Physiological Role of Lanthanide-dependent Methylotrophy in Plant Symbiotic Bacteria

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（植物共生細菌におけるランタノイド依存型メタノール代謝系の生理的役割）

2019

The United Graduate School of Agricultural Science, Gifu University

Science of Biological Resources

(Gifu University)

WANG LUN

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INTRODUCTION

Methylotrophic bacteria in sensu lato are a group of microorganisms that are able to utilize single-carbon compounds such as methane and methanol as their sole carbon source [1,2]. Methylotrophic bacteria in sensu stricto use methanol (and other methylated compounds including methylamine and chlorinated methane) for this purpose and are among the most abundant microorganisms in the phyllosphere, such like *Methylobacterium* and *Bradyrhizobium* of bacterial genera, are considered as plant symbiotic bacteria [3,4]. Those living on leaves utilize methanol that is transpired from the leaves [5] and provides some plant hormone-like functional compounds such as pyrroloquinoline quinone (PQQ) to their host plants [6]. *Methylorubrum extorquens* strain AM1, the first methylotrophic bacterium to be isolated from the air [7], has been investigated for more than half a century as the model strain of the Gram-negative methylotrophic bacteria [8]. In the case of *Methylorubrum extorquens* AM1, which was reclassified from the genus *Methylobacterium* [9], genetic mutations led to insufficient methylotrophic growth [10,11]. Moreover, since MxaFI requires Ca^{2+} as a cofactor [12], it has been believed that Ca^{2+} is an exclusive essential factor for methanol metabolism by methylotrophic bacteria. The pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (MDH) encoded by *mxaFI* is widely distributed in methylotrophic bacteria [13]. And also, in methanol metabolism of the Gram-negative methylotrophic bacteria, the PQQ-dependent methanol dehydrogenase (MDH) is one of the keys and essential enzymes [2,13,14].

It was formerly believed that Ca^{2+}-dependent MDH, which is encoded by the *mxa* operon, was the only active MDH in methanol metabolism, because (i) the *mxa* operon has been seen in many (but not all) methylotrophic isolates, and (ii) an *mxaF*-deficient mutant strain is unable to grow on methanol under laboratory conditions [15]. Another MDH gene homolog found in the strain AM1 genome, named *xoxF1*, was formerly regarded as a mysterious gene; its high homology to MxaF (50% in amino acid sequences) and its conservation of PQQ-binding amino acid residues suggested its function as an MDH, but its activity was very low and the gene deletion mutant had seemingly lost the ability to grow on methanol [16].

Recently, however, it was reported that the MDH purified from a *Methylobacterium* sp. grown in the presence of lanthanide (Ln) was XoxF-type MDH [17]. And that the MxaF-deficient mutant strain Δ*mxaF* from *Methylorubrum extorquens* AM1 was able to grow on methanol with La^{3+}, which is a member of the lanthanides, and that the mutant strain had a sufficient level of lanthanide-dependent MDH activity [18]. Moreover, it was shown that (i) the lanthanide-dependent MDH purified from methanol-grown *Methylobacterium* and *Methylorubrum* strains had sufficient MDH activity [17,18], (ii) these strains possessed La^{3+} as a cofactor in the enzyme molecule [17,18], and (iii) the lanthanide-dependent MDH was encoded by the *xoxF1* gene [18]. After these reports, lanthanide-dependent MDHs (XoxF) were discovered from several non-methylotrophic bacteria [19–21], and it was later established that *xoxF* is widely distributed in many methylotrophic/non-methylotrophic bacterial genomes and that the bacteria possessing it can be phylogenetically classified into five major clades (XoxF1–XoxF5) [22–25].

XoxF-type MDH was also found in a methanotroph, where it contained Ce^{3+} in its catalytic site [19]. These results mean that methylotrophs and methanotrophs have a novel type of methanol metabolic pathway depending on Ln in addition to their Ca^{2+}-dependent pathway. The XoxF was a dimer of the α-subunit only (XoxF1) [17,18]. The subsequent discovery of other examples of XoxFs from several methanotrophs [19,26–29] and from the *Bradyrhizobium* species of soybean-nodulating bacteria [21,28,29] established that the XoxF-type MDH is an Ln-dependent MDH. The *xoxF*-type genes can be found in the genomes of many bacterial species, including both methylotrophic and non-methylotrophic species [20,22–25,30–35], and it is already known that strains belonging to the genus *Bradyrhizobium*, which is one of the rhizobia, also possess a XoxF5-type MDH gene on their genomes [22–25].

There have already been some reports about the enzymatic properties of XoxFs in several strains of methylotrophic/non-methylotrophic bacteria [17–19,21,26–29,36]. In strain AM1, the regulation of *xoxF* gene expression has previously been clarified. Vu *et al.* showed through a promoter assay that *xoxF1* was expressed by only the four early Ln species, namely, La^{3+}, Ce^{3+}, Pr^{3+}, and Nd^{3+} [37]. Moreover, strain AM1 responded to low concentrations of La^{3+} and even to quantities under 100 nM, expressing *xoxF1* and repressing *mxaF* in response to La^{3+} even though Ca^{2+} also existed in the medium [37]. It means that strain AM1 preferentially expresses the *xoxF1* gene over *mxaF1* when Ln species co-exist with Ca^{2+}. In the natural environment, each Ln species co-exists with other Ln species. Their average abundance in the earth's crust ranges from 66 μg/g in Ce^{3+}, 40 μg/g in Nd^{3+}, and 35 μg/g in La^{3+} to 0.5 μg/g in Tm^{3+}, disregarding the extremely rare Pm^{3+} [38]. The first early Ln

species are more abundant in the soil than the others are [38]. Nevertheless, to date, there have been no reports on the specificity and preference for particular Ln species used for methylotrophic growth of strain AM1 or on the cofactors of XoxF.

XoxFs can utilize multiple Ln species as a cofactor [39–43]. XoxF in strain AM1 requires one of the light Ln species (La^{3+} to Nd^{3+}) as a cofactor, and a few research groups have reported the enzymatic properties of native and recombinant La^{3+}- and Nd^{3+}-XoxFs from strain AM1 [40,41]. In an artificial assay system using DCPIP as an electron acceptor, Featherston *et al*. have shown that native La^{3+}-XoxF had higher values of V_{max} and K_{cat} for methanol than Nd^{3+}-XoxF did, although the two XoxFs had similar K_m values for methanol [41]. Moreover, in an MDH assay using XoxG, which is a physiological electron acceptor, the K_m for XoxG was markedly higher in Nd^{3+} than in La^{3+}, although their catalytic rate constants were comparable [41,43]. Thus, there are several differences in the enzymatic properties of La^{3+}- and Nd^{3+}-XoxFs, but there have been few reports about the factors that determine these differences among the XoxFs.

On the other hand, it was reported that *Bradyrhizobium diazoefficiens* (synonym of *B. japonicum* [44] strain USDA110) also has the *xoxF* gene (*MxaF'*; blr6213), which is putatively an ortholog of *mxaF* in *Methylorubrum extorquens* strain AM1, in its genome, together with several genes related to a methanol oxidation pathway, *i.e.*, *fga,* glutathione-dependent formaldehyde activating enzyme (blr6216); *flhA*, glutathione-dependent formaldehyde dehydrogenase (blr6215); *fgh*, formyl-glutathione hydrase (blr6186), and *fdhF* encoding formate dehydrogenase (bll3136) [45,46]. However, the strain showed feeble growth in minimal medium containing methanol as a sole carbon

source, and the strain showed little MDH activity in laboratory conditions [46]. On the other hand, *Bradyrhizobium* sp. strain MAFF211645 was found to contain lanthanide-dependent MDH [21]. However, the individual enzymatic properties of the lanthanide-dependent MDH and its role in methanol utilization in *Bradyrhizobium* strains remain to be investigated.

In this work, we investigated the properties of XoxF-type MDH from *Bradyrhizobium diazoefficiens* USDA110 strain in detail and inspected the methanol metabolism changes because of different lanthanide species by used methylotrophic model strain *Methylorubrum extorquens* AM1. All the work showed that lanthanides have an important role in methylotrophic bacteria methanol metabolism.

CHAPTER 1

Preference for particular lanthanide species and thermal stability of XoxFs in *Methylorubrum extorquens* strain AM1

1. Introduction

In this chapter 1, I focused on the function of XoxFs in the methylotrophic bacterium, *Methylorubrum extorquens* AM1, and we aimed to show the rare earth elements specificities and molecular functions of XoxFs from both together with their enzymatic properties.

The rare earth elements requirement for methanol growth was observed using the model strain *M. extorquens* AM1. As a result, it was clarified that the AM1 strain could grow methanol depending on four kinds of rare earth elements from La to Nd. The AM1 strain showed the best methanol growth with La and showed the lowest methanol growth with Nd. Furthermore, XoxF encoded by xoxF1 was found to express MDH activity by using four rare earth elements as cofactors.

On the other hand, the AM1 strain can selectively use rare earth elements in the growth environment. The AM1 strain could choose La over Nd when La and Nd coexist, and the strain use La preferentially for methanol growth and for a cofactor of XoxF. It was also suggested that the methylotrophy of the AM1 strain depends on the selectivity of rare earth elements for the XoxF as a cofactor.

Next, it showed the enzymatic properties of La- and Nd-XoxFs. Both enzymes have the same K_m values for methanol, and the substrate specificity is very similar to each other, but La-XoxF had a higher Vmax value than Nd-XoxF. In other words, it was revealed that XoxF has different enzymatic properties depending on the rare earth elements as a cofactor. Furthermore, when the thermal stability of La- and Nd-XoxFs were observed, La-XoxF retained higher thermal stability than Nd-XoxF. La- and Nd-XoxFs did not change the zeta potential, but two different T_i values were observed in the thermal shift assay. La- and

Nd-XoxFs showed the same first T_i values, which means the dissociation for subunits of the XoxF dimer. On the other hand, the second T_i value meant the denaturation of subunits, and its value for La-XoxF was higher than Nd-XoxF. In other words, it is speculated that La-XoxF and Nd-XoxF have different conformations depending on the type of rare earth elements, which causes subunit denaturation.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Minimal medium with 0.5% (v/v) methanol or 0.5% (w/v) succinate as a carbon source [47] was used for cultivation of *Methylorubrum* strains. As necessary, the medium was supplemented with 30 μM Ln chlorides instead of Ca^{2+}. The concentrations of La^{3+} and Nd^{3+} were set to 20 and 10, 15 and 15, or 10 and 20 μM, respectively. The methanol/La^{3+}+Nd^{3+} media contained 20 μM $LaCl_3$ + 10 μM $NdCl_3$, 15 μM $LaCl_3$ + 15 μM $NdCl_3$ or 10 μM $LaCl_3$ + 20 μM $NdCl_3$, respectively. The growth test was performed aerobically at 30°C with rotary shaking using baffled flasks, and growth was monitored by measuring the optical density at 610 nm. Three biological replicates were measured for each condition.

For purification of the La^{3+}- and Nd^{3+}-XoxFs, strain Δ*xoxF2* was cultivated in 4 liters of methanol/La^{3+} or methanol/Nd^{3+} medium in a 5-liter wide-mouth bottle with airflow using a Laboport Mini Laboratory Pump (KNF Neuberger, Inc., Trenton, NJ, USA) at 30°C.

2.2 Construction of xoxF2 null mutant

Null mutants were generated in *xoxF2* (Mex_1p2757) using the allelic exchange vector pCM184 [48]. The following primers were used for the amplification of the up- and downstream regions of *xoxF2*: upstream of *xoxF2*; xoxF2up-fw (5'-GAATTCCGGCGACATCATGTACGTCC-3') and xoxF1up-rv (5'-GGTACCTGGCAGTGCCGTAGTAGAAG-3'); and downstream of *xoxF2*; xoxF2dn-fw (5'-CCGCGGAAAGGGTCGAAGAATTATGG-3') and xoxF1dn-rv (5'-GAGCTCCCTTGTGCGTGTAGGTCATC-3'). The PCR fragments were introduced into

*Eco*R I-*Kpn* I and *Sac* II-*Sal* I sites of pCM184, respectively. The resulting allelic exchange vectors were introduced into *M. extorquens* AM1 via conjugation using *E. coli* strain S17-1. The mutation was confirmed by diagnostic PCR.

2.3 Preparation of crude extracts and enzyme assays

Cells grown on methanol media were harvested by centrifugation (9,000 × *g* at 4ºC for 10 min). The cells were resuspended in 25 mM acetate buffer (pH 5.0) and disrupted with a 3110 BX mini Bead-Beater (Biospec Products, Bartlesville, OK, USA).

MDH activity was measured according to the methods of Day and Anthony [49] and Springer *et al*. [50] with minor modifications [21].

Protein concentration was determined according to the method of Bradford [50] with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard.

2.4 Purification of MDH

Strain AM1 Δ*xoxF2* cells grown on methanol/La^{3+}, methanol/Nd^{3+} or methanol/La^{3+}+Nd^{3+} media were suspended in a double volume of 25 mM acetate buffer (pH 5.0) and disrupted using a BSP-1107900 Bead-Beater (Biospec Products). Cell debris was removed by centrifugation (9,000 × *g* at 4°C for 15 min). The extracts were fractionated by the addition of solid ammonium sulfate ($AmSO_4$, 70% saturation). The precipitated protein was removed by centrifugation (9,000 × *g* at 4°C for 15 min). $AmSO_4$ was added to the supernatant to a final concentration of 1.2 M, and the sample was loaded into TOYOPEARL Butyl-650 S (φ26 mm

× 110 mm) (Tosoh, Tokyo, Japan) pre-equilibrated with 25 mM acetate buffer (pH 5.0) containing 1.2 M $AmSO_4$. The protein was eluted by a stepwise decrease in $AmSO_4$ concentration from 1.2 to 0.7 M. The active fractions were corrected, and the protein was loaded into a HiTrap SP HP (1 mL) (GE Healthcare, Little Chalfont, UK) pre-equilibrated with 25 mM acetate buffer (pH 5.0). The protein was eluted by a linear increase of $AmSO_4$ concentration from 0 to 100 mM. The active fractions were combined, desalted, and concentrated using an Amicon Ultra-50K (EMD Millipore, Billerica, MA, USA). The purity of the enzyme was then confirmed by SDS-PAGE (10% polyacrylamide gel).

2.5 Quantification of La^{3+} and Nd^{3+} contents in the XoxFs

The contents of La^{3+} and Nd^{3+} in the purified enzymes were determined using an Agilent 7500cx ICP-MS system (Agilent Technologies, Inc., Santa Clara, CA, USA).

2.6 Thermal stability assay

The purified enzymes were incubated at 65°C, 70°C, 75°C and 80°C for 5, 10 and 20 min, and these enzyme solutions were used for MDH assay. Three biological replicates were measured for each condition.

2.7 Thermal shift analysis

The thermal shift assay was performed using Tycho NT.6 (NanoTemper Technologies GmbH, Munich, Germany). The purified La^{3+}- and Nd^{3+}-XoxFs were diluted to the same concentration of 1.20 mg/mL with 25 mM acetate buffer (pH 5.0), and the enzyme solutions

were applied to capillary tubes in the Tycho NT.6. Intrinsic fluorescence was recorded at 330 and 350 nm while the sample was heated from 35 to 95°C at a rate of 3°C/min. The ratio of fluorescence (350/330 nm) and the T_i was calculated using the Tycho NT.6.

2.8 Differential Scanning Fluorimetry (DSF) analysis

DSF analysis of XoxFs was performed according to the method described by Niesen *et al*. [44]. Purified La^{3+}-XoxF (19.6 μM) and Nd^{3+}-XoxF (18.6 μM) were diluted in 25 mM acetate buffer (pH 5.0) with 150 mM NaCl. The samples were incubated at 0°C, 50°C, 60°C and 75°C for 10 min and then kept on ice. The fluorescent dye SYPRO Orange (Invitrogen, Carlsbad, CA, USA) was diluted to 10-fold, and La^{3+}- or Nd^{3+}-XoxFs were mixed with this diluted SYPRO Orange in a 1:1 ratio. Twenty μl of each XoxF/dye mixture was aliquoted into a 96-well PCR plate and sealed with an optical quality sealing film. Thermal unfolding was monitored using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) by heating from 25 to 95°C at a rate of 1°C per min. Fluorescence intensity was measured at every 1°C using a ROX filter at excitation and emission wavelengths of 490 and 600 to 630 nm, respectively. Fluorescence intensities in relative fluorescence units were plotted as a function of temperature. The T_m values were reported as the mean ± standard deviation.

2.9 Zeta potential analysis

La^{3+}- and Nd^{3+}-XoxFs at 1.54 and 1.63 mg/ml in 25 mM acetate buffer (pH 5.0) with 150 mM NaCl were incubated at 50ºC, 60ºC, and 75ºC for 10 min, and their zeta potentials were

measured using Zetasizer Nano ZS (Malvern Panalytical Ltd, Malvern, UK).

2.10 Simulation of XoxF1 from Methylorubrum extorquens AM1

From the RCSB Protein Data Bank (www.rcsb.org/) get the structure of *M. fumariolicum* SolV. The sequence of XoxF of *Methylorubrum extorquens* AM1 from NCBI (www.ncbi.nlm.nih.gov/). The amino acid sequences of XoxF1 is RQAHLLGLMLALGTTGALANEDVLKRTQDPNQQVLQTLDYANTRYSKLDQINASN VKNLQVAWTFSTGVLRGHEGSPLVVGDIMYVHTPFPNIVYALDLNNDSKILWKYEP KQDPSVIPVMCCDTVNRGLAYADGAIILHQADTTLVSLDAKTGKVNWSVKNGDSK VGETNTATVLPVKDKIIVGISGAEYGIRGHMTAYDAKTGKRVWRAYSVGPDDEMLV DPEKTTSLGKPIGKDSSLKTWEGDQWKTGGGATWGWYSYDPKLDLFYYGTANPST WNPKQRPGDNKWTMAIFARNPDTGQAKWIYQMTPHDEWDYDGINEMILTDQKVD GKERPLLTHFDRNGFAYTLDRANGEVLVAEKFDPVVNWASKVDLDKGSKNYGRPL VVSKYSTDQNGEDVNSKGICPAALGTKDQQPAAFSPKTQLFYVPTNHVCMDYEPFK VTYTPGQPYVGATLSMYPAPGGHGGMGNFIAWDNISGKIKWSNPEQFSVWSGALAT AGDVVFYGTLEGYLKAVDSKTGKELYKFKTASGVIGNVMTYTHKGKQYVGVLSGV GGWAGIGLAAGLTDPNAGLGAVGGYAALSQYTNLGGQLTVFALPN. The structure of XoxF1 was performed in SWISS-MODLE [51] (swissmodel.expasy.org/interactive) and RHYRE2 [52] (www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) .

3. Results

3.1 Preference among Ln species for methylotrophic growth of strain AM1

First, we observed methylotrophic growth of strain AM1 in the presence of various Ln mixtures that contained La^{3+} and Nd^{3+} in various proportions. On methanol, Strain AM1 could grow vigorously with La^{3+} but showed weak growth with Nd^{3+} (Fig. 1). When the methanol/Nd^{3+} medium was supplemented with La^{3+}, the strain showed luxuriant growth; likewise, it flourished on methanol/La^{3+} medium regardless of the ratio of Nd^{3+} to La^{3+} (Fig. 1).

These results indicate that strain AM1 preferentially utilizes La^{3+} for methylotrophic growth over Nd^{3+}, which demonstrates that methylotrophic bacterial strains can have preferences for certain Ln species for their methylotrophic growth.

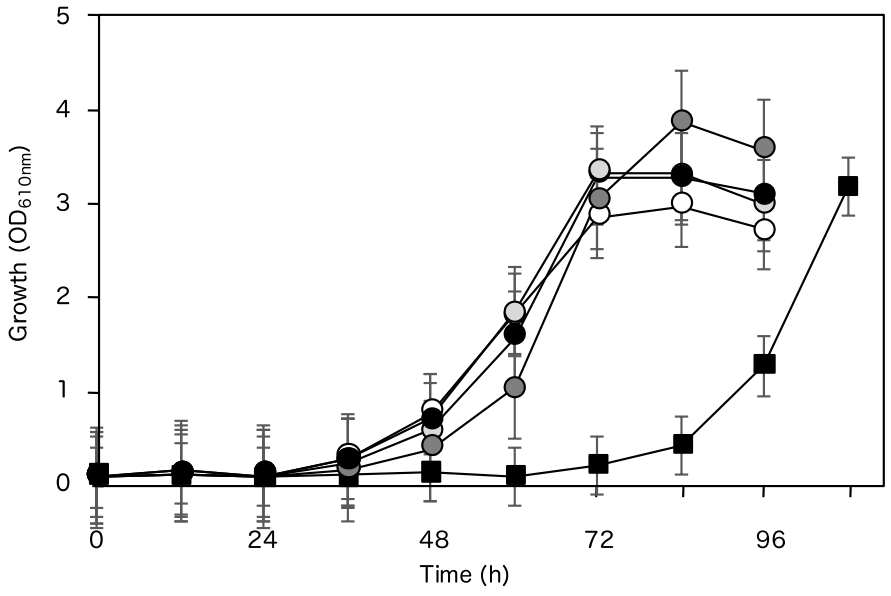


Fig. 1. Methylotrophic growth of strain AM1 depending on La^{3+} or/and Nd^{3+}. The growth of strain AM1 on methanol media supplemented with 30 μM La^{3+} (black

circles), with 20 μM La^{3+} + 10 μM Nd^{3+} (dark circles), with 15 μM La^{3+} + 15 μM Nd^{3+} (light circles), with 10 μM La^{3+} + 20 μM Nd^{3+} (white circles), and with 30 μM Nd^{3+} (black squares). Results are shown as means with standard deviations (n = 3).

3.2 XoxF preferentially requires La^{3+} as a cofactor compared with Nd^{3+}

It is already known that the first four light Ln species act as cofactors for XoxF [38,39]. We measured MDH activity in cells grown on methanol/La^{3+} and/or Nd^{3+} media. The former had higher MDH activity compared with the latter (Fig. 2). The supplementation of methanol/Nd^{3+} medium with La^{3+} enabled high MDH activity, whereas cells grown on methanol/Nd^{3+} medium without La^{3+} showed low MDH activity (Fig. 2). Taking these findings together with the growth data, we concluded that strain AM1 prefers La^{3+} to Nd^{3+} for its growth.

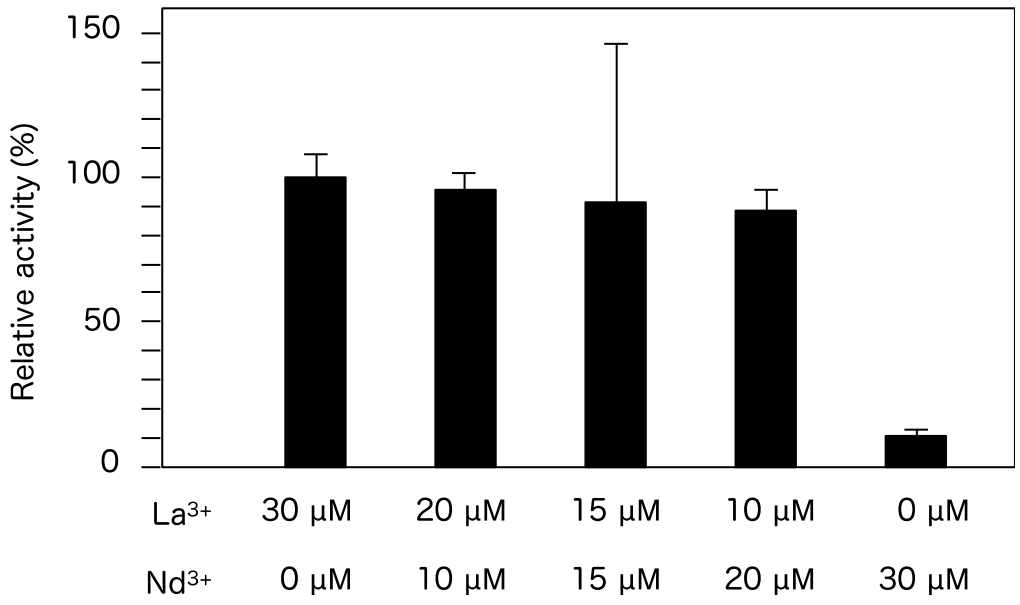


Fig. 2. MDH activity in the cell-free extract of strain AM1 grown on

methanol/La^{3+} and/or Nd^{3+} media. Cells were grown aerobically in methanol media with various concentrations of La^{3+} and/or Nd^{3+}. Results are shown as means with standard deviations (n = 3).

Next, we identified Ln content in the XoxF purified from the cells grown on methanol/La^{3+} and(or) Nd^{3+} media. The La^{3+}- and Nd^{3+}-XoxFs contained only La^{3+} or Nd^{3+} as a cofactor, with 0.45 and 0.69 atoms per subunit, respectively. However, all XoxF preparations purified from cells grown on methanol/La^{3+}+Nd^{3+} media contained mainly La^{3+} as a cofactor (>90%) (Fig. 3). These results indicated that XoxF preferentially requires La^{3+} as a cofactor over Nd^{3+}.

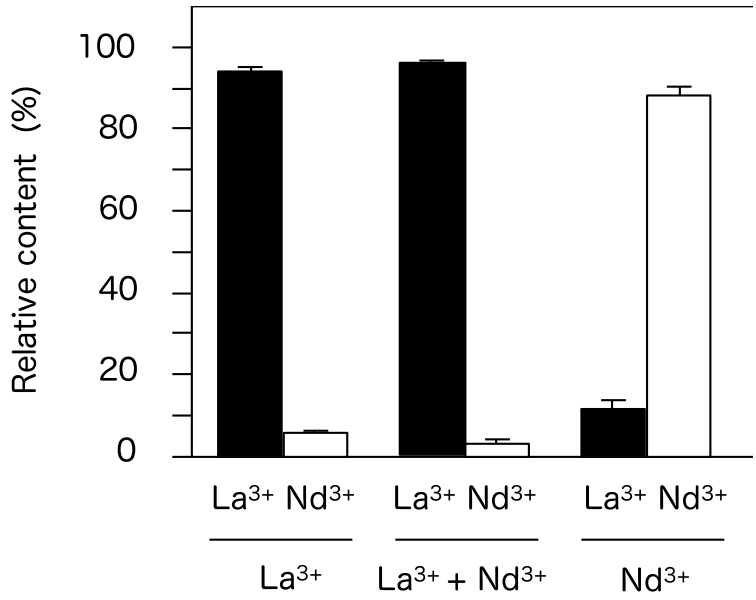


Fig. 3. Lanthanide contents of XoxFs purified from strain Δ*xoxF2* grown on methanol/La^{3+} and/or methanol/Nd^{3+} media.Results are shown as means with standard deviations (n = 3).

3.3. Enzymatic properties of the purified La^{3+}- and Nd^{3+}-XoxF1

The purified La^{3+}- and Nd^{3+}-XoxF1 were showed a single band with an apparent molecular mass of ca. 64 kDa (Fig. 4) without small protein corresponding to the β subunit.

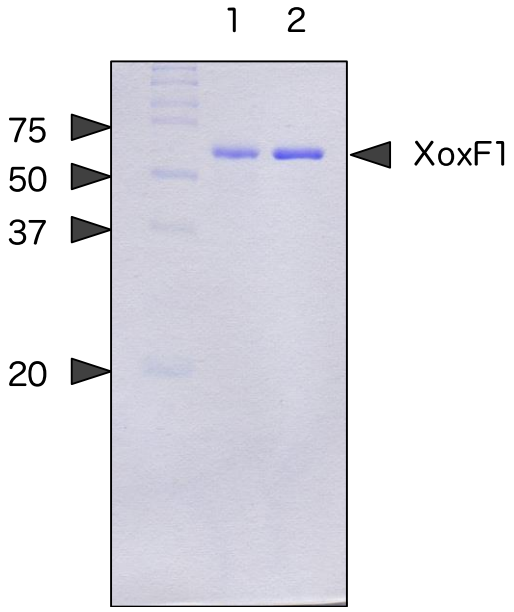


Fig.4 The SDS-PAGE analysis of purified MDH from strain AM1 grown on methanol/La^{3+} and methanol/Nd^{3+}. Lane 1, purified La^{3+}-XoxF1; Lane 2, purified Nd^{3+}-XoxF1.

Optimum pH of the purified La^{3+}- and Nd^{3+}-XoxF were 9.0 and 9.5 respectively. The V_{max} of La^{3+}-XoxF1 were value for methanol was 29.3 U/mg that almost three times than 11.0 U/mg of Nd^{3+}-XoxF1. The XoxF4-family MDHs (0.65 and 0.78 U/mg) [20], the XoxF2 (4.4 U/mg) and XoxF5 (5.97 U/mg) families [20,23]. Moreover, the La^{3+}-XoxF1 K_m value for methanol was 0.0677 mM and Nd^{3+}-XoxF1 with 0.0767 mM, which was similar to those of

the other XoxF4- (0.055 and 0.042 mM) and XoxF5-family MDHs (0.039 mM) [20], although the K_m value of XoxF2-family MDH was 0.0008 mM [23].

Table 1. Enzymatic properties of the purified La^{3+}- and Nd^{3+}-XoxFs from strain Δ*xoxF2*.

	La^{3+}-XoxF1	Nd^{3+}-XoxF1
Optimum pH	9.0	9.5
V_{max} (μM/min)	29.3	11.0
K_m (μM)	67.7	76.7

The substrate specificities of the La^{3+}- and Nd^{3+}-XoxF1 are shown in Table 2. Both XoxF exhibited activity toward short-chain primary alcohols such as methanol, ethanol, 1-propanol, and 1-butanol. On the other hand, the La^{3+}- and Nd^{3+}-XoxF1 could not oxidize secondary alcohols such as 2-propanol as substrates.

Table 2. Relative activities of La^{3+}- and Nd^{3+}-XoxF to various substrates.

Substrate	Relative activity (%)	
	La^{3+}-XoxF	Nd^{3+}-XoxF
Methanol	100	100
Ethanol	70.5 ± 2.69	72.9 ± 2.04

Substrate	Relative activity (%)	
	La^{3+}-XoxF	Nd^{3+}-XoxF
1-Propanol	72.4 ± 5.39	72.9 ± 7.35
2-Propanol	0.952 ± 1.35	0.00 ± 0.00
1-Butanol	52.4 ± 1.35	72.9 ± 4.08

3.4 Ln species affected the thermal stability of the La^{3+}- and Nd^{3+}-XoxF

As in previous reports, DCPIP-linked assay showed that La^{3+}- and Nd^{3+}-XoxF have similar enzymatic properties, including optimum pH, substrate specificities, and K_m values for methanol, but La^{3+}-XoxF had a higher V_{max} value for methanol than Nd^{3+}-XoxF did (Table .1). The optimum temperature of La^{3+}- and Nd^{3+}-XoxF were the same 45℃, but the Nd^{3+}-XoxF was sensitivity with temperature. The Nd^{3+}-XoxF was activity decreases markedly than La^{3+}-XoxF.

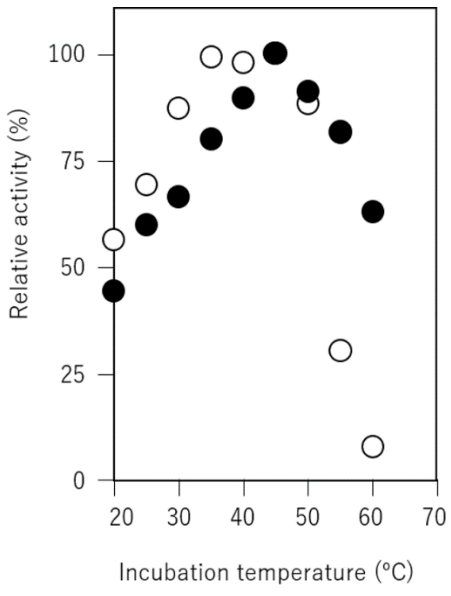


Fig. 5 The relative activity of the optimum temperature of La^{3+}- and Nd^{3+}-XoxF. Black dots, La^{3+}-XoxF; Circles, Nd^{3+}-XoxF.

Moreover, La^{3+}- and Nd^{3+}-XoxFs showed a definite difference in stabilities. Especially in the thermal stability test, La^{3+}-XoxF showed higher stability compared with Nd^{3+}-XoxF. After incubation at 75ºC for 20 min, Nd^{3+}-XoxF began to inactivate; after incubation at 80ºC for 20 min, it lost about 95% of MDH activity (Fig. 5). La^{3+}-XoxF, on the other hand, also began to inactivate after incubation at 75ºC for 20 min as Nd^{3+}-XoxF did, but La^{3+}-XoxF retained about 50% of it MDH activity after incubation at 80ºC for 20 min (Fig. 5).

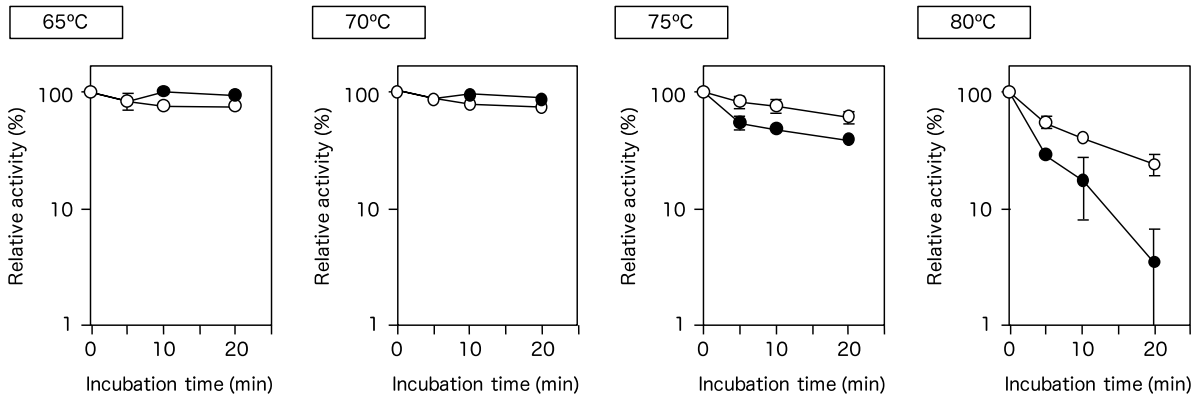


Fig. 6. Thermal stability of purified La^{3+}-XoxF and Nd^{3+}-XoxF. Purified XoxFs are incubated at the indicated temperatures for 10 and 20 min (material and methods). La^{3+}-XoxF (white circles) and Nd^{3+}-XoxF (black circles). Results are shown as means with standard deviations ($n = 3$).

3.5 La^{3+}-XoxF has higher Ti and Tm values for thermal stability than Nd^{3+}-XoxF

To quantify the thermal stability of La^{3+}- and Nd^{3+}-XoxF, we performed a thermal shift assay based on intrinsic fluorescence from aromatic amino acid residues. The fluorescence signals were plotted as a ratio (350 nm/330 nm) and used to calculate the midpoint unfolding inflection temperature (T_i). The thermal shift analysis showed two distinct T_i values for La^{3+}- and Nd^{3+}-XoxFs (Fig. 7). Although the first T_i values of La^{3+}- and Nd^{3+}-XoxFs were very similar to each other (58.5 and 58.7ºC, respectively), the second T_i values were notably different (72.8ºC and 70.5ºC, respectively) (Fig. 7).

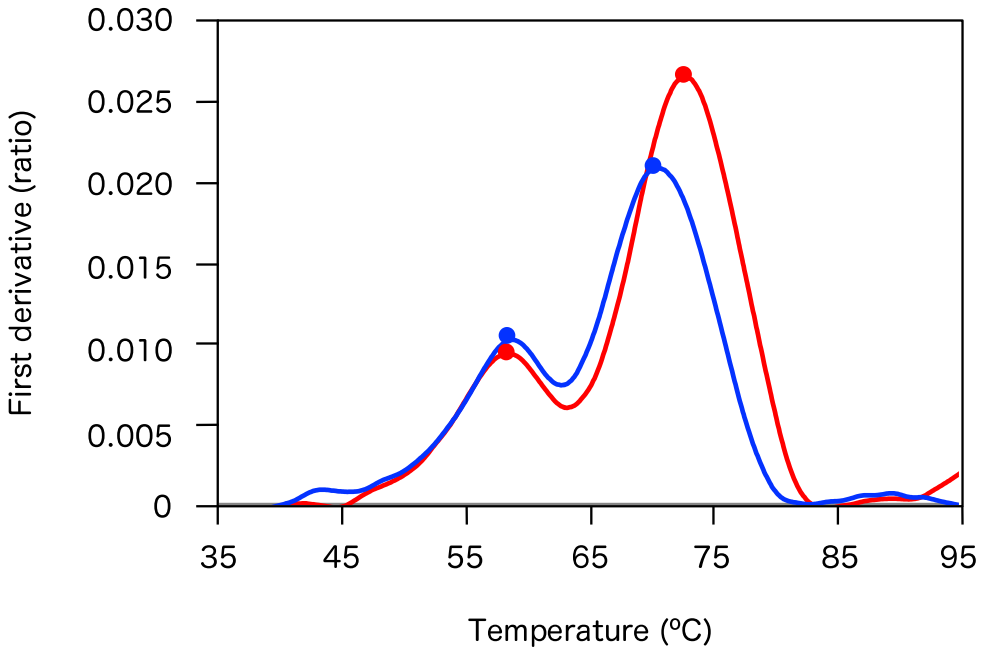


Fig. 7. Thermal unfolding midpoint temperature (T_i) of La^{3+}-XoxF (red line) and Nd^{3+}-XoxF (blue line). Peaks in the first derivative ratio (red and blue dots) correspond to the detected T_i of the purified XoxFs.

Next, we identified the melting temperature (T_m) of the XoxFs using DSF. In the DSF plot for La^{3+}-XoxF, there was a single inflection point showing a T_m value of 63 ± 0.095ºC (Fig. 8A). In the DSF plot for Nd^{3+}-XoxF, in contrast, there were at least two inflection points, one around 45ºC and the other around 58ºC, although we could not identify the T_m values of La^{3+}-XoxF (Fig. 8B). This result indicates that Nd^{3+}-XoxF is more easily denatured at lower temperatures compared to La^{3+}-XoxF.

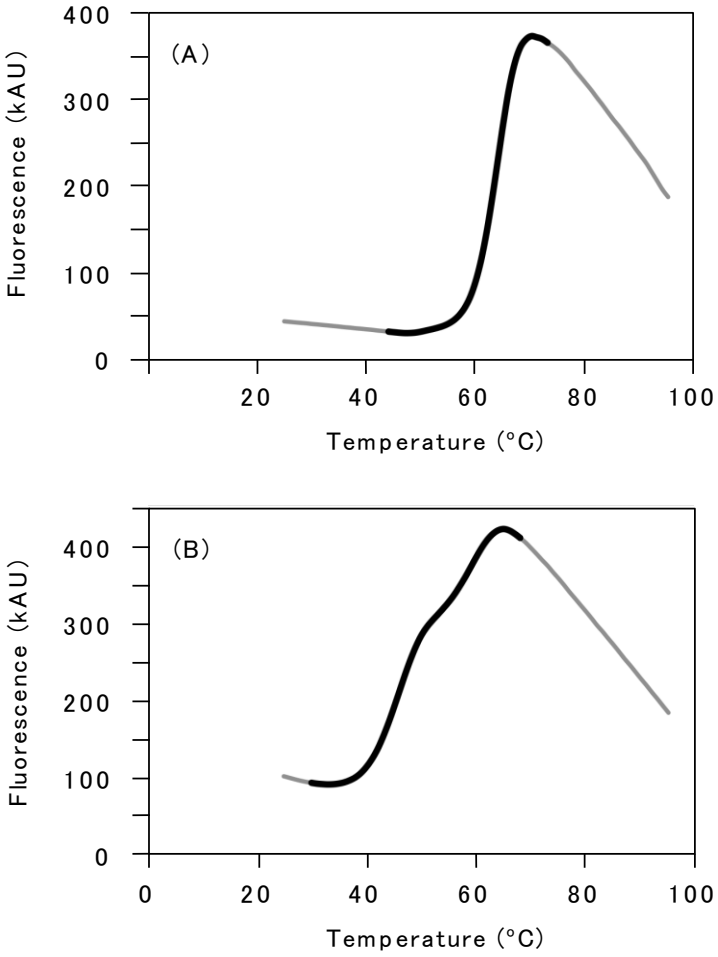


Fig. 8. Thermal stability analysis of La^{3+}-XoxF (A) and Nd^{3+}-XoxF (B). DSF data were measured with La^{3+}-XoxF (19.6 μM) and Nd^{3+}-XoxF (18.6 μM)

in 10× SYPRO Orange, 25 mM acetate buffer with 0.15 M NaCl (pH 5.0).

3.6 Zeta potential values of La^{3+}- and Nd^{3+}-XoxFs

We observed the zeta potential values of La^{3+}- and Nd^{3+}-XoxFs at different temperatures. The zeta potential values of La^{3+}-XoxF were +12.3, +13.3, and +13.1 mV at 50, 60 and 75ºC, respectively; these values were very similar to those of Nd^{3+}-XoxF (Table 3).

Table 3. Effect of temperature on zeta-potential values of La^{3+}- and Nd^{3+}-XoxFs

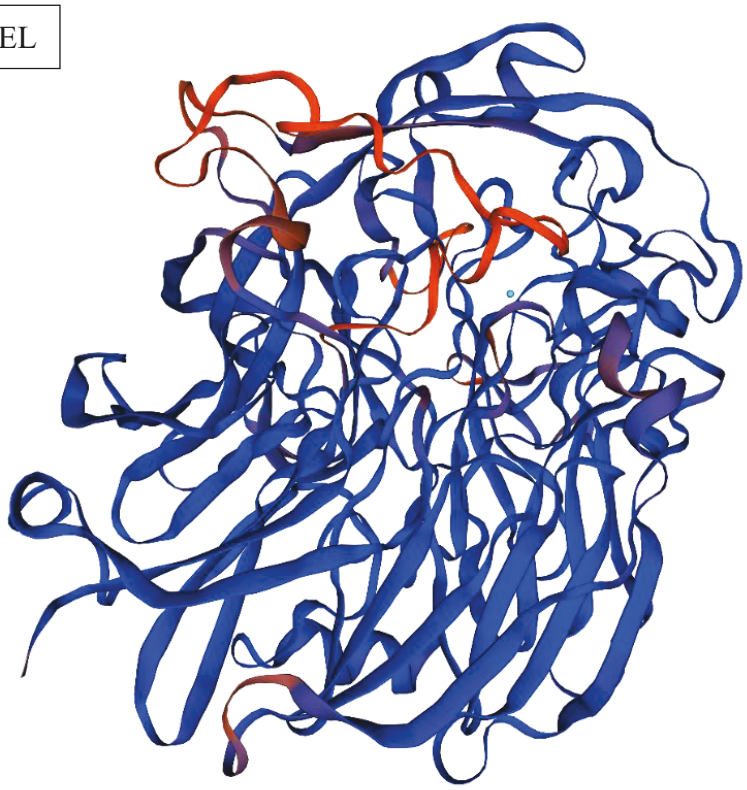
	Zeta-potential (mV)		
	50ºC	60ºC	75ºC
La^{3+}-XoxF	+12.3	+13.3	+13.1
Nd^{3+}-XoxF	+13.9	+14.4	+13.2

These results indicate that the difference in thermal stability between XoxFs is caused not by surface charges of the XoxFs but rather by other factors.

3.7 Simulation of XoxF1 structure

The XoxF1 structure was simulated with SWISS-MODEL and PHYRE2.

SWISS-MODEL



PHYRE2

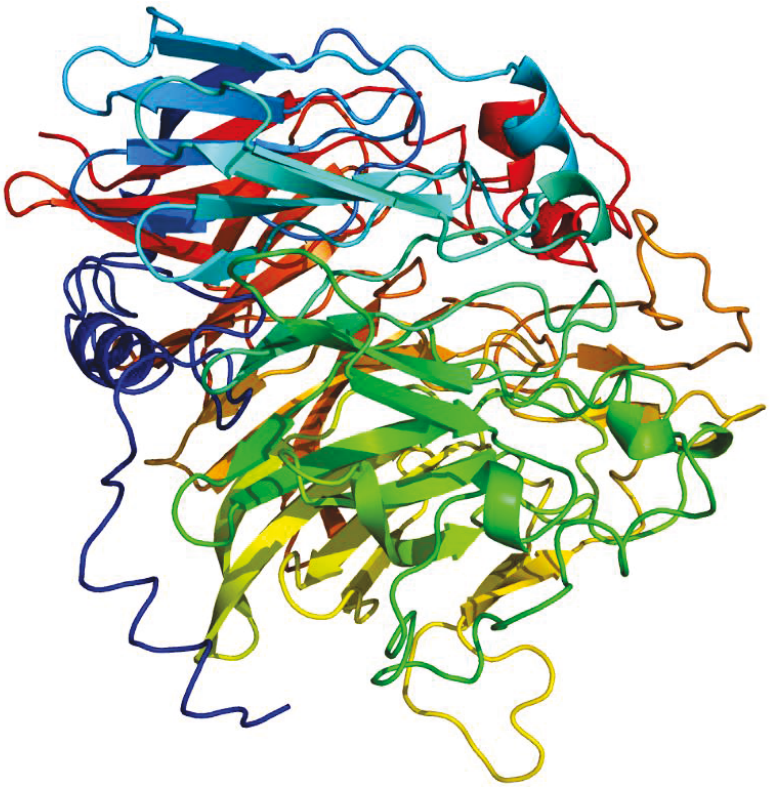


Fig. 7 The simulation expected the tertiary structure of XoxF1 from *Methylorubrum extorquens* AM1. A, SWISS-MODEL; B, PHYRE2.

4. Discussion

Strain AM1 can recognize and utilize four early Ln species, namely, La^{3+}, Ce^{3+}, Pr^{3+}, and Nd^{3+}, for methylotrophic growth, but it preferentially uses La^{3+} rather than Ca^{2+} in the methylotrophic growth condition [25]. In the natural environment, however, different Ln species coexist with other species such as Ca^{2+}; this led us to suppose that there was a preference for using light Ln species for methanol growth. In this study, we sought to confirm strain AM1's preference for particular Ln species for methanol utilization through experiments with La^{3+} and Nd^{3+}.

We showed that *M. extorquens* strain AM1 can distinguish among the various Ln species such as La^{3+} and Nd^{3+} and that the strain preferentially utilizes La^{3+} for its methylotrophic growth when it has a choice between La^{3+} and Nd^{3+}. The strain showed significantly slower growth on a methanol/Nd^{3+} medium than on a methanol/La^{3+} medium. Since all the light Ln species (La^{3+} to Nd^{3+}) induce comparable expression levels of the *xoxF1* gene [25], the strain's slower growth on methanol/Nd^{3+} medium is likely due to the lower activity and stability of Nd^{3+}-XoxF compared with La^{3+}-XoxF. Featherston *et al*. have reported that Ln species affect the interaction between XoxF and XoxG *in vivo* [40] and that the reduction potential (E_m) of the Ln-PQQ cofactor with increasing Ln Lewis acidity is one of the important factors affecting the interaction between XoxF and XoxG *in vivo* [42]. In other words, a strain's preference for a particular Ln species in the XoxF is one of the most important properties for activation of the Ln-dependent MDH *in vivo*, and it dominates the Ln-dependent methylotrophic growth of strain AM1.

XoxF is also able to choose a suitable Ln element to serve as a cofactor from

among a mixture of Ln species. In the case of a La^{3+}+Nd^{3+} mixture, XoxF preferentially utilizes La^{3+} as a cofactor as opposed to Nd^{3+}. One of the key determinants of the Ln specificity and stabilization of XoxF may be the ionic radius of each Ln species. It seems that XoxF has evolved to utilize La^{3+}, which has the largest ion radius of any Ln species, as a cofactor and is optimized to retain La^{3+} in the molecule. Though all the Ln species have similar ionic radii, the length of the ion radius decreases slightly and monotonically as the atomic number increases between La^{3+} and Nd^{3+} [53]. In fact, the difference in RI between La^{3+} (125.0 pm) and Nd^{3+} (117.5 pm) is only 7.5 pm [54]. The light Ln species (La^{3+} to Nd^{3+}), which have radii in the range of ca. 116-111 pm, appear to be of acceptable size for a cofactor of XoxF. Therefore, XoxF is able to utilize the light Ln species (La^{3+} to Nd^{3+}) and is able to choose La^{3+} as the best cofactor.

On the other hand, La^{3+}- and Nd^{3+}-XoxFs showed different levels of thermal stability, and La^{3+}-XoxF has higher T_i and T_m values than Nd^{3+}-XoxF does. Thermal shift analysis revealed that both XoxFs have two T_i values. XoxF has at least two stages in its denaturation process; the first is subunit dissociation from the dimer, and the second is subunit denaturation. In the latter process (subunit denaturation), La^{3+}-XoxF has a higher T_i value than Nd^{3+}-XoxF does (72.8ºC and 70.5ºC, respectively), although their T_i values in the first process (dissociation from the dimer) are similar. Furthermore, La^{3+}-XoxF had a higher T_m value than Nd^{3+}-XoxF did in the DSF plot. These results indicate that La^{3+}- and Nd^{3+}-XoxFs possess distinctly different degrees of thermal stability and that La^{3+}-XoxF has more stable conformation than Nd^{3+}-XoxF, although there is no difference in their zeta potential values. A similar pattern of subunit dissociation and subunit denaturation is seen in the envelope

glycoprotein of the dengue virus [55]. Jahn *et al*. reported that *Methylacidiphilum fumariolicum* SolV XoxF metallated with Eu^{3+} exhibits no significant structural changes but does exhibit lower catalytic efficiency compared to that metallated with La^{3+}/Ce^{3+}/Pr^{3+} [25]. However, the different degrees of thermal stability found in this study strongly suggested a slight difference in conformation between La^{3+}- and Nd^{3+}-XoxFs. Therefore, we recommend that XoxF has evolved so that the enzyme can utilize light Ln species with high Lewis acidity for higher catalytic activity and that its conformational rigidity is also a result of selection for the optimal Ln species.

CHAPTER 2

Lanthanide-dependent methanol dehydrogenase from the symbiotic nitrogen-fixing bacterium *Bradyrhizobium diazoefficiens* strain USDA110

1. Introduction

In this chapter 2, I focused on the physiological role of lanthanides in one of the model rhizobia, *Bradyrhizobium diazoefficiens* USDA110, and aimed to show the rare earth elements molecular functions of methylotroph in strain USDA110 and their enzymatic properties.

As we have known that the gene xoxF encoding XoxF is widely distributed not only in methylotrophic bacteria but also in rhizobia such as *Bradyrhizobium* and *Rhizobium*. However, so far, there is very little knowledge about XoxF derived from rhizobia, and its enzymatic properties are hardly clear. Therefore, we decided to clarify the property of methanol/lanthanide growth and XoxF enzymological characteristics using *B. diazoefficiens* USDA110 strain. The USDA110 strain was able to use methanol in a lanthanide dependent manner from La to Nd, like the AM1 strain, and the cells had sufficient MDH activity. Furthermore, the USDA110 strain showed the best methanol growth with La, and it also showed feeble methanol growth with Nd.

On the other hand, the USDA110 strain did not show any methanol growth with Ca. MDH was purified from USDA110 strain grown in methanol/Ce. The MDH was coded by blr6213 that found to belong to XoxF5 type MDH, and purified XoxF contained 0.58 Ce atoms per enzyme subunit. Moreover, enzymatic properties of the XoxF with the optimal temperature, the optimal pH, V_{max}, and K_m value for methanol is. Small-angle X-ray scattering (SAXS) analysis was performed to show the three-dimensional structure of the XoxF in solution, suggesting that the XoxF rotation radius (R_g) and maximum particle size (D_{max}), and that XoxF retains the structure in the solution.

2. Materials and Methods

2.1. Bacterial strain and cultivation conditions

B. diazoefficiens strain USDA110 was used in this study. For the cultivation of the strain, we used the minimal salts (MS) medium [55] for growth and MDH induction tests. The MS medium was supplemented with 0.5% methanol or 1% glucose as a sole carbon source and with 30 μM $CaCl_2$ or lanthanide chlorides; the resulting medium types were referred to as methanol/Ca^{2+}, methanol/La^{3+}, methanol/Ce^{3+}, methanol/Pr^{3+} and methanol/Nd^{3+} medium, respectively. *B. diazoefficiens* strain USDA110 was cultivated at 30ºC with reciprocal shaking, and the growth of the strain was monitored by measuring optical density at 610 nm.

2.2. Preparation of cell-free extracts

Strain USDA110 cells grown on methanol/La^{3+}, methanol/Ce^{3+}, methanol/Pr^{3+} and methanol/Nd^{3+} media were collected during the log phase by centrifugation at 12,000 x *g* for 10 min and washed three times with 25 mM acetate buffer, pH 5.0. The cells were resuspended with 25 mM acetate buffer, pH 5.0, then broken with a 3110BX mini-bead beater (Biospec Products, Bartlesville, OK, USA) or a bead beater (Biospec Products). Cell debris was removed by centrifugation at 12,000 x *g* for 10 min at 4ºC. The supernatants were used as cell-free extracts.

2.3. MDH activity and protein assays

MDH activity was measured according to the modified methods of Day and Anthony [49]. As auto-bleaching of the reaction mixture has been reported previously [19,37,56], the reaction

mixtures were incubated at 30°C until auto-bleaching disappeared, and methanol was then added to the reaction mixture.

Protein concentration was determined either according to the method of Bradford [58] with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard or according to the method of Hill and Straka [59]. SDS-PAGE was performed according to previously published methods under denaturing conditions [60,61].

2.4. Purification of Ce^{3+}-dependent MDH

The MDH from strain USDA110 grown on methanol/Ce^{3+} medium was purified in three steps. The cell-free extract was fractionated by the addition of solid ammonium sulfate (70% saturation). The precipitate was removed by centrifugation at 12,000 x *g* for 10 min at 4ºC. The supernatant, to which ammonium sulfate had been added (final concentration; 1.5 M), was applied to a Toyopearl Butyl-650S column (Tosoh Co., Ltd., Tokyo, Japan) pre-equilibrated with 25 mM acetate (pH 5.0) buffer containing 1.2 M ammonium sulfate. Stepwise decreasing concentrations of ammonium sulfate eluted the protein from 1.2 to 0.7 M. The fractions containing MDH activity were collected, and the enzyme fraction was applied to a HiTrap SP HP column (GE Healthcare UK Ltd., Buckinghamshire, England) pre-equilibrated with 25 mM acetate buffer (pH 5.0). The protein was eluted by stepwise increasing concentrations of 0 to 400 mM NaCl, and the active fraction showed a single protein band on SDS-PAGE.

2.5. Identification of the protein

The protein band detected by SDS-PAGE analysis was subjected to in-gel tryptic digestion followed by mass spectrometric analysis using a MALDI-TOF/MS (Ultraflex, Bruker Daltonics K.K., Japan). Peptide mass fingerprinting (PMF) analysis and ms/ms analysis were performed for two prominent peptide peaks (m/z 1011.55 and 1477.82) and identification was made using Mascot software (Matrix Sciences, Mount Prospect, IL, USA) using the available information on the genome of strain USDA110.

2.6. Quantification of Ce^{3+} in the purified enzyme preparation

The metal content in the purified protein (1.86 mg/ml) was analyzed by means of inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx, Agilent Technologies Inc., Santa Clara, CA, USA). The sample (100 µl) was mixed with 9.9 ml 5% nitric acid and subjected to ICP-MS analysis using lanthanide standard.

2.7. SAXS analysis

The buffer of the purified XoxF was exchanged for 20 mM HEPES buffer (pH 7.0) and concentrated to 4.3 mg/ml. All experiments were performed at room temperature using a Rigaku BioSAXS-1000 system using Cu-Kα radiation and a Rigaku FR-X SuperBright rotating anode X-ray generator (Rigaku Co., Ltd., Tokyo, Japan). The XoxF sample was placed in a quartz capillary (1 mm ID) and 15 min of exposure was taken during each of four repetitions. The molecular weight of XoxF was calculated using 5 mg/mL human serum albumin (HSA) as the standard. Subsequently, the data were analyzed using PRIMUS [62] in

the ATSAS 2.7.2 program package [63], followed by 10 runs of GASBOR 2.3 [64] for *ab initio* beads modeling. The final model was aligned to *ab initio* models and an averaged model was constructed by a DAMAVER program [65]. The structural model of XoxF from strain USDA110 was constructed by CPHmodels servers [66] with a template structure (XoxF from *Methylacidiphilum fumariolicum* SolV, PDB code: 4MAE), then superpositioned using SSM program [66] and manually fitted using COOT software [67] onto the resultant beads model.

3. Results and discussion

3.1. B. diazoefficiens USDA110 is able to utilize methanol in a lanthanide-dependent process

Sudtachat *et al*. reported that strain USDA110 showed feeble growth in minimal medium with methanol, although the strain has some genes related to a methanol oxidation pathway, especially *MxaF'* [46]. Our data indicated that the strain did not show any growth on methanol without lanthanides (Fig. 1). On the methanol/Ce^{3+} medium, however, strain USDA110 showed significant growth on methanol, and the strain was also able to grow on methanol with La^{3+}, Pr^{3+}, and Nd^{3+} (Fig. 1), but not with the other lanthanides tested. Strain USDA110 grown on methanol with La^{3+}, Ce^{3+}, Pr^{3+}, or Nd^{3+} showed significant MDH activity; methanol/Ce^{3+} grown cells had the highest MDH activity (Fig. 2).

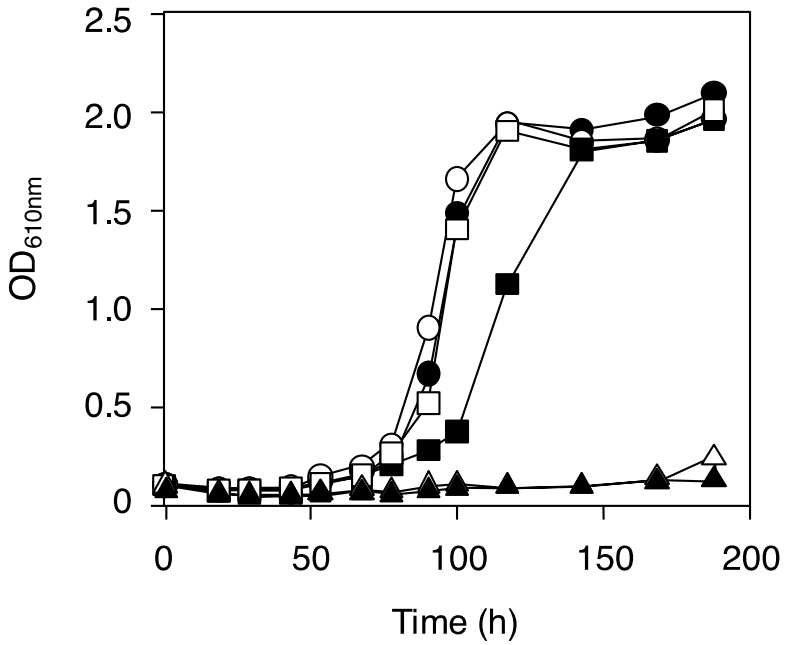


Fig. 1. Growth curve of strain USDA110 on methanol supplemented with

La^{3+} (○), Ce^{3+} (●), Pr^{3+} (□), Nd^{3+} (■), Eu^{3+} (△), or Ca^{2+} (▲). The concentrations of lanthanide and Ca^{2+} in the medium were 30 μM each.

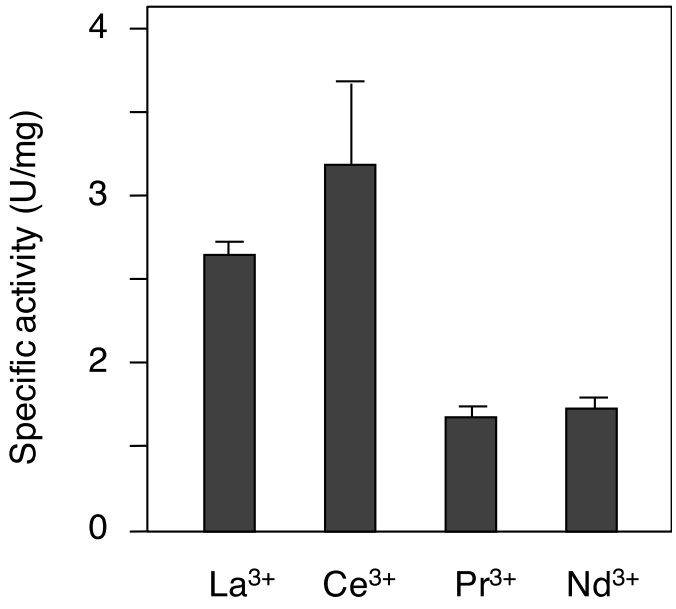


Fig. 2. MDH activity in the cell-free extract of strain USDA110 grown on methanol media in the presence of lanthanides. Cells were grown aerobically in methanol media with 30 μM of a lanthanide. Results are shown as means with standard deviations (n = 3).

This is the first report on methanol utilization for growth by a member of the symbiotic nitrogen-fixing bacterium other than the methylotrophic bacterium *Methylobacterium nodulans* [68]. The four lightest lanthanides, La^{3+}, Ce^{3+}, Pr^{3+}, and Nd^{3+}, are reported to support the growth of *mxaF* mutants of *Methylorubrum extorquens* AM1 [37] and *Oharaeibacter diazotrophicus* SM30 [69] and that of the newly described lanthanide-dependent methylotroph *Novimethylophilus kurashikiensis* La2-4 [69]. The

Methylacidiphilum fumariolicum strain SolV utilizes not only these four but also Sm, Eu and Gd for growth [19], and the XoxF from strain SolV could utilize a wide range of lanthanides as a cofactor [70]. This study revealed that strain USDA110 is capable of utilizing methanol depending on light lanthanides similar to methylotrophs. These lanthanides induce MDH activity in strain USDA110, and lanthanide-dependent MDH may have an important function in methanol utilization by the strain.

3.2. Characterization of purified Ce^{3+}-dependent MDH

The purified XoxF showed a specific activity of 13.2 U/mg of protein. On SDS-PAGE, the purified protein yielded a single band with an apparent molecular mass of ca. 64 kDa (Fig. 3). There was no small protein corresponding to the β subunit. BLAST analysis using *Methylorubrum extorquens* AM1 *mxaI* against the USDA110 genome resulted in no-hit (with >30% identity), suggesting that the genome of strain USDA110 does not harbor *mxaI*. This result was consistent with previous findings on XoxFs from *Bradyrhizobium* sp. strain MAFF211645, *Methylorubrum extorquens* strain AM1 and *Methylacidiphilum fumariolicum* strain SolV [18,19,21], none of which harbor MxaI. The purified MDH from strain USDA110 was identified as XoxF protein (blr6213) by both PMF analysis (mascot score 63.6) and ms/ms analysis (mascot score 79.68 for m/z 1477.816 and 9.06 for m/z 1011.508). It were valuing that the Ca^{2+}-dependent MxaFI-MDHs found in many methylotrophs are heterotetrameric proteins ($\alpha_2\beta_2$) [12] and that their β subunits can thus be seen in SDS-PAGE analyses [18]. It is becoming apparent that several bacterial species in different genera also possess *xoxF* genes in their genomes [22–25]; in particular, several strains belonging to genus

Bradyrhizobium and other Rhizobiales also have orthologous genes of *xoxF* that are categorized in the XoxF5 family, and the XoxF protein in strain USDA110 (blr6213) also belongs in the XoxF5 family [22–25].

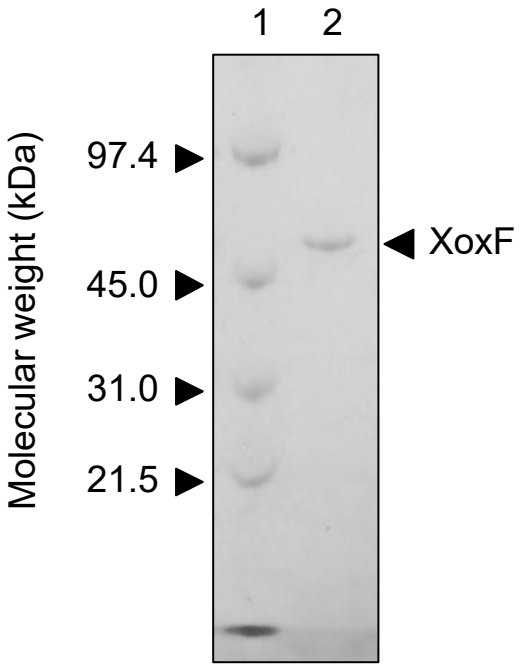


Fig. 3. The SDS-PAGE analysis of purified MDH from strain USDA110 grown on methanol/Ce^{3+}. Lane 1, molecular marker; Lane 2, purified MDH.

Moreover, the purified XoxF contained cerium, and its content in the purified protein (0.339 mg/ml) was 464 ppb, suggesting that 0.58 cerium atom is bound to each subunit. This value is similar to those of XoxFs from *Methylorubrum extorquens* AM1 XoxF1 (0.46 atom/subunit) and *Methylacidiphilum fumariolicum* strain SolV (0.6 atom/subunit) [18,19].

3.3. Enzymatic properties of the purified XoxF from strain USDA110

Optimum temperature and pH of the purified XoxF were 35ºC and 9.0 respectively. The V_{max} value for methanol was 12.9 U/mg, which was over ten times greater than that for the XoxF4-family MDHs (0.65 and 0.78 U/mg) [20], but its V_{max} value for methanol was only two or three times greater than that of the MDH categorized in the XoxF2 (4.4 U/mg) and XoxF5 (5.97 U/mg) families [20,23]. Moreover, the K_m value for methanol was 0.067 mM, which was similar to those of the other XoxF4- (0.055 and 0.042 mM) and XoxF5-family MDHs (0.039 mM) [20], although the K_m value of XoxF2-family MDH was 0.0008 mM [23].

The substrate specificities of the MDH are shown in Table 1. The XoxF exhibited activity toward short-chain primary alcohols such as methanol, ethanol, 1-propanol, and 1-butanol as well as toward formaldehyde. On the other hand, the XoxF could not oxidize secondary alcohols such as 2-propanol as substrates.

Table 1. Relative activity of MDH from strain USDA110 against various substrates

Substrate	Relative activity (%)
Methanol	100
Ethanol	93.0 ± 4.7
1-Propanol	85.3 ± 4.8
2-Propanol	0.775 ± 1.3
1-Butanol	78.3 ± 5.9
Formaldehyde	91.5 ± 2.4

From these results, we concluded that (i) the XoxF in strain USDA110 should be categorized in the XoxF5 family, (ii) the XoxF is able to function as a Ce^{3+}-dependent MDH in the cell, and (iii) MDH is involved in methanol metabolism by the strain.

3.4. Structural analysis of XoxF by SAXS analysis

SAXS analysis was performed to investigate the solution structure of Ce^{3+}-dependent MDH from strain USDA110. The radius of gyration (R_g) and maximum particle dimension (D_{max}) of XoxF were calculated to be 32.3 and 96.8 Å, respectively. The molecular weight of XoxF, calculated from SAXS data, was 136 kDa, which is consistent with a dimer of XoxF. The scattering plot for XoxF is shown in Fig. 4A. We attempted to dock a dimer structural model of XoxF into the SAXS beads model (Figs. 4C-D). The structural model fits well onto the *ab initio* bead model. These data indicated that XoxF adopts a dimer structure in solution. However, SAXS data indicates that the solution structure of XoxF from strain USDA110 did not have a symmetrical shape (Fig. 4). We assumed that the asymmetrical shape of XoxF would be particularly meaningful with respect to the enzymatical function of the XoxF. We are not at present able to explain the reason for the asymmetrical structure of XoxF, but it is possible that the higher-order structure of XoxF proteins may be changeable during the reaction stage.

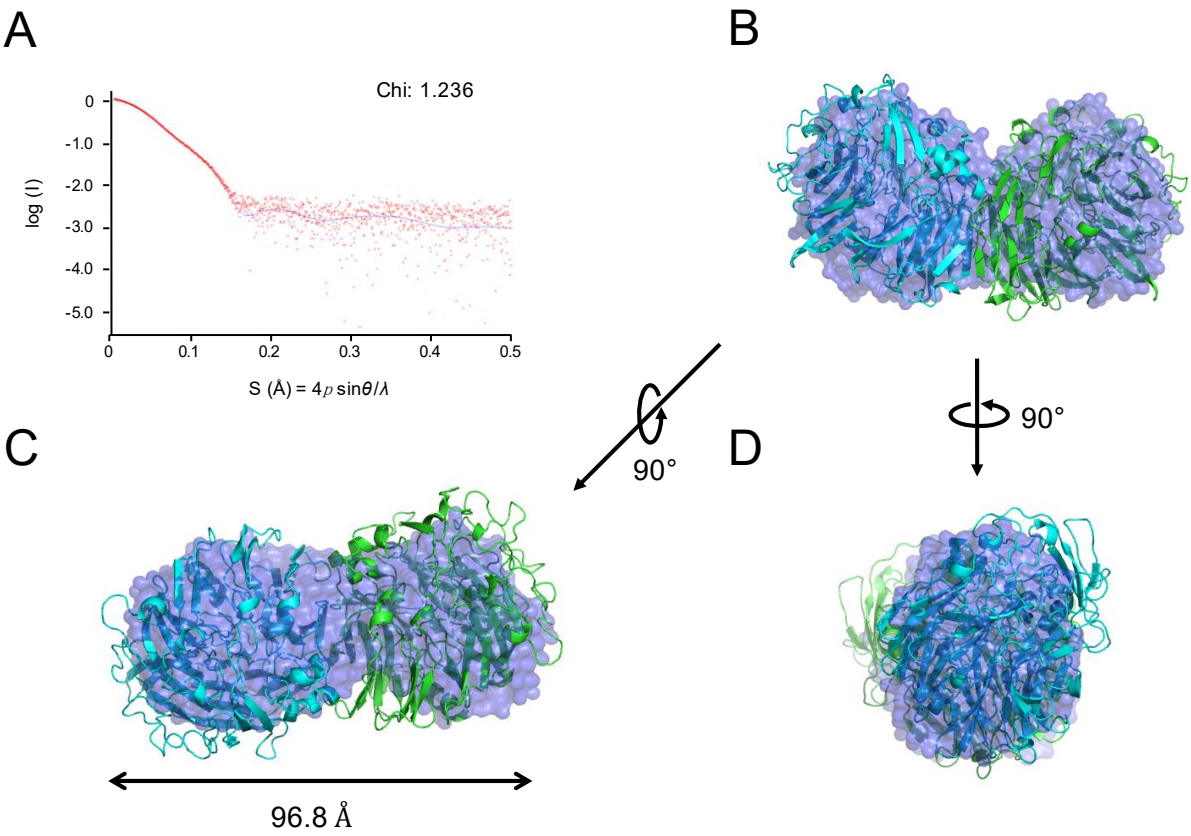


Fig. 4. Solution structure of XoxF. (A) The *ab initio* beads model obtained by SAXS data. The model is depicted using surface representation in blue. The structural model of a dimer of XoxF (blue and green) was fitted into the beads model as described in *Materials and Methods*. The beads models and structural models rotated 90 degrees along the *x*-axis and the *y*-axis is shown in panels B and C, respectively. (D) The experimental scattering curve for XoxF. Experimental X-ray scattering curve from the XoxF (red crosses) and the theoretical curve estimated from the crystal structure (blue solid line) are shown in panel D.

4. Conclusion

In this work, we showed that *B. diazoefficiens* strain USDA110 is capable of utilizing methanol depending on light lanthanides and that it possesses a XoxF5-type lanthanide-dependent MDH encoded by the *xoxF* gene (blr6213). Therefore, the XoxF of strain USDA110 is able to function as a Ce^{3+}-dependent MDH *in vivo*, and we suggest that the XoxF is involved in methanol metabolism by strain USDA110.

The wide distribution of the XoxF5-type MDH gene in the Rhizobiales strongly suggests its importance in plant-associated lifestyles, especially in the rhizosphere. Although methanol emission from leaves has been documented [71], there have been no reports on methanol exudation from plant roots. Given that methanol is a waste product in pectin degradation, methanol in the rhizosphere may be important as a nutrient for rhizospheric bacteria. Therefore, methanol utilization may be of importance in enabling Rhizobiales to outcompete other bacteria in the rhizosphere. The activity of MDHs could be supported by lanthanides, which are relatively abundant in the soil. The genome of strain USDA110 encodes genes related to methanol oxidation but not those related to carbon fixation of the serine pathway or the ribulose-monophosphate pathway [45,46]. According to a transcriptome analysis [72], methanol utilization genes are not up-regulated in the nodule, although the experiment indicating that was done in essentially lanthanide-free laboratory conditions. Therefore, the physiological role of lanthanide-dependent methanol oxidation in *Bradyrhizobium* with regard to nodule-forming symbiosis must be investigated in more detail.

DISCUSSION

In this work, I analyzed the lanthanide-dependent methanol metabolism of both strains using *M. extorquens* AM1 strain and *B. diazoefficiens* USDA110 strain. Both strains showed the best methanol growth with La but showed slow methanol growth with Nd. Since La-XoxF had a higher molecular function as MDH than Nd-XoxF, it seems that kinds of rare earth elements must determine the lanthanide-dependent methanol growth of both strains. How can XoxF recognize rare earth element species and preferentially choose La, as a better cofactor? Among rare earth elements, La has the highest Lewis acidity tendency and is presumed as the best activator of XoxF. Also, rare earth elements have very similar ionic radii, and it is known that the atomic radius decreases as the atomic number increases. In other words, XoxF5 type MDH has the highest molecular function with La, which has the largest atomic radius, and then it is considered that the XoxF molecule has evolved to utilize La as a cofactor. In contrast, rare earth elements, from La to Nd, which have similar properties and similar ionic radii, are deemed to be able to activate XoxF by being well within the pocket of the active center. In this way, since plant symbiotic bacteria actively utilize methanol, which is one of the major plant VOCs, XoxF has been molecularly evolved as requiring La, which is the best cofactor for expressing MDH activity among lanthanides.

As known, there are abound phyllosphere microorganisms living on the plant system, even cultivated vegetables, and they do affect plants all life, especially plant symbiotic bacteria. Pink-pigmented facultative methylotroph (PPMF) generally refers to

Methylorubrum, a group of microorganisms that can utilize single-carbon compounds such as methane and methanol [1,73,74]. *Methylorubrum* species, one of the most abundant methylotroph bacteria, is always present as the dominant microorganism on the phyllosphere [3,74]. As known, *Rhizobium* species are frequently associated with seeds of the soybean (*Glycine max* L.) [75] and bean (*Phaseolus vulgaris*) [76]. Furthermore, methylotrophs used methanol by plant produce [5] and promote plant growth [77–79] as symbiotic bacteria [80].

Moreover, *Methylorubrum* is usually to be a symbiotic bacterium of plants, always present as an abundance microorganism on the phyllosphere [3,81]. Also, some reports have shown that spinach can uptake part of the lanthanides like Ce^{3+} to replace part of Mg^{2+} to synthesize chlorophyll [82] and promote the rapid photosynthetic process. Also, lanthanides widely exist in plant systems [38]. Moreover, related researches show that *Methylorubrum* can use lanthanides transported by plants to achieve efficient metabolism through XoxF methanol dehydrogenase [18] and produce plant hormones to make a mutually-symbiotic relationship with plants.

It seems like *Methylorubrum* and *Bradyrhizobium* species benefit from the plants to provide methanol and lanthanides in environments or plants system. In nature, XoxF proteins existed in large quantities because methylotrophy bacteria use lanthanides, which widely distributed in the environment and many plant systems [38,83,84]. Because of the transportation of lanthanides by plants and the release of methylotrophy in plant symbiotic bacteria have established a good relationship with plants. And one of the essential physiological roles of lanthanides to XoxF for methylotrophy. The schematic of a plant ecosystem Fig.1.

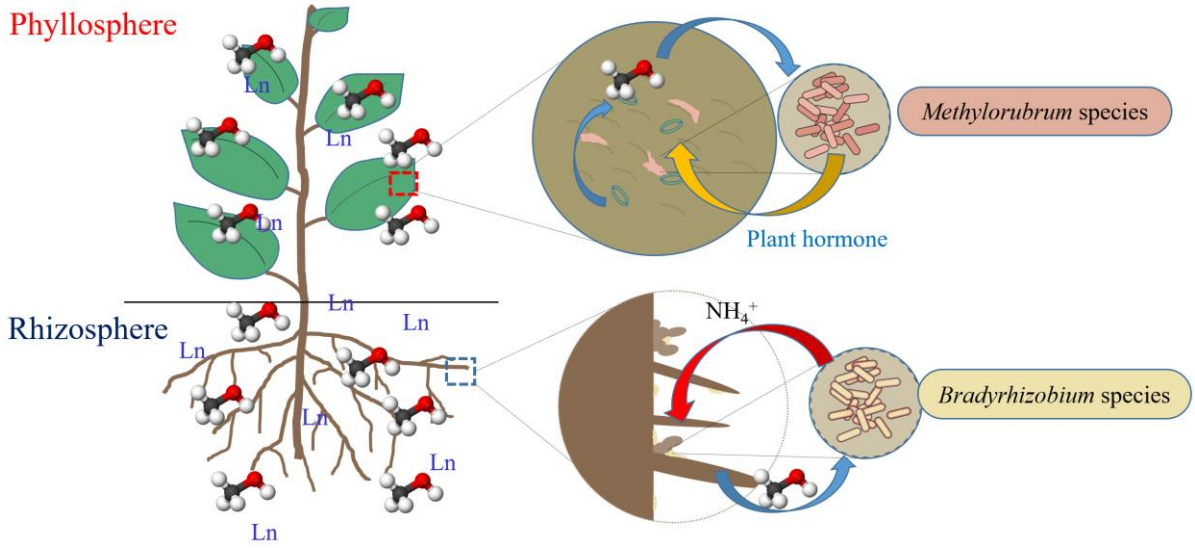


Fig. 1. Schematic of the symbiotic bacterial methylotrophy in a plant system.

In the rhizosphere and phyllosphere, enough levels of lanthanides exist. *Methylorubrum* and *Bradyrhizobium* species are able to utilize methanol emitted from plants and the lanthanides, which have the essential role for bacterial methylotrophy and activation of MDH.

However, it is still unclear that the mechanism of the lanthanide-dependent system in methylotrophy and no-methylotrophy bacteria. It can be expected that lanthanide as an intermedia to promoting agricultural production by plant symbiotic bacteria. And I also expect that the physiological role of lanthanides to the eukaryote will be revealed and the enzyme engineering base on lanthanide-dependent XoxFs will be developed.

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