A Catalytic Role of XoxF1 as La$^{3+}$-Dependent Methanol Dehydrogenase in Methylobacterium extorquens Strain AM1

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Abstract

In the methylotrophic bacterium Methylobacterium extorquens strain AM1, MxaF, a Ca$^{2+}$-dependent methanol dehydrogenase (MDH), is the main enzyme catalyzing methanol oxidation during growth on methanol. The genome of strain AM1 contains another MDH gene homologue, xoxF1, whose function in methanol metabolism has remained unclear. In this work, we show that XoxF1 also functions as an MDH and is La$^{3+}$-dependent. Despite the absence of Ca$^{2+}$ in the medium strain AM1 was able to grow on methanol in the presence of La$^{3+}$. Addition of La$^{3+}$ increased MDH activity but the addition had no effect on mxaF or xoxF1 expression level. We purified MDH from strain AM1 grown on methanol in the presence of La$^{3+}$, and its N-terminal amino acid sequence corresponded to that of XoxF1. The enzyme contained La$^{3+}$ as a cofactor. The Amxf mutant strain could not grow on methanol in the presence of Ca$^{2+}$, but was able to grow after supplementation with La$^{3+}$. Taken together, these results show that XoxF1 participates in methanol metabolism as a La$^{3+}$-dependent MDH in strain AM1.

Introduction

Methylotrophs are microorganisms with the ability to utilize reduced C$_1$-compounds, such as methane, methanol and methylamine as their sole carbon and energy source. They are ubiquitous in nature, and some of them are well-known plant epiphytes [1,2]. Among them, the genus Methylobacterium, an aerobic facultative methylotrophic α-proteobacterium, is one of the most abundant bacterial genera in the phyllosphere [3–5], with a titer between $10^4$ and $10^7$ colony-forming units (CFU) per gram fresh weight of plant material [6]. Over the past few decades, considerable work has been done on the methylotrophy of Methylobacterium and their symbiosis with plants, as Methylobacterium can metabolize the methanol released by plants and may also grow on other plant-derived carbon compounds [7–9]. M. extorquens strain AM1 serves as an important model organism for studying methylotrophy in bacteria [10,11], and the genome sequence of the strain is available [12].

In the methylotrophic metabolism of Methylobacterium, methanol is first oxidized to formaldehyde via methanol dehydrogenase (MDH) in the periplasm [13,14]. MDH is a heterotetrameric protein (α2β2) consisting of two 66-kDa large subunits (MxaF) and two small 8.5-kDa subunits (MxaI) [15], and contains Ca$^{2+}$ and pyroloquinoline quinone (PQQ) as a prosthetic group in the active site [15,16]. MxaF and MxaI are encoded by mxaFI genes located in the large mxa gene cluster [17], and both are essential for growth on methanol, as the loss of these genes in strain AM1 eliminates virtually all methanol dehydrogenase activity [18,19].

The genome of strain AM1 contains several homologs of MxaF, one of which is named XoxF1 [20]. XoxF1 is predicted to be a PQQ-dependent periplasmic MDH exhibiting 50% sequence identity to MxaF. Recently, Schmidt et al. reported that XoxF1 was found to be strongly expressed in bacterial phyllosphere communities [1], and that the xoxF1-deleted strain was less competitive than the wild-type during colonization in the phyllosphere, although XoxF1 had low MDH activity in strain AM1 [21]. Skovran et al. showed that the double mutant of both xoxF homologs (xoxF1 and xoxF2) was unable to grow on methanol and that the expression of the two-component regulatory systems MxcQE and MxbDM required for activation of the mxa genes is repressed in the double mutant strain [22]. From these facts, it is clear that XoxF functions in the regulation of methanol metabolism, but its catalytic function as an MDH has not been clear.

In our previous work, we showed that lanthanum (La), cerium (Ce), and praseodymium (Pr), all of which are belong to the rare earth elements (REE), increased MDH activity in cell extracts of M. radiotolerans and the non-methylotrophic bacteria Bradyrhizobium sp. [23,24]. Moreover, the MDHs purified from the cells grown in media containing these metal ions corresponded to XoxF1, while the MDH purified from Ca$^{2+}$-grown cells corresponded to MxaFI.
These results indicate that the MDHs dependent on La\(^{3+}\), Ce\(^{3+}\) or Pr\(^{3+}\) are products of xoxF and that these ions may have important physiological roles in C\(_1\) metabolism.

The REEs are a group of 17 elements, specifically, 15 lanthanoids plus Sc and Y, and are widely dispersed among many primary and secondary minerals, such as phosphates, carbonates, fluorides, and silicates, especially pegmatites, granites, and related metamorphic and igneous rocks [25]. They are regarded as “the vitamins of modern industry”, since many of them are utilized in a wide range of industrial products such as glass, catalysts, alloys, ceramics, and magnets. As for their effects on life forms, the REEs have not been characterized as either essential or strongly toxic elements in the environment [26], although some have negative effects as inhibitors of several enzymes and proteins [27–30], and some exert positive effects as growth promoters for various crops [27].

In this study, using M. extorquens strain AM1 as a model organism to investigate REE-dependent methylotherotrophy, we set out (i) to see whether La\(^{3+}\) is involved in methylothetic growth of the strain, (ii) to assess whether the strain has REE-dependent MDH activity, (iii) to identify the gene encoding REE-dependent MDH, and (iv) to validate the role of XoxF1 and La\(^{3+}\) in methanol metabolism. Our results suggest that XoxF1 is a La\(^{3+}\)-dependent functional MDH that may participate in methanol metabolism.

**Results**

* M. extorquens strain AM1 has a methanol-oxidation system independent of Ca\(^{2+}\).

Although MDH activity in *Methylobacterium* species has been shown to depend on Ca\(^{2+}\) [14], the growth of these strains on methanol without Ca\(^{2+}\) has never been examined. In our previous work, we showed that some REEs increased MDH activity in *M. radiotolerans* and the non-methylothetic *Bradyrhizobium* sp. [23,24]. These facts suggest that REEs may have some roles as activators or inducers of MDH. Thus, we examined whether *M. extorquens* strain AM1 could grow on methanol in the presence of La\(^{3+}\) instead of Ca\(^{2+}\). As shown in Fig. 1, strain AM1 could grow normally in methanol/Ca\(^{2+}\) medium. In methanol medium without Ca\(^{2+}\) and La\(^{3+}\), the strain showed very slow growth, because the medium contained a small amount of Ca\(^{2+}\) (0.867 μM). In methanol media containing La\(^{3+}\) and not Ca\(^{2+}\), the strain grew as well as it did in methanol/Ca\(^{2+}\) medium, and the addition of La\(^{3+}\) to methanol/Ca\(^{2+}\) medium had no effect on the growth of strain AM1 (Fig. 1). On the other hand, strain AM1 did not show any growth defect in succinate media even without Ca\(^{2+}\) and La\(^{3+}\). Ca\(^{2+}\) and La\(^{3+}\) have an important role in methanol metabolism but not in succinate metabolism, and strain AM1 has a novel methanol-metabolic pathway that depends on La\(^{3+}\) and not Ca\(^{2+}\).

**XoxF1 is a functional La\(^{3+}\)-dependent MDH**

The growth defect of strain AM1 in the methanol medium without Ca\(^{2+}\) was restored by the addition of La\(^{3+}\) to the medium. Next, in order to see whether MDH activity was induced by La\(^{3+}\), we measured MDH activity of strain AM1 grown on media containing La\(^{3+}\). When strain AM1 was grown in methanol media, MDH activity in the cell-free extract was ten times greater in methanol/La\(^{3+}\)+Ca\(^{2+}\) medium than in methanol/Ca\(^{2+}\) medium, and cells grown in methanol/La\(^{3+}\) medium showed levels of MDH activity similar to those in cells grown in methanol/La\(^{3+}\)+Ca\(^{2+}\) medium (Fig. 2). Cells grown on the succinate media also had enough MDH activity more than half of the activity in the methanol-grown cells, and the MDH activity induced on the succinate/La\(^{3+}\) medium was higher than that induced on the succinate/Ca\(^{2+}\) medium, as well as the methanol grown cells.

There are two possible explanations for this positive effect of La\(^{3+}\): one is that La\(^{3+}\) enhances MDH gene(s) expression and the other is that La\(^{3+}\) activates MDH protein.

To determine whether La\(^{3+}\) enhances MDH gene(s) expression, we quantified the gene expression levels of xoxF and xoxF1 using cells harboring the *xyle* reporter gene regulated by the predicted promoter regions, which are 220- and 227-bp upstream sequences of the *MxaF* and *XoxF1* genes, respectively. The reporter activities regulated by the *mxaF* and *xoxF1* promoters were detected in all cells grown on methanol or succinate, and the *xoxF1* promoter of the cells grown on methanol/Ca\(^{2+}\) medium exhibited the highest expression activity (Fig. 3). The activities of both promoters on the methanol grown cells exhibited always higher than those on the succinate grown cells (Fig. 3). Moreover, expression activity of the *xoxF1* promoter was always greater than that of the *mxaF* promoter on any media, irrespective of the presence of La\(^{3+}\) and/or Ca\(^{2+}\).

Xyle activity was not detected in cells harboring the promoterless control plasmid pCM130, irrespective of the carbon sources, as reported previously [31]. These results show that the increase in MDH activity caused by La\(^{3+}\) is due not to an increased expression of MDH genes but rather to post-translational activation of MDH.

We then purified MDH from strain AM1 cells grown in methanol/La\(^{3+}\)+Ca\(^{2+}\) medium in order to identify the La\(^{3+}\)-dependent MDH and to observe whether MxaF and XoxF are concurrently activated by La\(^{3+}\) and Ca\(^{2+}\) (Table 1). In all the purification steps, we observed only one fraction peak showing MDH activity (data not shown). The purified MDH had a specific activity of 10.0 U/mg of protein. The protein migrated as a single protein band on the SDS-PAGE gel with an apparent molecular mass of 61 kDa. A small protein corresponding to subunit α was not observed (Fig. 4), although the MDH purified from cells grown in methanol/Ca\(^{2+}\) medium showed two bands for α and β subunits (data not shown). Using gel chromatography with a Superdex G-200 GL column, the native molecular weight of the purified protein was estimated to be ca. 117 kDa (Fig. 4B). These results indicated that the purified MDH is a homodimer of only the α subunit. The purified enzyme contained 0.91 atoms of La\(^{3+}\) and 0.39 atoms of Ca\(^{2+}\) per dimer. After treatment with 50 mM EDTA, the La\(^{3+}\) and Ca\(^{2+}\) contents in the enzyme were shown to be 1.24 and 0.10 per dimer, respectively, suggesting that the La\(^{3+}\) is tightly bound to the enzyme. The N-terminal amino acid sequence of the MDH protein was NESVLKGVAN-ORF. The predicted cleavage site of the amino acid sequence of XoxF1 as predicted by SignalP version 4.0 [32] was Ala21-Asn22. This cleavage site coincides completely with the N-terminal amino acid sequence of the purified MDH. Taken together, our data show that the *xoxF1* gene encodes a functional La\(^{3+}\)-dependent MDH and that XoxF1 may have a signal peptide for periplasmatic localization.

**XoxF1 encodes an essential MDH for the methylotherotrophy depending on La\(^{3+}\)**

We next examined the growth behavior and MDH induction patterns of an MxaF-disrupted mutant. In succinate media containing La\(^{3+}\) and/or Ca\(^{2+}\), strain *ΔmxaF* exhibited normal growth comparable to that of the wild-type strain (Fig. 5). Strain *ΔmxaF* could not grow on methanol at all in the presence of Ca\(^{2+}\) as previously reported [22], but its growth was restored by supplementation with La\(^{3+}\) even without Ca\(^{2+}\) (Fig. 5).

Strain *ΔmxaF* grown in succinate/Ca\(^{2+}\) medium did not show detectable MDH activity (Fig. 6). Strain *ΔmxaF* grown in succinate or methanol media containing La\(^{3+}\), however, showed MDH activity similar to those in cells grown in methanol/La\(^{3+}\)+Ca\(^{2+}\) medium.
activity comparable to that of the wild-type strain grown in succinate or methanol media containing La$^{3+}$ (Fig. 6). These results suggest that the XoxF1 is able to function as the MDH in the cells in the presence of La$^{3+}$, in place of MxaF, which explains the growth of the mutant on methanol in the presence of La$^{3+}$.

**Discussion**

*Methylobacterium* species and various other bacteria harbor *xoxF*, which is homologous to *mxaF*, encoding the large subunit of MDH. Over the last few years, the function of XoxF has been the subject of controversy. Using strain AM1, Schmidt *et al.* showed that XoxF1 had low activities of methanol and formaldehyde dehydrogenase activity [21], and the work by Skovran *et al.* suggested that XoxF1 and XoxF2 might have some roles in the expression of the MDH genes [22]. Nevertheless, the function and physiological role of XoxF in methanol metabolism is not yet completely understood.

In this work, we showed that XoxF1 from strain AM1 functions as a La$^{3+}$-dependent MDH and has a role in La$^{3+}$-dependent methanol metabolism of the strain, because (i) purified XoxF1 from the strain grown on methanol in the presence of La$^{3+}$ contained La$^{3+}$ with significant MDH activity, (ii) strain AM1 could grow on the methanol/La$^{3+}$ medium even without Ca$^{2+}$, and (iii) the growth defect of strain ΔmxaF was completely restored by supplementation with La$^{3+}$. In our previous work, we have shown that MDHs purified from *M. radiotolerans* strain NBRC15690 grown in the presence of REEs and non-methylotrophic bacterium *Bradyrhizobium* sp. strain MAFF211645 have significant MDH activity and that the N-terminal amino acid sequences of both enzymes were identical to those of XoxF homologues [23,24]. These facts suggest that XoxF1 in strain AM1 has an important physiological role as an MDH in the methanol metabolism in the presence of La$^{3+}$, and that an REE-dependent methanol-metabolic pathway may be distributed among known methylotrophic bacteria as well as in other non-methylotrophic bacteria containing XoxF homologues.
It has been reported that XoxF1 purified from strain ΔxoxF harboring the pCM80-xoxF-His vector grown on Ca\(^{2+}\)-containing medium exhibits low MDH activity (V\(_{\text{max}}\) value for methanol is 0.015 U/mg) [21]. La\(^{3+}\)-dependent XoxF1, however, exhibited significant specific activity for methanol (10.0 U/mg), with levels over fifteen times higher than those in purified Ca\(^{2+}\)-induced MxaF1 from cells grown on Ca\(^{2+}\)-containing medium (0.66 U/mg) (data not shown). Strain Δmdh grown in the methanol/Ca\(^{2+}\) medium had little MDH activity despite high expression levels of the xoxF1 gene (Fig. 3). Moreover, Ca\(^{2+}\)-induced XoxF1 was a monomer [21], while La\(^{3+}\)-containing XoxF1 was a homo-dimer of α-subunits only (Fig. 4). Thus it can be hypothesized that La\(^{3+}\) can facilitate the dimerization of XoxF1 protein, which in the absence of La\(^{3+}\) is an inactive monomeric apo-enzyme. Similarly, since there was no fraction showing any MDH activity except for that containing XoxF1, MxaF1 may be inactive in cells grown in the presence of La\(^{3+}\), although their hetero-tetramerization has not been examined (data not shown). It has been reported that Ca\(^{2+}\)-dependent enzymes and other proteins, e.g., horseradish peroxidase, might be inhibited by La\(^{3+}\) [27–30]. Thus we hypothesize that La\(^{3+}\) may inhibit MxaF1 posttranslational activation and/or its activity. Taken together, our work suggests that the posttranslational activation of XoxF1 and that of MxaF1 require La\(^{3+}\) and Ca\(^{2+}\), respectively, and that strain AM1 has the ability to generate MDH, either XoxF1 or MxaF1, depending on which metal is present, for methanol metabolism.

La\(^{3+}\) is one of the REEs, which are relatively abundant in the earth’s crust (35 μg/g for La\(^{3+}\), 66 μg/g for Ce\(^{3+}\), and 40 μg/g for Nd\(^{3+}\)); in fact, the abundance of Ce\(^{3+}\) is almost equal to those of much more commonly studied elements in the environment, such as Cu and Zn [26]. La\(^{3+}\) exists in all plants examined, with levels of around 0.178–3.1 μg/g dry mass in leaves, which is in the same range as Mn (0.5–15.6 μg/g dry mass) and Fe (1.33–2.5 μg/g dry mass) [25]. Meanwhile, Delmotte et al. have reported that XoxF is highly expressed in bacterial phyllosphere communities in situ, with a prevalence similar to that of MxaF as demonstrated by shotgun proteomics [1]. Therefore, the Methylobacterium species, as major plant epiphytes, would be readily able to access La\(^{3+}\) and Ca\(^{2+}\) on plant surfaces in the natural environment, and it is highly possible that XoxF1 is active on plant leaf surfaces together with MxaF1, because XoxF1 and MxaF are induced by methanol regardless of the presence of La\(^{3+}\) and/or Ca\(^{2+}\).

In this paper, we showed that XoxF1 is a functional MDH that depends on La\(^{3+}\). As far as we know, this is the first report of a metabolic pathway and enzyme dependent on an REE as a cofactor. Recently, XoxF was reported to be involved in a complex regulatory cascade of a MxcQE two-component system [22]. Taking our data together with these reports, it appears that XoxF may play a dual role in both regulation of MDH genes and catalysis of methanol oxidation.

**Materials and Methods**

**Bacterial strains, media, and cultivation**

*M. extorquens* strains and plasmids used in this study are described in Table 2. *M. extorquens* strains were cultivated in minimal salts (MS) media [33] supplemented with 0.5% methanol or 0.4% succinate as a carbon source. MS medium with 0.5% methanol is referred to as methanol/Ca\(^{2+}\) medium, methanol/Ca\(^{2+}\)+La\(^{3+}\) medium containing 30 μM LaCl\(_3\) is referred to as methanol/Ca\(^{2+}\)+La\(^{3+}\) medium, and MS medium with 0.5% methanol containing 30 μM LaCl\(_3\) instead of CaCl\(_2\) is referred to as methanol/Ca\(^{2+}\)+La\(^{3+}\)-dependent MDH.

**Table 1.** Purification scheme of the La\(^{3+}\)-dependent MDH isolated from *M. extorquens* strain AM1.

| Step          | Total activity (Unit) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---------------|-----------------------|--------------------------|---------------------|-----------|
| Cell free extract | 46                    | 0.62                     | 1.0                 | 100       |
| PD-10        | 33                    | 0.74                     | 1.2                 | 71        |
| Hi-trap SP HP Sepharose HP | 15                  | 14                       | 22                  | 32        |
| MonoS 5/50 GL | 4.5                   | 10                       | 17                  | 18        |

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**Figure 4.** SDS–PAGE analysis (A) and molecular weight (B) of purified MDH from strain AM1 grown on methanol/Ca\(^{2+}\)+La\(^{3+}\) medium. A, Lane 1, marker proteins; lane 2, purified MDH. B, Marker proteins were: 1, ovoalbumin (43 kDa); 2, conalbumin (75 kDa); 3, aldolase (158 kDa); and 4, ferritin (440 kDa).

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Methanol/La³⁺ medium. For the cultivation of strain AM1 to purify La³⁺-dependent MDH, a 1/10 nutrient medium supplemented with 0.5% methanol and 30 μM LaCl₃ was used [23]. In this medium, the content of Ca²⁺ was 31.8 μM. When appropriate, antibiotics were added at the following concentrations: tetracycline (Tc), 10 μg/ml, and kanamycin (Km), 50 μg/ml.

Cultivation of M. extorquens strains was done in 200 μl of MS media in 96 well round bottom microplates (Asahi Glass Co., Ltd., Chiba, Japan) at 28°C with reciprocal shaking, and growth was monitored by measuring the optical density at 610 nm in the a HTS BioMicroplate reader (Scinics co, Ltd., Tokyo, Japan). This medium, the content of Ca²⁺, was measured according to the methods of Day and Anthony [36] and/or La³⁺, and kanamycin (Km), 50 μg/ml.

Construction of null mutants

Null mutants were generated in mxaF using the allelic exchange vector pCM184 [35]. The following primers were used for the amplification of the up- and downstream regions of mxaF: upstream of mxaF, mxaFup-fw (5’-GGATCCGTCTCAGATGCCACCTTCG-GATG-3’) and mxaFup-rv (5’-GGATCCGTCTCAGATGCCACCTTCG-GATG-3’); downstream of mxaF, mxaFdn-fw (5’-GGATCCGTCTCAGATGCCACCTTCG-GATG-3’) and mxaFdn-rv (5’-GGATCCGTCTCAGATGCCACCTTCG-GATG-3’). Each PCR fragment was introduced into pCM184. The resulting allelic exchange vectors were introduced into M. extorquens strain AM1 via conjugation using E. coli strain S17-1. Mutations were confirmed by diagnostic PCR.

Construction of promoter fusions

mxaF and mxaF promoter fusions with sulE encoding catechol 2,3-dioxygenase were constructed in vector pCM130 [31]. The following primers were used for amplification of the promoter regions of mxaF and mxaF: mxaF promoter, PmxaF-fw (5’-GGATCCGTCTCAGATGCCACCTTCG-GATG-3’) and PmxaF-rv (5’-GGATCCGTCTCAGATGCCACCTTCG-GATG-3’); sulE promoter, PsulE-fw (5’-GGATCCGTCTCAGATGCCACCTTCG-GATG-3’) and PsulE-rv (5’-GGATCCGTCTCAGATGCCACCTTCG-GATG-3’). The resulting PmxaF-sulE and PsulE-sulE fusions were transferred into strain AM1 via conjugation.

Purification of La³⁺-dependent MDH

Cells were grown on each medium for 36 h, then harvested and resuspended in 20 mM Tris–HCl buffer, pH 8.0. Cells were broken with a 3110BX mini-beadbeater (Biospec Products, Bartlesville, OK, USA) or an Ultrasonic Disruptor UD-201 (Tomy Seiko Co., Ltd., Tokyo, Japan). Cell debris was removed by centrifugation at 12,000 × g for 10 min at 4°C.

MDH and catechol dioxygenase (XoxF1) activities were measured according to the methods of Day and Anthony [36] and Springer et al. [37], respectively. Protein concentration was determined according to the method of Bradford [38] with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) by using bovine serum albumin as the standard.

Preparation of crude extracts and enzyme assays

Cells were grown on each medium for 36 h, then harvested and resuspended in 20 mM Tris–HCl buffer, pH 8.0. Cells were broken with a 3110BX mini-beadbeater (Biospec Products, Bartlesville, OK, USA) or an Ultrasonic Disruptor UD-201 (Tomy Seiko Co., Ltd., Tokyo, Japan). Cell debris was removed by centrifugation at 12,000 × g for 10 min at 4°C.

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Table 1. Strains and plasmids used in this study.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| Strains           |             |                     |
| AM1               | Wild type (JCM 2805) | JCM                 |
| ΔmxAF             | mxAF::Km    | This study          |
| pCM184            | Allelic exchange suicide vector (Km\(^r\) Tc\(^r\) Ap\(^r\)) | 35                   |
| pΔmxAF            | pCM184 with mxAF upstream and downstream flanks (Km\(^r\) Tc\(^r\) Ap\(^r\)) | This study           |
| pCM130            | Promoter-less xylE fusion vector (Tc\(^r\)) | 31                   |
| pRM246            | pCM130 with mxAF promoter region (Tc\(^r\)) | This study           |
| pRM248            | pCM130 with xoxF1 promoter region (Tc\(^r\)) | This study           |

Determination of La\(^{3+}\) and Ca\(^{2+}\) contents of the media and the purified enzyme

The contents of La\(^{3+}\) and Ca\(^{2+}\) in the media were determined using an Agilent 7500cx ICP-MS system (Agilent Technologies, Inc., Santa Clara, CA, USA). The buffer containing purified enzyme was re-equilibrated in 25 mM Tris-HCl buffer, pH 8.0, using a PD-10 column. The enzyme (2.6 μM) was then incubated with 50 mM EDTA, pH 8.0, at 30°C for 2 h, after which it was desalted and concentrated with an Amicon Ultra-0.5 mL 3 K concentrator.

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Author Contributions

Conceived and designed the experiments: TN RM AT KK. Performed the experiments: TN RM AT TI TH KK. Contributed reagents/materials/analysis tools: TN RM AT KK. Wrote the paper: TN RM AT.

References

1. Delmotte N, Knief C, Chaffron S, Innererburger G, Roschitzki B, et al. (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc Natl Acad Sci U S A 106: 16428-16433.
2. Kawaguchi K, Yurimoto H, Oku M, Sakai Y (2011) Yeast methylotrophy and autophagy in a methanol-oscillating environment on growing *chlorella vulgaris* leaves. PLoS ONE 6: e25257.
3. Knief C, Delmotte N, Chaffron S, Stark M, Innererburger G, et al. (2012) Metaproteogenomic analysis of microbial communities in the phylloplane and rhizosphere of rice. ISME J: 6. doi:10.1038/ismej.2011.192.
4. Knief C, Frances L, Cantet F, Vorholt JA (2008) Cultivation independent characterization of *Methylotobacterium populations* in the plant phylloplane by automated ribosomal intergenic spacer analysis. Appl Environ Microbiol 74: 2218-2228.
5. Knief C, Ramette A, Frances L, Alonso-Blanco C, Vorholt JA (2010) Site and plant species are important determinants of the *Methylotobacterium community composition in the plant phyllosphere*. ISME J 4: 719-728.
6. Holland MA, Long RLG, Polacco JC (2002) *Methylobacterium spp.*: Phylloplane bacteria involved in cross-talk with the plant host. In: Lindow SE, Hecht-Poinar EI Elliott VJ, editors. Phyllosphere Microbiology. APS Press. pp. 125-133.
7. Abande-Napcott D, Musch M, Tschiersch J, Boettner M, Schub W (2006) Molecular interaction between *Methylotobacterium extorquens* and seedlings: growth promotion, methanol consumption, and localization of the methanol emission site. J Exp Bot 57: 4025-4032.
8. Gourion B, Rossignol M, Vorholt JA (2006) A proteomic study of *Methylotobacterium extorquens* reveals a response regulator essential for epiphytic growth. Proc Natl Acad Sci U S A 103: 13106-13109.
9. Sy A, Timmers AC, Knief C, Vorholt JA (2005) Methylotrophic metabolism is advantageous for *Methylotobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. Appl Environ Microbiol 71: 7245-7252.
10. Chistoserdova L, Chen SW, Lapidas A, Lidstrom ME (2003) Methylotrophy in *Methylotobacterium extorquens* AM1 from a genomic point of view. J Bacteriol 185: 2980-2987.
11. Schrader J, Schilling M, Holmman D, Sell D, Filho MV, et al. (2009) Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. Trends Biotechnol 27: 107-115.
12. Vuilleumier S, Chistoserdova L, Lee MC, Bringel F, Lajas A, et al. (2009) *Methylotobacterium* genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources. PLoS One 4: e3384.
13. Anthony C (2000) Methanol dehydrogenase, a PQQ-containing quinoprotein dehydrogenase. Subcell Biochem 35: 73-117.
14. Anthony C, Williams P (2003) The structure and mechanism of methanol dehydrogenase. Biochim Biophys Acta 1647: 18-23.
15. Williams PA, Coates E, Mohammed F, Gill R, Eskin PE, et al. (2005) The atomic resolution structure of methanol dehydrogenase from *Methylotobacterium extorquens*. Acta Crystallogr D Biol Crystallogr 61: 75-79.
16. Anthony C, Zatman LJ (1964) The methanol-oxidizing enzyme of *Pseudomonas sp.* M 27. Biochem J 92: 614-621.
17. Lidstrom ME, Anthony C, Briville F, Gasser F, Goodwin P, et al. (1994) New unified nomenclature for genes involved in the oxidation of methanol in Gram-negative bacteria. FEMS Microbiol Lett 117: 103-106.
18. Nunn DN, Lidstrom ME (1980) Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylotobacterium* sp. strain AM1. J Bacteriol 146: 581-590.
19. Nunn DN, Lidstrom ME (1996) Phenotypic characterization of 10 methanol oxidation mutant classes in *Methylotobacterium* sp. strain AM1. J Bacteriol 166: 591-597.
20. Chistoserdova L, Lidstrom ME (1997) Molecular and mutational analysis of a DNA region separating two methylotrophy gene clusters in *Methylotobacterium* AM1. Microbiology 143: 1729-1736.
21. Schmid M, Chislenko P, Kief P, Vorholt JA (2010) Functional investigation of methanol dehydrogenase–like protein XoxF in *Methylotobacterium extorquens* AM1. Microbiology 156: 2575-2586.
22. Skovran E, Palmer AD, Rountree AM, Good NM, Lidstrom ME (2011) XoxF is required for expression of methanol dehydrogenase in *Methylotobacterium extorquens* AM1. J Bacteriol 193: 6032-6038.
23. Fittiyanto NA, Fushimi M, Matsumaga M, Pertinawingrum A, Iwama T, et al. (2011) Molecular structure and gene analysis of *Ce*?-induced methanol (Millipore, Billerica, MA, USA) concentrator and diluted with Milli Q water (Millipore). The *La*\(^{3+}\) and *Ca*\(^{2+}\) contents in the enzyme were determined using an ULTIMA 2 ICP-OES spectrometer (Horiba Ltd., Kyoto, Japan).

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Author Contributions

Conceived and designed the experiments: TN RM AT KK. Performed the experiments: TN RM AT SS TI TH KK. Analyzed the data: TN RM AT TI. Contributed reagents/materials/analysis tools: TN RM AT KK. Wrote the paper: TN RM AT.
dehydrogenase of *Bradyrhizobium* sp. MAFF211645. J Biosci Bioeng 111: 613–617.

24. Hibi Y, Asai K, Arafuka H, Hanajima M, Iwama T, et al. (2011) Molecular structure of La\(^{3+}\)-induced methanol dehydrogenase-like protein in *Melphotbacterium radiotolerans*. J Biosci Bioeng 111: 547–549.

25. Tyler G, Olson T (2005) Rare earth elements in forest-floor herbs as related to soil conditions and mineral nutrition. Biol Trace Elem Res 106: 177–191.

26. Tyler G (2004) Rare earth elements in soil and plant systems—a review. Plant Soil 267: 191–206.

27. Brawn PH, Rathjen AH, Graham RD, Tribe DE (1990) Rare earth elements in biological systems, In: Gschneidner KA Jr, Eyring L, Editors, Handbook on the physics and chemistry of rare earths, vol. 13. North-Holland, Elsevier Science Publishers BV, pp. 423–452.

28. Erdmann F, Jung M, Eyrisch S, Lang S, Helms V, et al. (2009) Lanthanum ions inhibit the mammalian Sec61 complex in its channel dynamics and protein transport activity. FEBS Lett 583: 2359–2364.

29. Liu P, Liu Y, Lu Z, Zhu J, Dong J, et al. (2004) Study on biological effect of La\(^{3+}\) on *Escherichia coli* by atomic force microscopy. J Inorg Biochem 98: 68–72.

30. Wang L, Zhou Q, Lu T, Ding X, Huang X (2010) Molecular and cellular mechanism of the effect of La(III) on horseradish peroxidase. J Biol Inorg Chem 15: 1063–1069.

31. Marx CJ, Lidstrom ME (2001) Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. Microbiology 147: 2065–2075.

32. Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785–786.

33. Harder W, Attwood M, Quayle JR (1973) Methanol assimilation by *Hyphomicrobium* spp. J Gen Microbiol 76: 155–163.

34. Simon R, Priefer U, Huhler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Nature Biotechnol 1: 784–779.

35. Marx CJ, Lidstrom ME (2002) Broad-host-range cre-lox system for antibiotic marker recycling in gram-negative bacteria. Biotechniques 33: 1062–1067.

36. Day DJ, Anthony C (1990) Methanol dehydrogenase from *Methylbacterium extorquens* AM1. Methods Enzymol 188: 210–216.

37. Springer AL, Morris CJ, Lidstrom ME (1997) Molecular analysis of MsdD and MsdM, a putative sensor-regulator pair required for oxidation of methanol in *Methylbacterium extorquens* AM1. Microbiology 143:1737–1744.

38. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72: 248–254.