×), 10 nM (○), 25 nM (△), 50 nM (◇), 100 nM (□), 250 nM (●), 500 nM (▲), 1 µM (◆), and 30 µM La^{3+} (■); (B) 0.5% (v/v) methanol plus an additional 100 µM Ca^{2+} (×), 30 µM Ce^{3+} (□), La^{3+} (◇), Nd^{3+} (△), and Pr^{3+} (○); (C) 0.1% (v/v) methylamine with (●) or without (○) 30 µM La^{3+}. Data are presented as an average of technical triplicate preparations with standard deviation.

Table 2-6. dDDH and ANI values between genomes of strain La2-4^T and closely related species.

Reference strains	Accession number	dDDH (Formula: 2)	ANI
Methylophilus methylotrophus DSM 46235^T	NZ_KB905141	16.30%	67.50%
Methylotenera mobilis JLW8^T	CP001672	18.70%	66.80%
Methylobacillus glycogenes JCM 2850^T	NZ_BAMT01000001	17.50%	69.00%
Methylovorus glucosetrophus SIP3-4	NC_012969	19.30%	69.80%

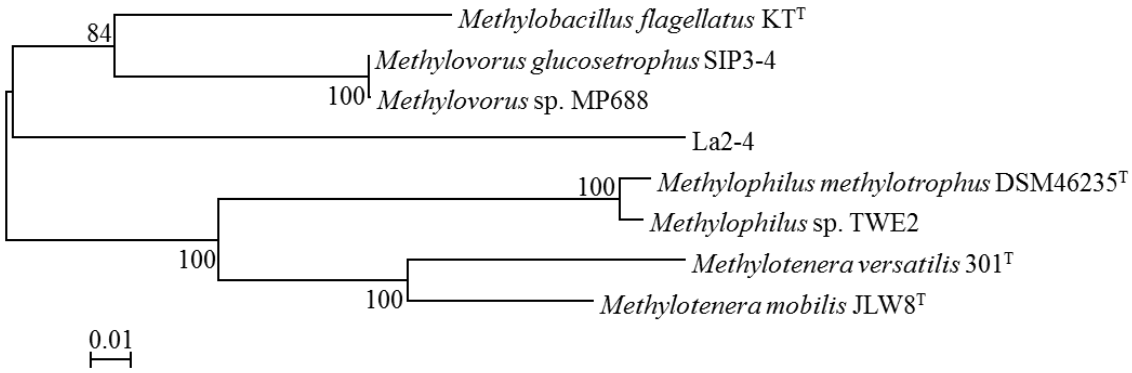

Fig. 2-8. MLSA-based maximum likelihood phylogeny of concatenated partial amino acid sequences of RpoB, GyrB, InfB, and AtpD of strain La2-4^T and other related type strains using the JTT algorithm (Accession numbers are listed in Table 2-3). Bootstrap values greater than 70% are listed as percentages at the branching points. Bar, 0.01 substitutions per site.

Table 2-7. Methylotrophy islands of *Novimethylophilus kurashikiensis* La2-4^T

Cluster	Scafold	Locus_tag (NMK_)	Start	End	Direction	Gene	Product
XoxF1	2	359	104529	104867	1		diguanylate cyclase
	2	360	104911	105159	-1		uncharacterized protein
	2	362	105355	108960	-1		diguanylate cyclase
	2	363	108957	109952	-1		NitT/TauT family transport system substrate-binding protein
	2	364	110109	110969	-1		multidrug transporter
	2	365	110992	111426	-1		glycolate utilization protein
	2	366	111502	111954	-1		thiosulfate transporter subunit
	2	367	111951	112574	-1	*coxC*	cytochrome c oxidase subunit III
	2	368	112567	114519	-1	*coxA*	cytochrome c oxidase subunit I
	2	369	114529	115527	-1	*coxB*	cytochrome c oxidase subunit II
	2	370	115529	116041	-1	*xoxG1*	cytochrome C
	2	371	116052	116897	-1		ABC transporter substrate-binding protein
	2	372	116894	118762	-1	*xoxF1*	methanol dehydrogenase
	2	373	118840	120261	1	*cydA*	cytochrome d ubiquinol oxidase subunit I
	2	374	120272	121273	1	*cydB*	cytochrome d ubiquinol oxidase subunit II
XoxF2	6	1555	26433	26645	-1		ABC transporter
	6	1556	26647	28569	1	*xoxF2*	methanol dehydrogenase
	6	1557	28672	29385	1		malonyl-(acyl-carrier protein) O-methyltransferase
	6	1558	29382	29810	1		polysaccharide synthesis protein GtrA
	6	1559	29807	30751	1		glycosyl transferase family 2
	6	1560	30755	32251	1		cyclopropane-fatty-acyl-phospholipid synthase
	6	1561	33581	34852	1	*metY*	O-acetylhomoserine (thiol)-lyase
	6	1562	35621	36808	-1		TonB-dependent receptor
XoxF3	9	2263	48317	48742	-1		GCN5 family acetyltransferase
	9	2264	48898	49704	1	*modE*	molybdate transport system regulatory protein
	9	2265	49764	50663	1		cytochrome C biogenesis protein
	9	2266	50787	51548	1	*modA*	molybdate transport system substrate-binding protein
	9	2267	51566	51994	1		glutamine--fructose-6-phosphate aminotransferase (isomerizing)
	9	2268	51984	52649	1	*modB*	molybdate transport system permease protein
	9	2269	52646	53521	1	*modC*	molybdate transport system ATP-binding protein
	9	2270	53771	55642	1	*xoxF3*	methanol dehydrogenase
	9	2272	56087	57157	-1		2-alkenal reductase
XoxF4	18	2900	19329	20612	-1		membrane protein
	18	2901	20609	21592	-1	*pip*	proline iminopeptidase
	18	2902	21833	23710	1	*xoxF4*	methanol dehydrogenase

Continued Table 2-7

Cluster	Scafold	Locus_tag (NMK_)	Start	End	Direction	Gene	Product
XoxF4	18	2903	23829	24755	1		ABC transporter substrate-binding protein
	18	2904	24851	25393	1	*xoxG4A*	cytochrome C
	18	2905	25508	26041	1	*xoxG4B*	cytochrome c-L
	18	2906	26063	27400	1	*ndh*	NADH dehydrogenase
	18	2907	27451	28623	-1		major facilitator transporter
	18	2908	28687	30087	-1	*phoH*	PhoH-like ATPase
	18	2909	30164	30619	-1	*bcp*	peroxiredoxin Q/BCP
	18	2910	30797	31237	1	*ligT*	2'-5' RNA ligase
	18	2911	31218	31973	-1	*tonB*	periplasmic protein TonB
	18	2912	32067	32996	-1	*gloB*	beta-lactamase
	18	2913	33118	33717	1	*cybB*	cytochrome b561
	18	2914	33779	34579	-1	*soxY*	sulfur-oxidizing protein SoxY
XoxF5	21	3451	65630	66313	-1		uncharacterized protein
	21	3452	66395	66931	-1		ubiquinone/menaquinone biosynthesis C-methyltransferase UbiE
	21	3453	66931	68205	-1		uncharacterized protein
	21	3456	68850	69215	-1	*xoxG5*	cytochrome C
	21	3457	69248	71071	-1	*xoxF5*	methanol dehydrogenase
	21	3458	71432	71710	1		uncharacterized protein
	21	3459	72179	73078	-1	*fdhE*	FdhE protein
	21	3460	73095	73733	-1	*fdoI2*	formate dehydrogenase subunit gamma
	21	3461	73730	74602	-1	*fdoH2*	formate dehydrogenase iron-sulfur subunit
	21	3462	74605	77151	-1	*fdoG2*	formate dehydrogenase major subunit
	21	3463	77200	77889	-1	*fdoG3*	formate dehydrogenase major subunit
PQQ synthesis	1	85	96502	97365	1		pyrroloquinoline-quinone synthase
	2	3674	60522	60593	1	*pqqA*	pyrroloquinoline quinone biosynthesis protein A
	2	315	60728	61642	1	*pqqB*	pyrroloquinoline quinone biosynthesis protein B
	2	316	61661	62416	1	*pqqC*	pyrroloquinoline-quinone synthase
	2	317	62394	62681	1	*pqqD1*	pyrroloquinoline quinone biosynthesis protein D
	2	318	62678	63868	1	*pqqE1*	pyrroloquinoline quinone biosynthesis protein E
	12	2526	26594	26884	1	*pqqD2*	pyrroloquinoline quinone biosynthesis protein D
	12	2527	26853	27989	1	*pqqE2*	pyrroloquinoline quinone biosynthesis protein E
	18	3201	135055	136413	1	*pqqF*	zinc protease
	18	3202	136403	137731	1	*pqqG*	zinc protease

Continued Table 2-7

Cluster	Scafold	Locus_tag (NMK_)	Start	End	Direction	Gene	Product
glutamate-mediated methylamine utilization pathway	3	1000	303944	304651	-1		glutamine amidotransferase
	3	1009	309815	311161	-1		glutamate synthase
	3	1010	311175	311858	-1		putative N-methyl glutamate synthase subunit B
	3	1011	311846	312742	-1		glutamine amidotransferase
	3	1012	312777	314111	-1	*glnA*	glutamine synthetase
	3	1013	314142	314819	-1	*soxC*	sarcosine oxidase subunit gamma
	3	1014	314821	317718	-1	*soxA*	sarcosine oxidase, subunit alpha
	3	1015	317715	317978	-1	*soxD*	sarcosine oxidase, subunit delta
	3	1016	317991	319232	-1	*soxB*	sarcosine oxidase, subunit beta
	3	1079	378142	379551	-1	*glnA*	glutamine synthetase
	3	1291	598396	603003	1	*gltB*	glutamate synthase (NADPH/NADH) large chain
	3	1292	603006	604469	1	*gltD*	glutamate synthase (NADPH/NADH) small chain
	3	1414	715945	717306	1	*glnA*	glutamine synthetase
	4	1440	50	904	-1	*purU*	formyltetrahydrofolate deformylase
	10	2487	69641	70384	-1		glutamate synthase
	18	2943	62638	63279	-1	*hisH*	glutamine amidotransferase
	19	3223	157675	158382	1		glutamine amidotransferase
H_4MPT-linked formaldehyde oxidation	2	336	79004	79810	1	*fwdC*	formylmethanofuran dehydrogenase subunit C
	2	337	79807	80727	1	*ftr*	formylmethanofuran--tetrahydromethanopterin N-formyltransferase
	2	338	80724	82400	1	*fwdA*	formylmethanofuran dehydrogenase subunit A
	2	339	82425	83705	-1	*fwdB*	formylmethanofuran dehydrogenase subunit B
	2	342	86419	87315	-1	*mtdB*	methylene-tetrahydromethanopterin dehydrogenase
	2	344	88402	89382	-1	*mch*	methenyltetrahydromethanopterin cyclohydrolase
	2	348	91992	92528	1	*fae*	5,6,7,8-tetrahydromethanopterin hydro-lyase
	2	349	92607	93233	1	*fae*	5,6,7,8-tetrahydromethanopterin hydro-lyase
	7	1930	76308	76844	1	*fae*	5,6,7,8-tetrahydromethanopterin hydro-lyase
	18	3017	130639	131553	-1	*mtdB*	methylene-tetrahydromethanopterin dehydrogenase

Continued Table 2-7

Cluster	Scafold	Locus_tag (NMK_)	Start	End	Direction	Gene	Product
RuMP cycle	2	498	264566	265477	1	*pgd*	6-phosphogluconate dehydrogenase
	2	499	265481	266962	1	*g6pd*	glucose-6-phosphate 1-dehydrogenase
	2	585	353312	354955	-1	*gpi*	glucose-6-phosphate isomerase
	3	1117	416129	416827	1	*hxlA*	3-hexulose-6-phosphate synthase
	22	3512	25826	26467	1	*hxlA*	3-hexulose-6-phosphate synthase
	22	3513	26559	27200	1	*hxlA*	3-hexulose-6-phosphate synthase
	22	3514	27326	27865	1	*hxlB*	6-phospho-3-hexuloisomerase
formate oxidation	7	1897	56116	56370	-1		formate dehydrogenase
	18	2922	43911	44132	-1	*fdsD*	formate dehydrogenase subunit delta
	18	2923	44176	45009	-1	*fdhD*	FdhD protein
	18	2924	45014	47884	-1	*fdoG1*	formate dehydrogenase major subunit
	18	2925	47899	49452	-1	*fdoH1*	formate dehydrogenase iron-sulfur subunit
	18	2926	49449	49934	-1	*fdoI1*	formate dehydrogenase subunit gamma
	21	3459	72179	73078	-1	*fdhE*	FdhE protein
	21	3460	73095	73733	-1	*fdoI2*	formate dehydrogenase subunit gamma
	21	3461	73730	74602	-1	*fdoH2*	formate dehydrogenase iron-sulfur subunit
	21	3462	74605	77151	-1	*fdoG2*	formate dehydrogenase major subunit
	21	3463	77200	77889	-1	*fdoG3*	formate dehydrogenase major subunit

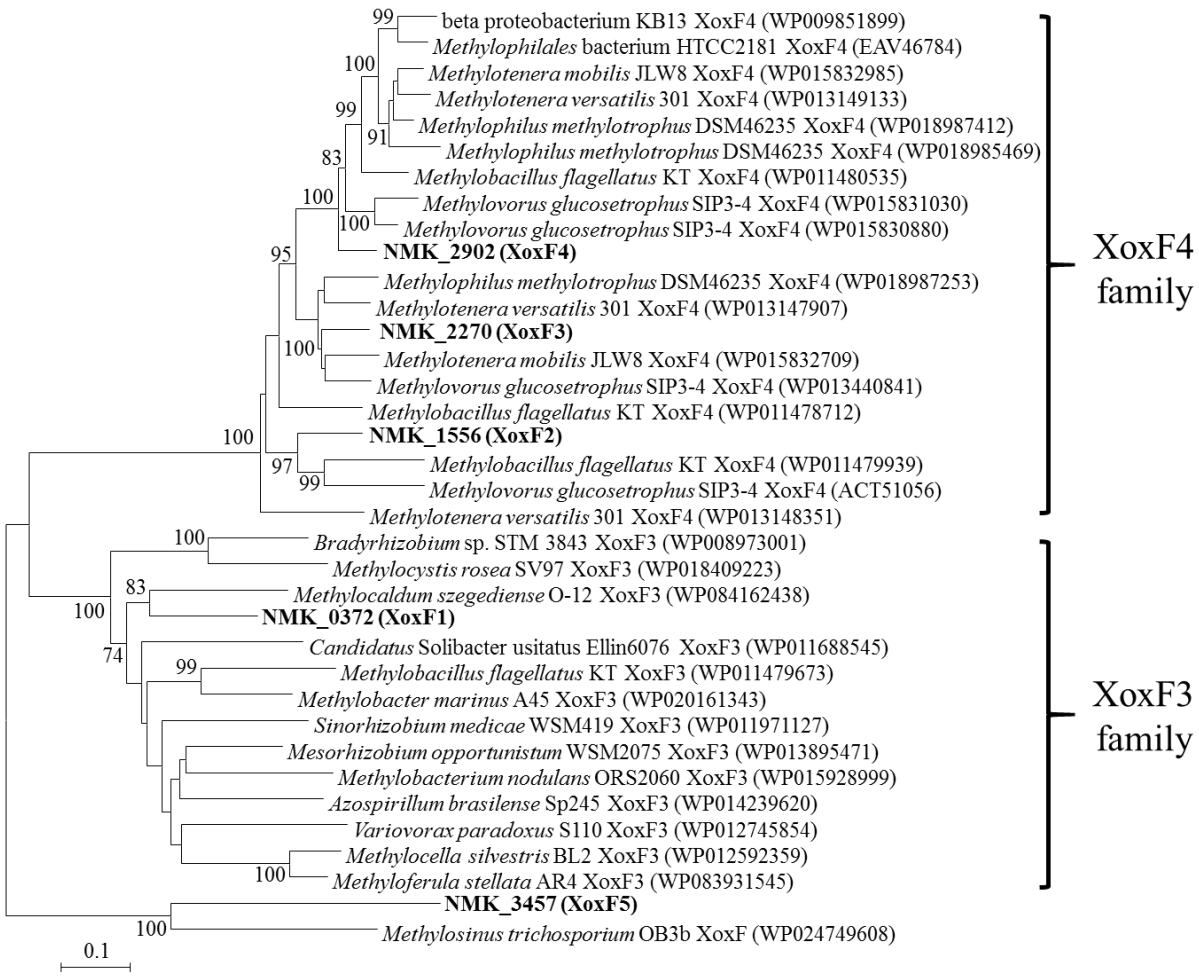

Fig. 2-9. Neighbor-joining phylogeny using the JTT algorithm of XoxF amino acid sequences of strain La2-4^T and other related type strains. Bootstrap values greater than 70% are listed as percentages at the branching points. Bar, 0.1 substitutions per site.

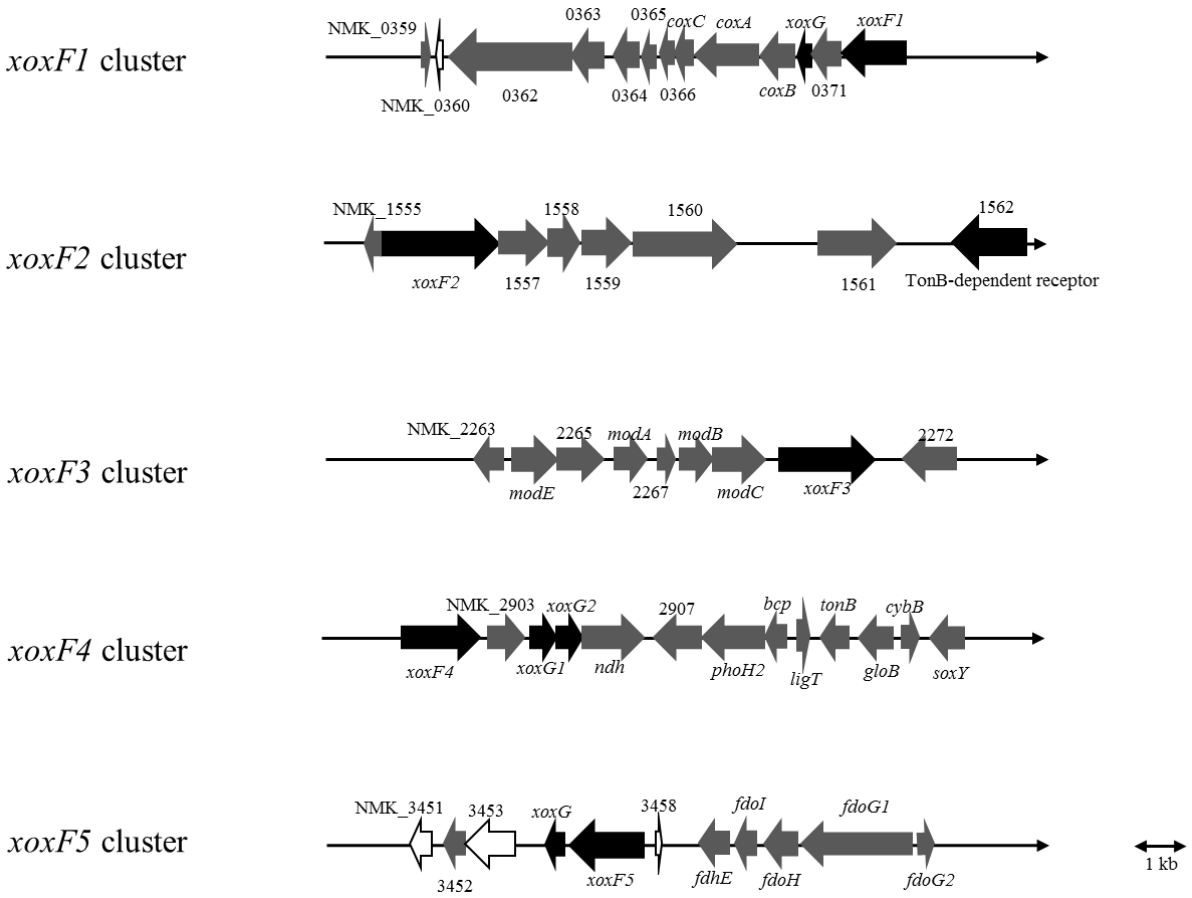

Fig. 2-10. Genomic organization of *xoxF* genes in strain La2-4^{T}. Black arrows show genes for structural proteins for MDH, gray arrows show genes for proteins with known function, and white arrows show genes for protein with unknown function.

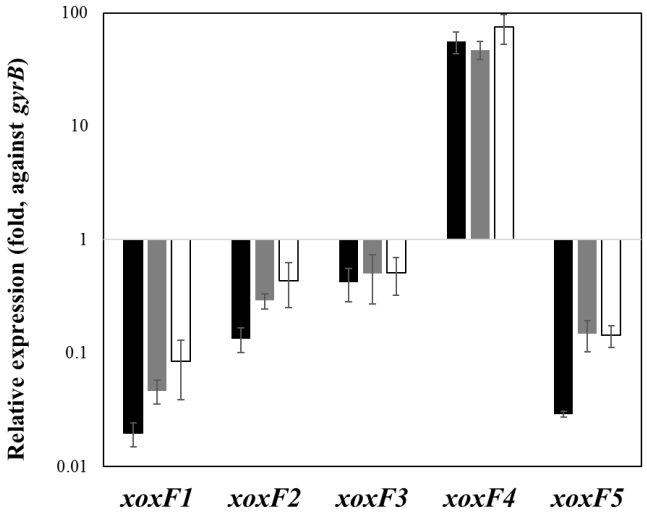

Fig. 2-11. Relative mRNA levels of *xoxF* genes in La2-4^T grown in liquid NMS medium containing 0.5% (v/v) methanol and 30 µM La^{3+} (black bar), 0.1% (v/v) methylamine and 30 µM La^{3+} (gray bar), or 0.1% (v/v) methylamine without 30 µM La^{3+} (white bar) measured by real-time PCR. Their expression level was normalized to that of *gyrB*. Values are average values ± standard deviation (biological replicates, n=3).

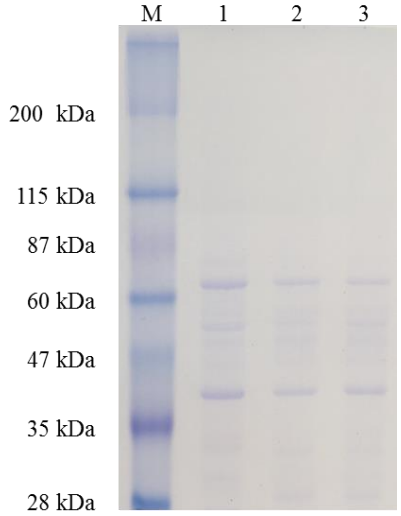

Fig. 2-12. SDS-PAGE analysis of La2-4^T cell-free extracts growing under three conditions: 1 (NMS+MeLa), 2 (NMS+MALa), and 3 (NMS+MA). M, protein marker (nacalai tesque). The total protein amount of each sample was 0.544 µg.

Table 2-8. LC-MS analysis of major protein bands of La2-4^{T} growing in methanol in the presence of La^{3+}.

Protein	Hits	Annotation	Score [a]	Mass (Da)
63 kDa	NMK_1763	chaperonin GroEL	828	57673
	NMK_2902	methanol dehydrogenase	726	67898
	NMK_1079	glutamine synthetase	355	52085
	NMK_2206	type II and III secretion system protein	50	53648
	NMK_3103	histidine kinase	38	50142
	NMK_1306	F-type H^{+}-transporting ATPase subunit alpha	29	55243
	NMK_2655	hydroxyacid dehydrogenase	27	39515
	NMK_1091	uncharacterized protein	27	29729
	NMK_0974	histidine kinase	25	51748
	NMK_1126	phosphoglycerate kinase	25	43094
	NMK_2080	N-acetyl-gamma-glutamyl-phosphate reductase	25	30452
40 kDa	NMK_0924	uncharacterized protein	1418	41602
	NMK_0161	transaldolase	1010	34863
	NMK_0156	peroxiredoxin	144	21736
	NMK_2702	methyl-accepting chemotaxis protein	38	59991
	NMK_1079	glutamine synthetase	32	52085
	NMK_2270	methanol dehydrogenase	28	68487
	NMK_0916	hypothetical protein	27	11160
	NMK_1497	thiazole synthase	26	27600
	NMK_0059	uncharacterized protein	25	38244
	NMK_2036	iron-sulfur cluster carrier protein	23	78285

a, score $\geq$ 23 is statistically significant hit.

Table 2-9. Metabolic modules of one carbon metabolism between La2-4^T and related species.

Strains	Genome	Methanol oxidation		H_4MPT pathway	RuMP cycle	Methylamine dehydrogenase	Glutamate-mediated methylamine utilization pathway
		mxaF	*xoxF*				
La2-4^T	BDOQ01000001 to BDOQ01000032	-	+	+	+	-	+
Methylotenera versatilis 301^T	NC_014207	-	+	+	+	-	+
Methylotenera mobilis JLW8^T	NC_012968	-	+	+	+	+	-
Methylobacillus flagellatus KT^T	NC_007947	+	+	+	+	+	+
Methylovorus glucosetrophus SIP3-4	NC_012969	+	+	+	+	-	+
Methylophilus sp. TWE2	CP012020	+	+	+	+	+	+
Methylovorus sp. MP688	NC_014733	+	+	+	+	-	+

Table A-1. Kimura B nutrient solution (Ma et al., 2001).

Macronutrients	Concentration (mM)
$(NH_4)_2SO_4$	0.18
$MgSO_4 \cdot 7H_2O$	0.27
KNO_3	0.09
$Ca(NO_3)_2 \cdot 4H_2O$	0.18
KH_2PO_4	0.09

Micronutrients	Concentration (µM)
$NaEDTA\text{-}Fe \cdot 3H_2O$	20
$MnCl_2 \cdot 4H_2O$	6.7
H_3BO_3	9.4
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.015
$ZnSO_4 \cdot 7H_2O$	0.15
$CuSO_4 \cdot 5H_2O$	0.16

Final pH 5.5, without adjustment.

Fig. A-1. Experimental setup

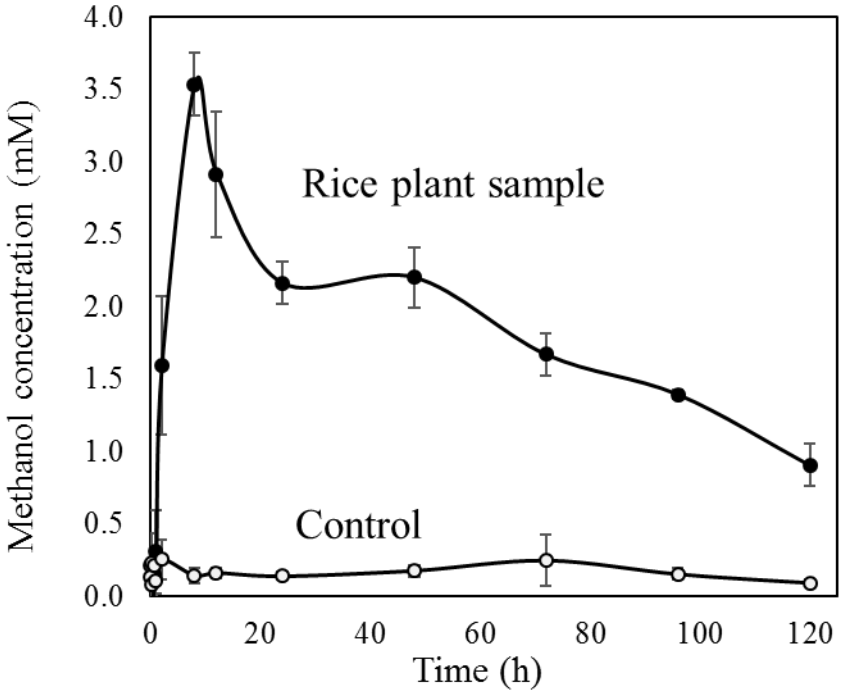

Fig. A-2. Methanol exudation from rice roots.

Table A-2. The soluble concentration of 16 Ln^{3+} and 23 common metals in soil sample of IPSR.

	Concentration (nM)			
	soil : water			
REEs	1:5	1:25	1:125	1:625
Sc	15	9.3	5.3	0.7
Y	11.2	5.8	3.0	0.9
La	5.5	3.6	2.7	1.7
Ce	11.4	6.1	3.0	0.4
Pr	1.4	0.8	0.4	0.1
Nd	5.5	2.7	1.2	0.1
Sm	1.2	0.8	0.4	0.1
Eu	0.3	0.1	0.1	0
Gd	1.2	0.6	0.4	0.1
Tb	0.2	0.1	0.1	0
Dy	1.0	0.5	0.3	0
Ho	0.4	0.3	0.2	0.2
Er	0.7	0.3	0.1	0
Tm	0.1	0.1	0	0
Yb	0.8	0.4	0.1	0
Lu	0.1	0.1	0	0

	Concentration (nM)			
	soil : water			
Metals	1:5	1:25	1:125	1:625
Ca	162000	71900	56000	43800
Si	106000	45800	68600	15100
K	106000	42300	19300	7980
Na	94400	8530	181000	0
Al	25500	16600	13800	1770
Mg	25200	8480	3480	2430
P	24000	17500	11100	4300
Fe	10100	5710	3390	930
Mn	1800	500	116	60.6
Zn	781	265	132	116
Cu	651	346	155	81.7
B	455	0	32700	834
Ti	443	254	262	73.4
V	188	220	146	35.3
Li	184	0	0	0
Cr	172	151	148	136
Ba	162	46.9	29.7	7.3
Ni	83.8	56.9	44.1	39.4
Co	19.4	6.4	2.2	1.2
Pb	16.8	8.4	4.4	0.5
Sb	11.1	4.5	1.7	0.6
Cd	2.6	0.8	0.2	0.1
Mo	1.5	0	0	0