Molecular mechanisms of rare earth element utilization by methane-oxidizing bacteria and protease-producing bacteria

（メタン酸化細菌およびプロテアーゼ生産菌のレアアース元素利用機構）

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Abbreviations

REE……..……..……rare earth element

LREE……..……..…light rare earth element

HREE……..……..……..…heavy rare earth element

MMO……..……..……..……methane monooxygenase

MDH…….……methanol dehydrogenase

REE-MDH……..……REE-dependent methanol dehydrogenase

Ca-MDH……Ca-dependent methanol dehydrogenase

ADH……..……..……alcohol dehydrogenase

MB……methanobactin

LanM…….……lanmodulin

ABSTRACT

Rare earth elements (REEs) form a chemically uniform group and include scandium (Sc), yttrium (Y), and 15 lanthanides. Despite the name, their abundance in the earth's crust is not low. Their bioavailability had long been overlooked until their discovery as a co-factor in the active site of alcohol dehydrogenases, such as XoxF-type methanol dehydrogenase (REE-MDH), whereas their well-characterized counterparts MxaF-type (Ca-MDH) are Ca-dependent. Since then, the response in various methane/methanol-oxidizing bacteria to REEs has been researched and it showed REEs can readily enhance and suppress the expressions of Xox-MDH and Mxa-MDH, respectively. This regulation, the so-called REE switch has only been investigated in a limited number of species. Furthermore, most REEs exist in the natural environment as insoluble oxide forms. However, the utilization mechanism of such REEs is remaining unclear. In addition, there are no known cases of REEs being used by enzymes other than alcohol dehydrogenases.

In this study, REE switch and the differences from other species were confirmed in one of the model strains of less studied gamma-proteobacterial methane-oxidizing bacterium *Methylococcus capsulatus* Bath. Moreover, the molecular mechanisms of their utilization of insoluble REE were investigated. Besides, as a Ca-containing enzyme, protease has drawn attention. The REE effect on protease-producing bacteria is examined.

1. REE switch in *Methylococcus capsulatus* Bath

REE switch in Bath was investigated by quantitative RT-PCR and transcriptome analysis, with up-and down-regulation on the expressions of REE-MDH and Ca-MDH respectively by the supplement of Ce as chlorides, one of the most abundant REEs. Other than Ce, results also showed the REE switch in Bath can be triggered by light REEs like La readily and by Nd slightly but not by non-lanthanide REEs (Sc, Y) or heavy REEs like Dy. Compared to other

reported species, Bath responds to lower REE concentrations at 10 nM.

2. Putative molecular mechanism of insoluble REE utilization by Bath

Bath responds to not only soluble REE chlorides but insoluble forms of REE oxides. When insoluble REE oxides (CeO_2) were suspended with Bath culture supernatant, elution of Ce ions was observed, indicating the presence of REE chelators in the culture supernatant. The production of REE chelators was also confirmed by the Chrome Azurol Sulfonate (CAS) assay, which changes color depending on the presence of metal chelator compounds. It was also found that Bath produces the REE chelators only under conditions of insufficient REEs (<0.1 μM soluble $CeCl_3$). Further, gene expression was compared under chelator-producing and non-chelator-producing conditions. Twenty-six genes were highly expressed in the chelator-producing condition. Among them, we found two genes that may be related to chelator production, namely, a non-ribosomal peptide synthetase gene and a c-type cytochrome gene. Knockout mutants of these genes are now under construction and their functions are being analyzed.

3. REE effect on protease-producing bacteria

Since REEs especially the Ln^{3+} are showing chemical properties similar to Ca^{2+}, REE dependence in other enzymes containing Ca^{2+} such as protease was hypothesized. The results of the accumulation and cultivation of protease-producing bacteria on REE-containing and non-REE-containing media showed different dominant microorganism communities. Three species from *Stenotrophomonas* and *Chryseobacterium* were subsequently isolated from REE-containing media. In which, high protease activities were observed in *Stenotrophomonas*, produced proteases were detected with casein substrate SDS-PAGE. Further identification and the metal content analysis of those proteases are currently underway.

GENERAL OVERVIEW

1. Biological roles of REEs

1.1 Introduction of REEs

Rare earth elements (REEs) comprising yttrium, scandium, and 15 lanthanides, form a chemically uniform group as shown in Figure 1.1A [1]. According to the mean atomic mass (weight) and larger effective radius, lanthanides are further grouped into LREE(s) including lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), samarium (Sm) and europium (Eu), and HREE(s) including gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb) and lutetium (Lu), disregarding the almost non-existing Pm [2]. In numerous advanced technologies, REEs are widely used as critical components from permanent magnets in wind turbines and electric car batteries, to lasers and phosphors, to medical imaging agents [3-5]. These chemically close elements are mostly found in the natural environment as phosphate or carbonate minerals, such as bastnaesites, monazites, and xenotime [6, 7]. Their chemical similarity makes them difficult to separate, and their insolubility and uneven distribution have made these metals to be considered rare and biologically inactive for a long time. In fact, REEs are relatively abundant in the Earth's crust disregarding the extremely rare Pm. The abundance of Ce (66 $\mu g\ g^{-1}$) is almost the same as environmentally much more studied elements, such as Cu and Zn. And the scarcest lanthanides, Lu and Tm (0.5 $\mu g\ g^{-1}$) are more abundant in the Earth's crust than Cd and Se (see Figure 1.1B).

During the last decade, along with the increasing knowledge about the ICP-MS/ICP-OES technique, quantitative analysis of REEs widened the research perspective to their biological roles [1, 2]. For example, some lanthanides (La, Ce, Pr, Nd) from LREEs perform similar chemistry to other biologically useful metals such as calcium and iron. Possessing the

sufficiently distinct coordination chemistry allows those lanthanides to be selectively uptake, trafficked, and incorporated into enzymes, they are showing even more enzymic efficiency due to the higher Lewis acidity [3]. These properties make REEs, which are otherwise considered to be biologically inactive, have been utilized biologically.

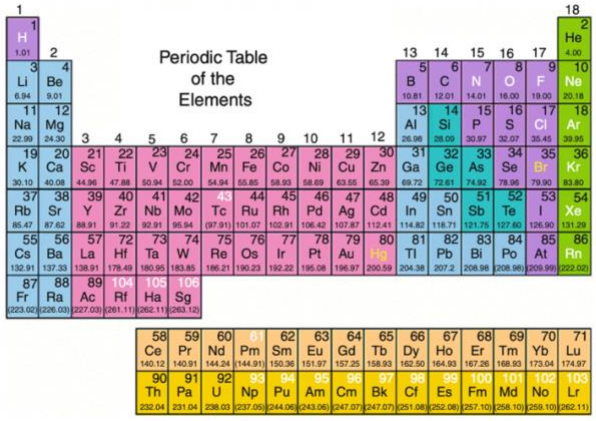


Figure 1.1A Periodic table of the elements. The REEs Sc, Y, and 15 lanthanides including La to Lu [8].

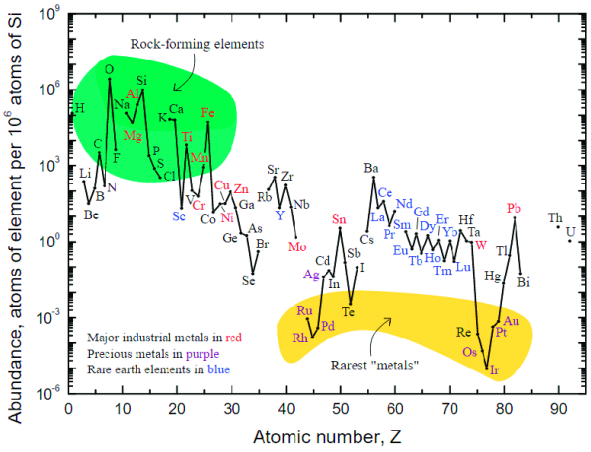


Figure 1.1B Crustal abundances of the REEs relative to silicon (adapted from USGS by Kim, 2018 [9]).

1.2 Effect of REEs on seed germination, growth, and photosynthesis in plants

REEs are reported to be applied in agriculture as micro-fertilizers to improve crop nutrition in China for recent decades [10-12]. The specific effects of rare earth elements on plants have only been intensively studied in recent decades. Mainly effects were demonstrated on photosynthesis,

germination, biomass production, secondary metabolites, or enzymes.

A study about the photosynthetic characteristics of soybean seedlings showed Ce can alleviate the inhibition of UV-B radiation on the photosynthesis in soybean seedlings to a certain extent [13]. It's also reported that Ce^{3+} could obviously stimulate the growth of spinach and increase its chlorophyll contents and photosynthetic rate [14]. On the contrary, REEs are not always having a positive effect, under magnesium deficiency, Ce^{3+} exhibits inhibition of chlorophyll biosynthesis of maize [15]. Research showed in *Salvia miltiorrhiza*, 30 mg/L of La^{3+} solution displays the optimal promotion effect on germination rate and germination potential, and 20 mg/L of La^{3+} solution optimally promotes the germination index and vigor index. Besides, the contents of soluble sugar, soluble protein, and chlorophyll also got increased by La^{3+}, while the high concentration of La^{3+} can inhibit the plant growth [16]. The significant decrease in root and plant height and biomass also reflected the inhibition of rice growth by 0.5 mg/L and 1 mg/L REEs [17].

As for the uptake of REEs, some evidence showed that La^{3+} affected the expression of plant calmodulin in endocytosis and low concentration of La^{3+} can interact and bind to two Ca-binding sites of plant calmodulin, providing insights into REEs uptake mechanisms in plants [18, 19].

1.3 Biological effect of REEs on bacteria and fungi

Although REE has a relatively long history of utilization in agriculture, its specific roles remain uncovered. Notably, the specific utilization by enzymes was first established in bacteria in 2011, Ce (one of the light REEs) was found to increase the protein level of several proteins in *Bradyrhizobium* sp. MAFF211645 and one of them was identified to be similar to the well-characterized quinoprotein containing pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (MDH) [20, 21]. Further, it was found the REE-dependent MDH (REE-MDH)

was encoded by *xoxF*, with a counterpart of Ca-dependent MDH (Ca-MDH) encoded by *mxaF*. Both Ca-MDH and REE-MDH function as MDH catalyzing the methanol oxidation in *Methylobacterium extorquens* AM1 [22]. The XoxF-type MDHs were then investigated in various methane/methanol oxidizing bacteria (methanotrophs and methylotrophs respectively) [23-25]. After then, the example of REE-dependent alcohol dehydrogenases has been reported in non-methylotrophic bacteria [26]. Although the important role of REEs in MDH was not discovered until recent decades, reports since then suggest that XoxF-type MDH is abundantly present. commonly found in nature. The isolation of *Methylacidiphilum fumariolicum* SolV and *Methylosinus* sp. strain Ce-a6, which harbor only the *xoxf*, shows the REE-dependent growth and absolute REE requirement [23, 27].

Besides methylotrophs and methanotrophs, the investigation of REEs effect on other microbes has been much less yet increased recently. The growth of *E. coli* BW25113 was monitored in various concentrations of in total 16 types of REEs. Results showed that all the tested REEs are toxic to microbial cells in a concentration-dependent manner. Especially the HREEs and scandium were more toxic than LREEs. Bacteria injury assessment showed that REEs brought cell membrane damage involving lipid peroxidation and enhanced membrane permeability and depolarization, resulting the impaired ATP production [28]. Similar toxicities were found by the above 16 REEs in *E. coli* K-12, the survival rate of K-12 significantly dropped after treatment of REEs, though their toxicities were weaker than that of Zn and Cu. As for fungi, the toxicity of REEs was weaker than that of silver but comparable to that of copper [29].

1.4 Effect of REEs on higher organisms

After in-feed growth-promoting antibiotics were banned in 2006, REEs have been studied as an alternative feed additive, and research about their effect on farm animals was increased recently. As a feed additive, REE is still very controversial, as it has not been thoroughly studied

toxicological and ecotoxicological [30]. Evaluation of the external and internal parameters showed that 150 mg/kg of CeCl and CeO diet brought improved egg quality of laying hens [31]. However, REEs have been reported to be detected in human hair, blood, and male sperm [32-35], and exhibit relations to various functional impairments and diseases in humans, such as children's IQ levels, breast cancer, and genotoxicity [36, 37]. Children living in mining areas were detected to contain REEs in their urine, which may affect their health, while lower detection rates of REEs in their drinking water indicate that REEs entered the body through other sources [38]. Considering the absorption and accumulation of REEs in some plants, REEs have the possibility to enter the food chain which emerged further research about REEs, and organisms is needed.

2. Aerobic methanotrophs and their metal utilizations

2.1 Aerobic methanotrophs diversity and their central metabolic pathway

Aerobic Methanotrophs consume methane as the sole carbon and energy source and have been found to ubiquitously distribute in diverse natural environments such as paddy fields, landfill soil, forest soil, and peat dogs [39-45]. They are also phylogenetically diverse, so far found in Proteobacteria, *Verrucomicrobia*, and *candidate phylum NC10* [46, 47]. Quite a similar pathway shown in Figure 2.1 was followed in diverse methanotrophs, they oxidize the methane to methanol initially by methane monooxygenase (MMO), then convert methanol to formaldehyde using methanol dehydrogenases (MDHs). Formaldehyde is then converted to formate either using a ribulose monophosphate way by Type I and Type X methanotrophs or a serine cycle by Type II methanotrophs respectively [48, 49]. Some other non-proteobacterial methanotrophs assimilate carbon through the Calvin-Benson-Bassham (CBB) cycle,

represented by *Methyloacidiphilum fumariolicum* SolV, *Methylacidimicrobium* spp., and "*Candidatus* Methylomirabilis oxyfera" [50-53].

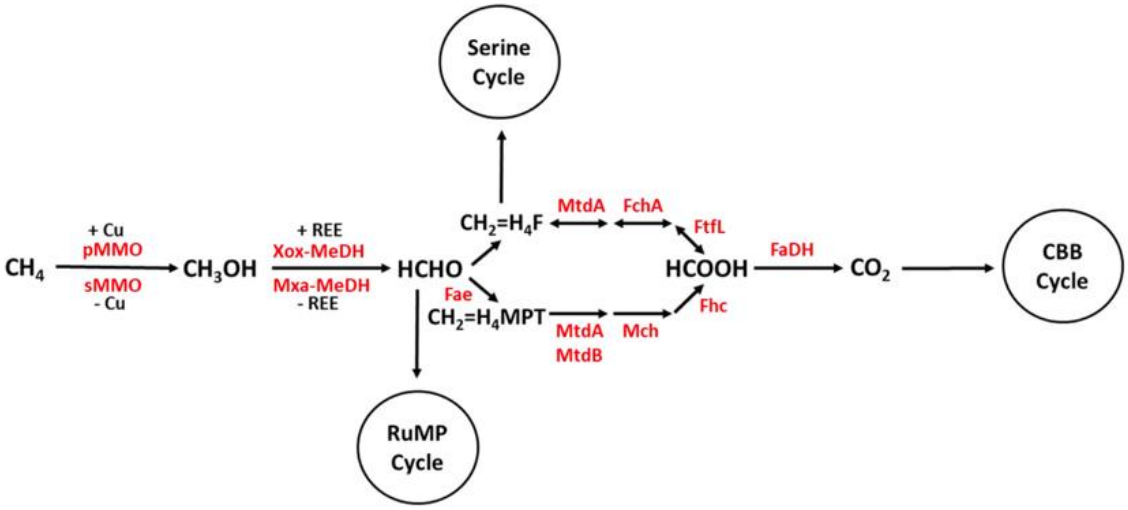


Figure 2.1 General pathway of methane oxidation by aerobic methanotrophs. Enzymes are noted in red [53].

2.2 Two types of MMOs and their regulated expressions in methanotrophs

The initial oxidation step of methane in methanotrophs is catalyzed by methane monooxygenases (MMO), two types of MMO have been characterized. One is a membrane-bound methane monooxygenase, known as particulate MMO (pMMO). Another one is a cytoplasmic type, known as soluble MMO (sMMO), which is evolutionarily separate from pMMO [49]. Figure 2.2A is showing the X-ray crystal structure of pMMO from *M. capsulatus* Bath, which consists of three subunits (PmoA, PmoB, and PmoC) and copper as cofactors [54, 55]. Figure 2.2B is showing three components in sMMO, this enzyme system requires a hydroxylase MMOH (*mmoXYZ*), a reductase MMOR (*mmoC*), and a regulatory or effector protein MMOB (*mmoB*) for optimal activity [56-58].

Most methanotrophs harbor pMMO, and part of them harbor sMMO at the same time, such as *M. capsulatus* Bath which is the object of this study [47, 53]. Copper and iron are required by pMMO and sMMO respectively for enzymic activities, methanotrophs which can express both

MMOs have alternative expressions of two types of MMOs. This regulation was described as the “copper switch” that showed suppressed expressions of sMMO and promoted expressions of pMMO with the presence of copper [59].

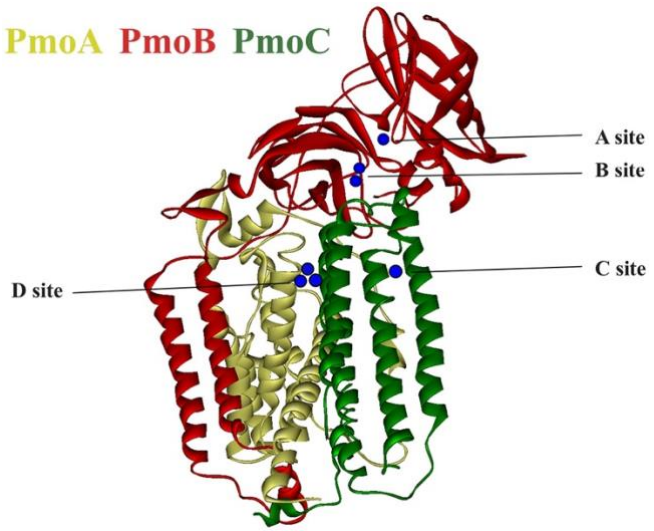


Figure 2.2A X-ray crystal structure of pMMO from *M. capsulatus* Bath [54]. The membrane topology of the PmoB subunit (red) is highlighted vis a vis the PmoA (yellow) and PmoC (green) subunits. The locations of the known copper sites are shown by blue atoms, four sites were established, or putative copper sites described previously [55, 60, 61].

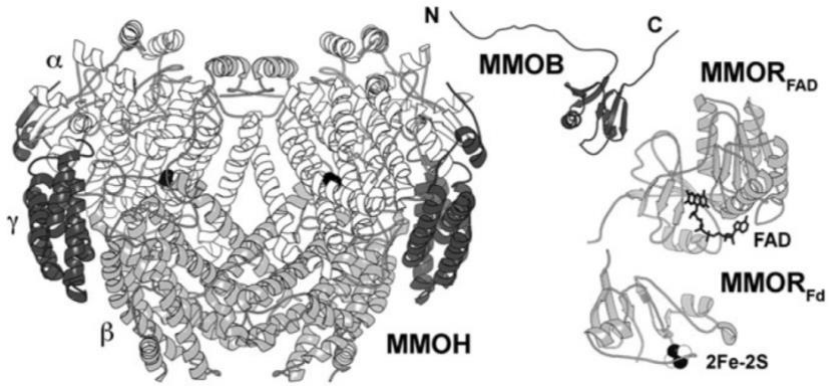


Figure 2.2B Three components (MMOH, MMOB, and MMOR) of soluble methane monooxygenase from *M. capsulatus* Bath[56].

2.3 Copper acquisition in methanotrophs

The protein CopC found in *Pseudomonas syringae*, was reported to be capable of binding both copper(I) and copper(II) at two different sites [62, 63]. The protein CopD, which is an inner membrane transporter that may take the copper donated from CopC, was suggested to function with CopC in the uptake of copper to the cytoplasm in *Pseudomonas syringae* [64, 65]. These proteins also distribute in other bacteria, such as in methanotroph *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath [66, 67]. However, yet no definitive findings on the role of these proteins in these two strains or their specific functions in copper acquisition.

Instead, some *Alphaproteobacterial* methanotrophs were found to secrete methanobactin (MB), a copper-binding compound or chalkophore, for copper uptake and these characterized MBs are in low molecular sizes, less than 1,300 Da [68-71]. The MBs can be structurally divided into two groups, where group I MBs have two oxazolone groups, differ from group II MBs have a C-terminal oxazolone ring, with the other ring being either an imidazolone or a pyrazinedione moiety [53]. The MB was subsequently demonstrated to be a ribosomally synthesized and posttranslationally modified peptide and the gene cluster (*mbnIRTABCMNPH*) was found to include genes encoding the precursor polypeptide or other proteins involved or putative involved in MB regulation, synthesis, secretion, or uptake in *M. trichosporium* OB3b [72-75]. Especially, the function of gene *mbnT* which encodes a TonB transporter was discovered by the construction of a mutant, resulting in the loss of the ability to internalize MBs in *M. trichosporium* OB3b [73].

However, a distinguished copper uptake system was utilized in *Gammaproteobacterial* methanotrophs. In *M. capsulatus* Bath, one of the model strains of *Gammaproteobacterial* methanotrophs, secreting an extracellular Cu-binding protein MopE for Cu acquisition [76, 77]. This protein was expressed corresponding to copper concentrations and has the highest

expression when under copper stress, besides, it was reported to have an affinity with both Cu(I) and Cu(II) [78, 79]. The MopE was confirmed with the copper-binding ability and efficiently transports copper into the cell, while the mechanism of MopE transport remains unclear [80]. Another cell surface-located protein CorA, found in *Gammaproteobacterial Methylomicrobium albus* BG8, is sharing amino acid similarity and has an overall fold similar to MopE, suggested to be crucial for copper acquisition in *M. album* BG8 [81, 82].

Comparative transcriptome analysis of the presence and absence of copper in *M. trichosporium* OB3b and *M. capsulatus* Bath showed that, besides the MB cluster, *mopE* and *corA*, some other genes are differentially expressed in response to copper [83, 84]. Such as genes encoding recently identified copper storage proteins and copper efflux system in *M. trichosporium* OB3b, and *c*-type cytochromes associated with the cell surface in *M. capsulatus* Bath [85, 86]. And some of the methanotrophs, synthesizing neither MBs nor MopE/CorA, are employing other yet elusive copper acquisition strategies. These findings suggested a more flexible copper homeostasis system was present in methanotrophs for adapting to varying copper concentrations in the environment.

2.4 Two types of MDHs and their regulated expressions in methanotrophs

The following oxidation step of methanol to formaldehyde in methanotrophs is catalyzed by the enzyme named methanol dehydrogenases (MDH). MDHs belong to the large class of eight-bladed β propeller (PQQ-containing) quinoproteins, which are localized in the periplasm with about 600 amino acids in size [87, 88]. Two types of MDHs have been characterized in methylotrophs and methanotrophs, and just like MMOs, MDHs are also acquiring metals for their activities. As shown in Figure 2.4, MxaF-type MDHs (showing as a well-characterized MxaFI-MDH, which is composed of two large MxaF catalytic subunits and two small MxaI subunits) and XoxF-type MDHs are containing either Ca or REE (showing as Ce here) in the

active sites as co-factor [23, 87, 89]. Research on *M. extorquens* AM1 suggested that more than 25 genes clustered in five groups (*mxa, mxb, mxc, pqqABCDE*, and *pqqFG*) were involved in the functional expression of MxaFI-MDH [90]. These genes, especially the *mxa* gene cluster, were found to be highly conserved in other methylotrophs and methanotrophs [87, 91, 92]. In *mxa* cluster, *mxaF* encodes the MDH large (α) subunit, *mxaJ* encodes a polypeptide with yet unknown functions but is required for functional MDH [93, 94]. The other two genes namely *mxaG* and *mxaI* encode a cognate physiological electron acceptor of methanol oxidation (cytochrome c_L) and the MDH small (β) subunit respectively [87]. Functional genes for XoxF-type MDH expressions are much less than that for MxaF-type ones. Exhibiting the absence of gene *mxaI* encoding the small subunit, as well as the gene *mxaACKLD* encoding for Ca^{2+} insertion and gene *mxaRSEH* for further protein maturation [20, 22, 87].

Having either the Ca or REE in the active site as a co-factor, the MxaF-type and XoxF-type of MDHs are expressed alternatively depending on the metal supplementations. Generally, with the presence of REE, expressions of MxaF-type MDH will be suppressed while expressions of XoxF-type MDH will be promoted, even when the concentration of Ca is much higher than that of REE. This regulation was found ubiquitous in many methylotrophs and methanotrophs, described as the "REE switch" [95-97]. Although the REEs show chemical similarities, not all the REEs can induce the "REE switch". In *Methylosinus trichosporium* OB3b, one of a model strains of *Alphaproteobacterial* methanotroph, light REE of Nd can comparatively regulate the expressions of two MDHs as La does, but Sm and Pr are not able to trigger the switch [98]. In *Methylobacterium extorquens* AM1, which is an *Alphaproteobacterial* methylotroph, Pr and Nd can significantly regulate two MDHs expressions, while Sm was not able to bring the regulations [96]. Part of the specificity comes from the chemical nuances among the REEs. Although they show chemical similarities to each other, the ionic radium, coordination number,

and Lewis acidity make them cannot be easily substituted [99, 100]. Besides, few reports stated that methylotrophic bacteria are producing diverse REE chelator and lanthanide-binding proteins and these siderophores and proteins may be involved in the acquisition and utilization of REEs [101-104]. The diversity of REE binding products may explain the different utilization of REEs among species since each of them has various binding sites and binding affinities.

Interestingly, in methanotrophs, copper governs the expression levels of sMMO and the pMMO in prior methane oxidation, "copper switch" was found to override the "REE switch" in Type II methanotrophic *Methylosinus trichosporium* OB3b. Representing the barely affected MxaF-type MDH expressions and weakened promotion level of XoxF-type MDH expressions in the presence of both Ce and Cu. While the regulations of sMMO and pMMO were not affected by the presence of Ce [84]. However, in one of the Type I methanotrophic bacteria, the "copper switch" and "REE switch" were reported to operate independently in *Methylomicrobium buryatense* 5GB1C [24]. Yet this interaction has only been demonstrated in limited strains, no convincing explanation can be concluded if this phenomenon occurred differentially in Type I and Type II methanotrophs.

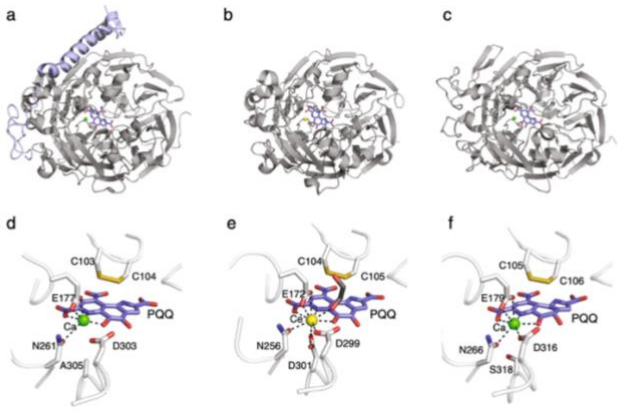


Figure 2.4 Structures of methanol and ethanol dehydrogenase quinoproteins. Bottom views of a MxaFI-MDH of *M. extorquens* AM1 [89], b XoxF-MDH from *M. fumariolicum* SolV [23], and c ethanol dehydrogenase from *P. aeruginosa*, d-f Detailed view of the PQQ catalytic site of MxaFI-MDH, XoxF-MDH, and ethanol dehydrogenase, respectively [87].

2.5 REEs acquisition in methylotrophs

When low-solubility metals are utilized in microorganisms, the process is often accompanied by the assistance of other proteins used for transmembrane metal chelating and transport, metal recognition, and storage. Such as iron, plays a crucial role in biological metabolism, and was reported to be required in a micromolar level of total iron for growth. However, iron concentrations in the environment such as the surface waters of the oceans are only 0.01-2 nM. Thus, iron-requiring microbes are known to be evolved multiple pathways designed to extract iron from their surrounding environments [105]. Recently research has intensively reported the utilization of REEs in methylotrophs and methanotrophs, further, the strains actually require REEs for growth were isolated [23, 27]. The thermoacidophilic methanotroph *Methylacidiphilum fumariolicum* SolV required at least 320 nM of Ce^{3+} for optimal growth, the acidic environment makes it possible for SolV to get sufficient REE from the environment. While in the circumneutral environment, REEs are mostly in mineral forms which poorly soluble with a low concentration to sub-nanomolar. A methanotroph named *Methylosinus* sp. strain Ce-a6 isolated from a circumneutral environment required 30 nM of $CeCl_3$ for growth, suggesting the efficient strategy for REEs acquisitions employed by REEs-requiring strains.

Although the unequivocal utilization of rare earth elements has only recently been discovered, there has been a long history of research on microbial utilization of other metal mechanisms, which has led to a more directional approach to the study of the utilization mechanisms of rare earth elements. In the last 5 years, related studies have been reported (Figure 2.5), and the utilization mechanism of rare earth elements is becoming clearer, but there are still many ambiguities that need to be resolved. Existing studies show that interspecies differences are also evident, and such studies need to be explored in more strains, especially in methanotrophs, for a more complete understanding.

2.5.1 Transmembrane REEs uptake in methylotrophs and methanotrophs

For iron acquisition, iron-chelating compounds (siderophores) are commonly produced by microbes. Those metal-chelating compounds were recently reported to be involved in Ln uptake in methylotrophs. An eight-gene cluster encoding the staphyloferrin B-like (sbn) siderophore found in *Methylobacterium aquaticum* Strain 22A suggested a crucial function of siderophore for Ln uptake, besides, an exclusive TonB-dependent receptor (TBDRs) was discovered to be in charge of Ln uptake [106]. The Ln-chelator was also reported in methylotroph *Methylorubrum extorquens* AM1. A cluster encodes a TonB-dependent receptor and NRPS biosynthetic enzymes which are predicted to produce a metal-chelating compound were found to be promoted expressed when AM1 grew on poorly soluble Nd_2O_3. Further, this gene production was predicted to be similar to the siderophore named aerobactin. These studies provided insight into the Ln uptake mechanism strategy in methylotrophs, which through metal-chelating compounds and TonB-dependent receptors like the mechanism for iron uptake.

2.5.2 Lanthanide-binding protein

In one of the most studied methylotrophs *Methylobacterium extorquens*, a highly selective Ln^{III}-binding protein was found in 2018, which was the first report of lanthanide-binding protein after the found of REE-MDH. The newly found lanthanide-binding protein was localized to the periplasm, which is the location of REE-MDH. This protein was characterized to possess four metal-binding EF-hand motifs which were commonly found in the Ca^{2+}-binding protein calmodulin, thus it was named lanmodulin (LanM). The conformation changes in LanM from a largely disordered state to a compact, ordered state in the presence of picomolar concentrations of all Ln^{3+}, while in the case of Ca^{2+}, millimolar concentrations were required to trigger this response. This phenomenon suggested LanM has a high selectivity to Ln^{3+}. It

provides evidence of efficient lanthanides recognition in lanthanides-utilizing bacteria. However, this protein is not ubiquitous in all methylotrophs and methanotrophs and is not required for growth, suggesting the species without harboring LanM may possess other lanthanide-binding proteins for the same functions [103].

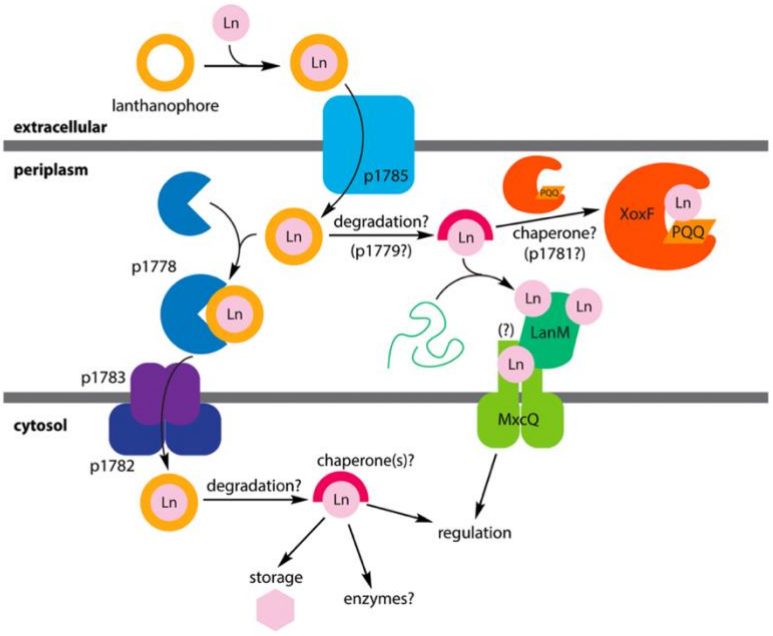


Figure 2.5 Model for lanthanide uptake and utilization in *M. extorquens* [3].

3. Effect of REEs on enzymes other than MDHs

Rare earth elements especially the light ones are chemically similar to calcium. Such as Ce^{3+} and Ca^{2+} exhibit similarities in ionic radius, bonding, and preferences for donor atoms [107]. However, some properties confer REEs more potential for bioavailability, such as the high charge of Ln^{3+} ions makes them good Lewis acids and more robust Lewis acid catalysts, their distinctive coordination chemistry facilitating the selective recognition [3].

Besides methanol dehydrogenases, yet no other enzymes were reported to be REE-dependent. However, REEs did show effects on other enzymes than MDH, especially those characterized to be Ca-dependent, such as proteases and amylases. By adding the scandium to the media for

Bacillus subtilis, the activity of amylase, protease, and antibiotic got promoted [108]. Besides, the artificial replacement of Ca with REEs promoted the thermal stability of proteinase K [109].

CHAPTER 1: REE switch in *Methylococcus capsulatus* Bath

1.1 Introduction

Methylotrophs and methanotrophs are taking reduced carbon substrates such as methane or methanol as their sole carbon and energy sources [110]. As an important part of the carbon cycle, methylotrophs and methanotrophs are found to be ubiquitously distributed in the environments even those with extreme conditions. Varieties of metals play inextricably roles in the metabolic pathways of methylotrophs, which has drawn more and more concern in recent decades. Methylotrophs and methanotrophs are taxonomical divers among *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Verrucomicrobia* and *NC10*, in which *Alphaproteobacteria* and *Gammaproteobacteria* possess the majority of methylotrophs and methylotrophs [47, 111-116]. Available reports indicate methylotrophs and methanotrophs from different classes are showing distinct features for carbon assimilation as well as their responses to metals.

In methanotrophs, the initial oxidation from methane to methanol is catalyzed by two alternative methane monooxygenases. The well-known "copper-switch" conserved in most methanotrophs describes the regulation of two alternative methane monooxygenases overseen by copper availability, presenting as the enhanced gene expressions of particulate methane monooxygenase (pMMO, *pmo* encoded) under copper sufficient condition and prior gene expressions of soluble methane monooxygenase (sMMO, *mmo* encoded) when copper is deficient [117]. The sequent methanol oxidizing step in methylotrophs and methanotrophs is catalyzed methanol dehydrogenases (MDH), which were believed to be Ca-dependent enzymes (Ca-MDH or Mxa-MDH). However, recently the REE-dependent counterpart (REE-MDH or Xox-MDH) was discovered [20].

REEs form a chemically uniform group including scandium, yttrium, and 15 lanthanides [118]. Despite being named rare, some of them such as lanthanum and cerium, have relatively high abundance in the earth's crust and can be comparable to that of copper and zinc, while in most natural environments, REEs exist in the forms of barely soluble ores and the ion concentrations in water are extremely low (nM or less) [2]. Although REEs play irreplaceable roles in advanced industries, their bioavailability had long been overlooked due to their poor solubility and the difficulties in purification. However, their recently discovered involvement in the catalyzation of methanol oxidation has broken the stereotype. The specific type of methanol dehydrogenases (Xox-MDHs, *xox* encoded) was found to contain REEs in their active sites as co-factors whereas the well-characterized counterparts are containing calcium (Mxa-MDH, *mxa* encoded). Metagenome studies had shown that Xox-MDHs are even more prominent in nature than Mxa-MDHs [20, 23, 87]. The stronger Lewis acidity of REEs makes it easier for Xox-MDHs to remove electrons from the substrates compared to that for Mxa-MDHs. In *Verrucomicrobial* methanotroph *Methylacidiphilum fumariolicum* SolV, the high affinity and specific activity for formaldehyde of Xox-MDH make a direct conversion of methanol to formate. This direct conversion seems to confer Xox-MDHs with superior catalytic efficiency [23, 119], however, such direct conversion has not been reported in other strains.

Several methylotrophs and methanotrophs were reported to actively sense and respond to REEs availability, activities, and gene expressions of two alternative MDHs are notably regulated by REEs. In the presence of REEs, the expressions of *mxa* and *xox* genes were downregulated and upregulated respectively versus in the absence of REEs. This regulation is known as “REE-switch” and has commonly been observed when REEs are supplied to media in micromolar level concentration with soluble forms [24, 73, 120]. Reported research showed that in *Methylobacterium extorquens* AM1, one of the most well-studied *Alphaproteobacterial*

methylotrophs, not all REEs could trigger the "REE-switch", common explanation for this phenomenon was believed as heavier REEs with larger atomic radii are less bioavailable [96]. Such selectivity also occurred in other strains yet with nuances on regulation degrees of Mxa-MDHs and Xox-MDHs. It is evident that whether the "REE-switch" can be activated or not is not only influenced by the type of REEs but also the concentration of REEs and the sensitivities of methylotrophs to REEs concentrations varies among strains. Moreover, in many reports of *Alphaproteobacterial* methanotrophs, the presence of copper weakened the regulatory effect by REEs. For example, expressions of *mxa* could only be significantly suppressed by REE in the absence of copper in *Methylosinus trichosporium* OB3b [84]. Whereas in *Methylomicrobium buryatense* 5GB1C, a *Gammaproteobacterial* methanotroph, the presence of copper did not have much effect on the regulation of REEs [24]. However, the number of studies on the co-effect of different metals in *Gammaproteobacterial* methanotrophs is much less than that of *Alphaproteobacterial* ones, wider studies are needed to comprehensively understand the methanotrophs response to various metals. Especially the methanotrophs, which are responsible for the oxidation of biologically generated methane [92]. Methane has about 26 times more greenhouse effect than that of carbon dioxide, metals are closely involved in the oxidation of methane, more detailed studies should be carried out in methanotrophs. In this chapter, one of the model strains of *Gammaproteobacterial* methanotrophs *Methylococcus capsulatus* Bath was used to expand the understanding of "REE-switch", and the co-effect of REEs and copper was investigated in Bath. Various types and concentrations of REEs were supplemented for Bath culture and the nuances of the "REE-switch" between reported strains and Bath were demonstrated.

1.2 Materials and methods

1.2.1 Cultivation conditions

Wild type *M. capsulatus* Bath was cultured in vial bottles of 125 mL capacity. Each vial bottle was filled with 24 mL calcium- and copper-free inorganic basal medium and 10 mL L^{-1} each of a trace element solution ($CuCl_2$ and $CaCl_2$ were omitted) and vitamin solution as described previously [121], pH was adjusted to 7.0 using 6N KOH. Bottles were sealed with butyl rubber stoppers and aluminum seals before autoclaving. After autoclaving, culture substrates were supplemented with 20 mM filter sterilized methanol. Copper and calcium were supplemented from filter-sterilized stock solutions with the final concentrations of 10 μM and 20 μM respectively when desired. The culture was carried out at the temperature of 37℃ with 180 rpm shaking. OD_{600} was measured using DeNovix DS-11 Spectrophotometer to monitor the growth of *M. capsulatus* Bath under different conditions. REEs were supplemented using $CeCl_3$, $ScCl_3$, YCl_3, $LaCl_3$, $NdCl_3$, and $DyCl_3$ (Wako) when desired. Triplicate was used for all cultivations.

1.2.2 Total RNA extraction and purification

After 18 h of incubation to reach the mid-log phase (OD_{600} of 0.1~0.2), culture fluid was cooled on ice for 10 min and then transferred to 50 mL centrifuge tubes (Corning) and centrifuged at 4700 ×g for 15 mins at 4 ℃. After decanting the supernatant, centrifugation was performed again, and the aqueous portion was carefully removed with a pipette. The cell pellet was subsequently frozen using liquid nitrogen and stored at -80 ℃. RNA extraction was carried out by adding 1 mg/ml of lysozyme solution to the cell pellet and incubating for 5 min at RT, subsequent steps followed the previously described bead-beating method [121]. Briefly, a mixture of cell pellet and lysozyme solution was resuspended in 0.9 mL of Isogen II reagent and mixed with 200 μl of chloroform in a 2 mL lysing matrix tube (MP Biomedicals). The

slurry was homogenized using FastPrep-24™ Classic Instrument (MP Biomedicals). After centrifugation at 15,000 ×g for 5 min at 4℃, the aqueous layer was collected. RNA was then precipitated in isopropanol and washed with 70% ethanol. RNA purification, as well as DNase treatment, was performed respectively according to the manufacturer's instructions for RNeasy Mini kit (Qiagen) and RNase-free DNase set (Qiagen). The concentration of purified RNA was quantified by Qubit 2.0 fluorometer (Thermo Fisher Scientific), and samples were processed following the manufacturer's instructions of Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific). Isolated RNA was stored at -80℃ for further use.

1.2.3 Quantitative RT-PCR (qRT-PCR)

Primers used for quantitative reverse transcription-PCR targeting *mxaF* (Bath-mxaF-725f; 5'-ACA AGG ACA ACC CGC ATT AC-3', Bath-mxaF-922r; 5'-TGG TCA TGG TCC ATT TGT TG-3'), *xoxF* (Bath-xoxF-108f; 5'-TCC GAA GAA CTT TGC GAC TT-3', Bath-xoxF-308r; 5'-GCG TAG ACC TTG TGG GGA TA-3'), *mopB* (Bath-mopB-57f; 5'-ACA GGC CGA AGA GAC TTT CA-3', Bath-mopB-246r; 5'-GGT GGT GTT GCC TTC GTA AT-3') were designed using Primer3 software [122]. Quantitative gene expression analysis was performed by one-step real-time RT-PCR using LightCycler® 96 Instrument (Roche Life Science). RNA samples were processed using RNA-direct SYBR Green Realtime PCR Master Mix (Toyobo) as described previously [121]. The gene encoding a constantly expressed outer membrane protein (*mopB*) was used for normalizing the expression values. The purity of PCR products was checked by melting curve analysis. Preparation of the standard and generation of standard curves were performed following the previously described method [121].

1.2.4 RNA-sequencing

RNA sequencing was performed using DNBSEQ-G400 sequencer by Bioengineering Lab. The

raw reads were trimmed by Trimmomatic v0.39 and mapped to the genome of *M. capsulatus* Bath (GCA_000008325) using BWA v0.7.17. The gene expression level was calculated as transcripts per million (TPM) by using StringTie v2.2.1 and then normalized to the average ribosomal protein of each sample [123, 124].

1.3 Results and discussion

1.3.1 "REE switch" triggered by various REEs in *M. capsulatus* Bath

Most known methanotrophs and methylotrophs possess both Ca-MDH and REE-MDH, and the expressions of two MDHs are readily regulated by the presence of REE, representing up-regulated REE-MDH expressions and down-regulated Ca-MDHs. This regulation was described as the "REE-switch". Here to discover the specificity of the "REE switch" response, 20 μM of Sc or Y from the non-Lanthanide group, La, Ce or Nd from the light REE group, Dy from the heavy REE group were applied to cultivate *M. capsulatus* Bath in the presence of 20 μM of $CaCl_2$ and 10 μM of $CuCl_2$. Under each condition, RNA was extracted and subjected to qRT-PCR to analyze the regulated expressions of two MDHs. The results (Figure 1.3.1) showed that light REEs such as La and Ce can readily trigger the "REE-switch" in *M. capsulatus* Bath, while Nd, could only bring attenuated regulations to *mxaF* and *xoxF* expressions. Non-Lanthanide REEs Sc and Y, heavy REE Dy didn't show significant regulatory effects on the expressions of two MDHs in *M. capsulatus* Bath. Nuances are showing among strains about the regulations brought by various REEs. In *Methylosinus trichosporium* OB3b, a model strain of alphaproteobacterial methanotroph, light REE of Nd can comparatively regulate the expressions of two MDHs as La does, but Sm and Pr are not able to trigger the switch [98]. In *Methylobacterium extorquens* AM1, which is an alphaproteobacterial methylotroph, Pr and Nd can significantly regulate two MDHs expression, while Sm was not able to bring the regulations

[96]. Part of the specificity comes from the chemical nuances among the REEs. Although they show chemical similarities to each other, the ionic radius, coordination number, and Lewis acidity make them cannot be easily substituted [99, 100]. Besides, few reports stated that methylotrophic bacteria are producing diverse REE chelator and lanthanide-binding proteins and these siderophores and proteins may be involved in the acquisition and utilization of REEs [101-104]. The diversity of REE binding products may explain the different utilization of REEs among species since each of them has various binding sites and binding affinities.

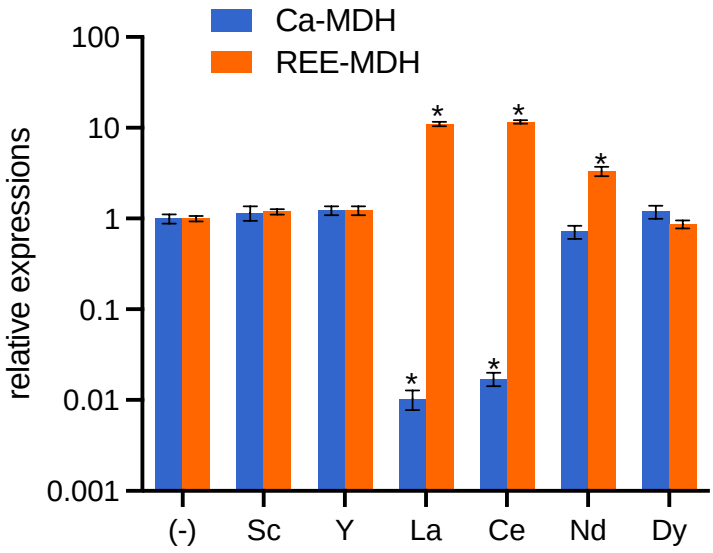


Figure 1.3.1 Expressions of two MDHs regulated by different REEs. Error bars are showing the standard deviations, triplicate was used in this analysis. Asterisks represent a significant difference (> 2-fold changes, $p < 0.05$) from the $CaCl_2$-supplemented control cultures.

1.3.2 Growth of *M. capsulatus* Bath in different concentrations of $CeCl_3$

$CeCl_3$ was confirmed to induce the "REE-switch" in 1.3.1. As one of the most abundant REEs in the earth's crust, Ce was commonly used in other REE- and MDHs- related studies. For further demonstration of the "REE-switch" in *M. capsulatus* Bath, and comparison of the nuances of "REE-switch" with other reported strains, Ce was used as REE supplementation for further investigation. To monitor the growth of *M. capsulatus* Bath in different concentrations of soluble REE source, $CeCl_3$ was applied with the concentration range from 0~1μM in the

presence of 20μM of Ca and 10 μM Cu. As shown in Figure 1.3.2, all concentrations tested here allowed ordinary growth of *M. capsulatus* Bath, only modest alterations were observed in growth rate and cell yield. Some methanotrophs reported to possess only the REE-MDH, the *Methylacidiphilum fumariolicum* SolV, and *Methylosinus* sp. strain Ce-a6 showed an REE concentration-dependent growth. Higher concentrations can achieve a higher growth rate and cell yield [23, 27]. While the methanotrophs and methylotrophs possess both REE-MDH and Ca-MDH like *M. capsulatus* Bath, have a more flexible metabolism, and their growth is less dependent on the concentrations of REE.

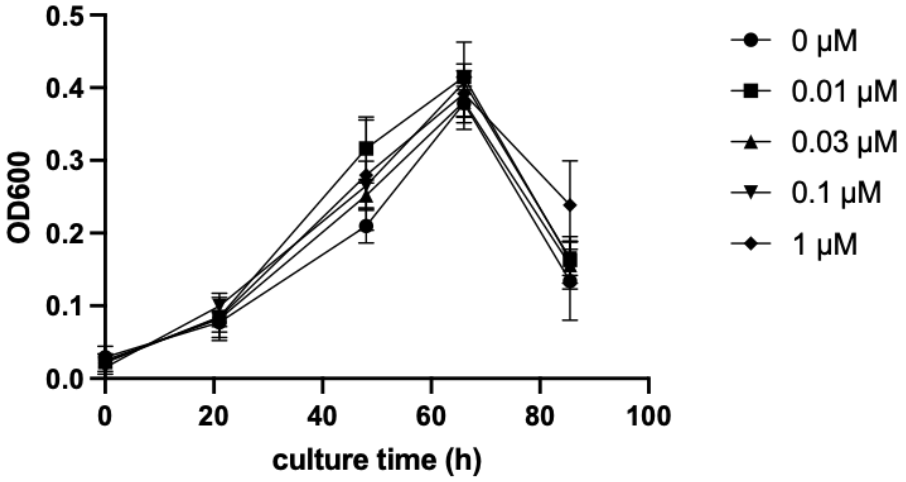


Figure 1.3.2 Growth f *M. capsulatus* Bath in different concentrations of $CeCl_3$. Error bars are showing the standard deviations, triplicate was used in this culture experiment.

1.3.3 "REE-switch" induced by different concentrations of $CeCl_3$

The regulation, the so-called "REE switch" was observed in some strains listed in Table 1.3.3. The reported effective concentrations to trigger the "REE switch" differ on species. Here in this study, quantitative RT-PCR was performed to reveal the regulations of gene expressions under different $CeCl_3$ concentrations ranging from 0~10 μM in the presence of 20 μM of Ca and 10 μM Cu. The results suggested that cerium supplements in tested concentrations all induced the

"REE-switch" in *M. capsulatus* Bath. As shown in Figure 1.3.3, *M. capsulatus* Bath could sense and respond to $CeCl_3$ from the concentration of 0.01 μM even when the concentration of calcium was approximately 200- to 650-fold higher than cerium. When the concentrations of $CeCl_3$ were no more than 0.1 μM, higher $CeCl_3$ concentrations brought more pronounced regulatory effects on *xoxF* and *mxaF* expressions. For *M. capsulatus* Bath, 0.03 μM of $CeCl_3$ is sufficient to completely promote the expression of *xoxF*, and 0.1 μM of $CeCl_3$ was required to completely suppress the expression of *mxaF*. This concentration-dependent regulation suggested that *M. capsulatus* Bath prefers to use REE-MDH if REE is available, it is also found in other methanotrophs such as *Methylomicrobium buryatense* 5GB1C. In addition, XoxF mutants grow more poorly than MxaFI mutants under unfavorable culturing conditions, which suggested that XoxF acts as the predominant methanol dehydrogenase in a methanotroph [24].

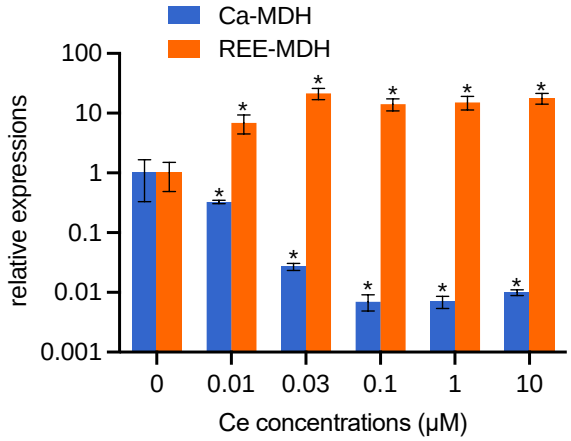


Figure 1.3.3 Regulations on the expression of two MDHs under different $CeCl_3$ concentrations. Error bars are showing the standard deviations, triplicate was used in this analysis. Asterisks represent a significant difference (> 2-fold changes, $p < 0.05$) from the no lanthanide control cultures.

Phylum or class	Strains	Type	"REE switch"	Cu override on "REE switch"	Tested REE concentrations	References
Gammaproteobacteria	*M. capsulatus* Bath	methanotroph	Yes	No	10 nM~	This study
	M. buryatense 5GB1C	methanotroph	Yes	No	600 nM~	[24]
	M. alcaliphilum 20Z	methanotroph	Yes	nt	nt	[125]
	M. tundripaludum 31/32	methanotroph	Yes	nt	nt	[126]
	Methylomonas sp. LW13	methanotroph	Yes	nt	300 nM~	[127]
	P. putida KT2440	Non-C1 bacterium	Yes	nt	1 nM~	[26]
Alphaproteobacteria	*M. trichosporium* OB3b	methanotroph	Yes	Yes	1 mM~	[98]
	M. extorquens AM1	methylotroph	Yes	nt	25 nM~	[96]
	Methylobacterium sp. MAFF211642	methylotroph	Yes	nt	30 μM~	[20]
	M. extorquens PA1	methylotroph	Yes	nt	~500 nM	[128]
	M. aquaticum 22A	methylotroph	Yes	nt	5 μM~	[97]
	Bradyrhizobium sp. MAFF211645	Rhizobiales	Yes	nt	30 μM	[21]

Table 1.3.3 Reported "REE-switch" in various strains, "nt" indicated not tested.

1.3.4 The "copper switch" and "REE switch" are independent in *M. capsulatus* Bath

M. capsulatus Bath was reported to have regulated expressions of two methane monooxygenase sMMO and pMMO by the presence of Cu [67, 129-131] as reported in other strains [84, 132, 133]. Cu is crucial for the upstream methane oxidation, to evaluate if Cu has an effect on the "REE switch" that occurs in the subsequent methanol oxidation, RNA-seq was performed under different metal supplementation. Transcriptome analysis results revealed that the expressions of *xoxF* and *mxaF* were regulated by the presence of 0.1 μM of $CeCl_3$ regardless of the presence of 10 μM of Cu, as shown in Figures 1.3.4A, B, and C. The two types of MMOs were also regulated by 10 μM of Cu with no effect by $CeCl_3$ addition. It is evidenced in *M. capsulatus*

Bath, both the “copper switch” and “REE switch” operate independently. This independent regulation was also found in *Methylomicrobium buryatense* 5GB1C, a *Gammaproteobacterial* methanotroph [24]. While in *Methylosinus trichosporium* OB3b, one of the *Alphaproteobacterial* methanotrophs, Cu overrides the regulations of REE, resulting same expression levels of Ca-MDH and REE-MDH in the presence of both REE and Cu. At this time, limited relative research about the overriding was performed, however, from the available results, it appears that it defers between gammaproteobacterial (Type I) and alphaproteobacterial (Type II) methanotrophs. Knowing these two classes are assimilating the formaldehyde in different ways, the RuMP pathway and serine pathway [134], the difference in metal switches may imply a more complicated role of Cu and REEs in terms of overall metabolism.

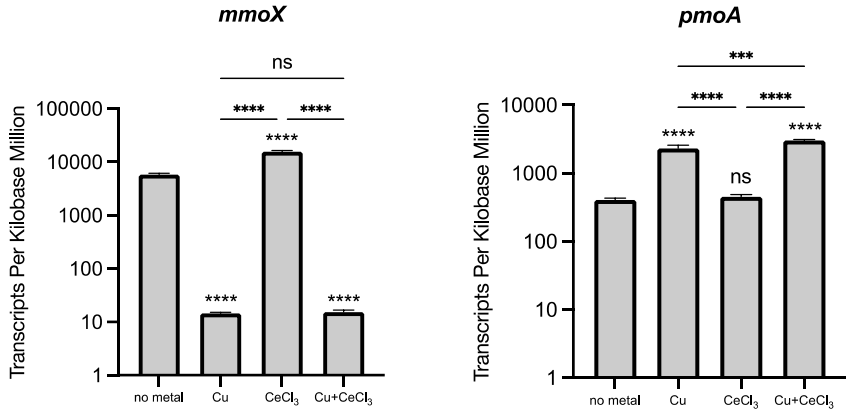


Figure 1.3.4A Expression of *mmoX* and *pmoA* in each metal supplementation. Error bars indicate standard deviations from triplicate. Indicated P values are from a one-way analysis of variance (ANOVA).

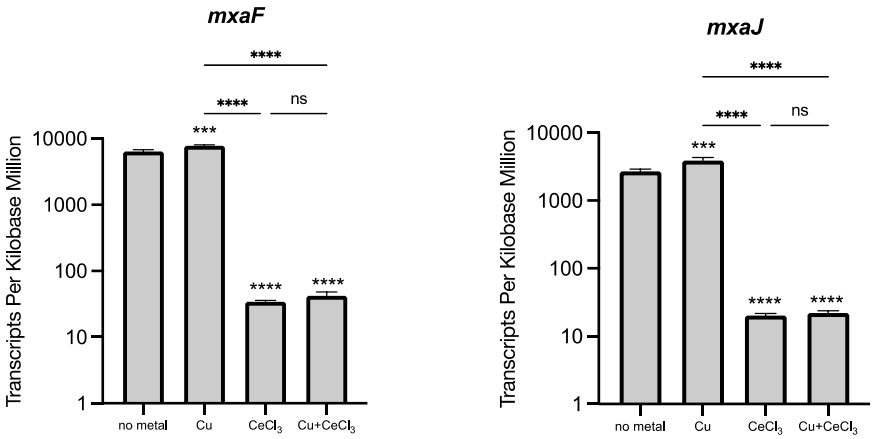


Figure 1.3.4B Expression of *mxaF* and *mxaJ* in each metal supplementation. Error bars indicate standard deviations from triplicate. Indicated P values are from a one-way analysis of variance (ANOVA).

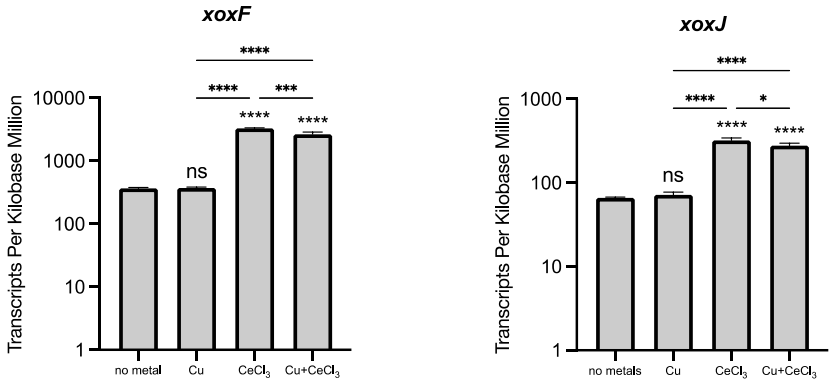


Figure 1.3.4C Expression of *xoxF* and *xoxJ* in each metal supplementation. Error bars indicate standard deviations from triplicate. Indicated P values are from a one-way analysis of variance (ANOVA).

1.3.5 Copper acquisition in *M. capsulatus* Bath

In *M. capsulatus* Bath, MopE is produced to bind Cu under the growth of copper starvation, however, the transport system of it remains unknown [78, 80, 135]. MopE production is readily repressed by the addition of Cu. Through the comparative transcriptome analysis between Cu sufficient and deficient conditions, cluster MCA0438-0447 comprising TonB-dependent receptor and other transporters as well as cluster MCA2169-2177 comprising ABC transporters showing down-regulated expressions by the addition of Cu. Similar regulation was reported earlier [67], both of these two clusters were suggested to be possibly involved in the transport

of MopE and the uptake of copper in *M. capsulatus* Bath. However, in this study, it was found that in the absence of Cu, the expressions of *mmo* were upregulated by the presence of $CeCl_3$ (see Figure 1.3.4A). At the same time, expressions of MCA0438-0447 were upregulated slightly, which implies this cluster may contribute to the metabolism along with *mmo* cluster. More detailed information about cluster MCA2169-2177 about the protein prediction is listed in Table 1.3.5. Protein structure and function predicting tool Phyre2 predicted the MCA2170 encoding a copper-binding protein sharing 33% amino acid sequence similarity of the product of *copC* from *Methylosinus trichosporium* [66]. Based on these analyses, the cluster of MCA2169-2177 was considered a promising cluster for the uptake of copper and transport of MopE in *M. capsulatus* Bath.

Gene ID	phyre2 prediction	similarity	coverage	confidence	Gene Name retrieved from cDNA information
MCA2169	hydrolase	22%	66%	99.9	BNR repeat domain protein
MCA2170	copper binding protein CopC	33%	68%	100	putative copper resistance protein
MCA2171	transferase	23%	31%	18.5	DUF4440 domain-containing protein
MCA2172	abc transporter	36%	99%	100	ABC transporter, ATP-binding family protein
MCA2173	abc transporter permease subunit	18%	98%	100	ABC transporter permease protein domain-containing protein
MCA2174	metal receptor	25%	90%	100	adhesion lipoprotein

Table 1.3.5 Cluster MCA2169-2174 with gene function predicted in Phyre2

1.3.6 Other regulated genes under different metal supplementations

When comparing the transcriptome of *M. capsulatus* Bath grown with 10 μM cerium + 10 μM copper vs. 0 μM cerium + 10 μM copper, a gene MCA2279 encoding a DNA-binding response regulator and a gene MCA2280 encoding a zinc resistance-associated protein, were highly expressed under the presence of cerium. Further function prediction by Phyre 2 tool suggested that the gene product of MCA2279 was similar to a putative two-component system response

regulator and the protein encoded by MCA2280 was similar to a metal-binding protein. These two genes may be involved in the sensing of REEs and regulation of two MDHs. Besides, the gene MCA2618 encodes a c-type cytochrome and showed similarity to an electron transport cytochrome c XoxG, however the expression of MCA2618 was down-regulated when REE was present. The previous report in *Methylomicrobium alcaliphilum* $20Z^R$, one of the *Gammaproteobacterial* methanotrophs, revealed the cytochrome is correlated with methane limitation rather than with La-growth [125]. Besides, genes for formate dehydrogenase (FDH) (MCA2576-2577) and its downstream ABC transporter (MCA2578-2580) were down-regulated in the Ce-supplemented condition. Down-regulation of FDH by lanthanide addition has been reported in a methylotroph *Methylobacterium extorquens* AM1, likely to remodel its metabolic flux to reduce formate oxidation and balance NAD(P)H production [136].

The genes up- or down-regulated (>2-fold expression change and $p<0.05$) in the Ce-supplemented culture (supplemented with 0.1 μM $CeCl_3$) compared to the control culture (supplemented with 0.1 μM $CaCl_2$) with the presence of 10 μM of Cu were listed in Appendix 1. And the genes up- or down-regulated (>2-fold expression change and $p<0.05$) in the Cu-supplemented culture (supplemented with 10 μM $CuCl_2$) compared to the control culture (without Cu-supplemented) with the absence of 0.1 μM of Ce were listed in Appendix 2.

1.4 Conclusion

The response of *Methylococcus capsulatus* Bath to various types and concentrations of REE was observed in this study. Bath was found to possess the “REE-switch” as other reported methylotrophs and methanotrophs. Like other strains, Bath preferred the light REEs and regulated the expressions of two MDHs under light REEs supplemented conditions. Compared to other reported strains, Bath showed the ability to sense and responded to regulate the expressions of two MDHs to extremely low concentrations of REEs to 10 nM, and 100 nM of

REEs was considered as a sufficient concentration for Bath. Consistent with the report of another gammaproteobacterial methanotroph *Methylomicrobium buryatense* 5GB1C, Bath also has an independent "REE-switch" which operates the same under the presence and absence of copper. Further, a gene cluster that may function for copper transport was found to be downregulated under Cu-sufficient conditions, suggesting the more complex mechanism employed by *M. capsulatus* Bath.

CHAPTER 2: Putative molecular mechanism of insoluble REE utilization by Bath

2.1 Introduction

REE utilization was characterized in methylotrophs and methanotrophs, REE were supplemented with chlorides which were soluble and convenient for the utilization by microbes. However, most of the REEs in the environment are in the form of ores, and their uneven distribution and insolubility make them difficult for microorganisms to utilize. Less was studied about the utilization of insoluble REEs by methylotrophs and methanotrophs. The isolation of a REEs-dependent methanotroph named *Methylosinus* sp. strain Ce-a6 from a circumneutral environment, which acquired at least 30 nM for optimal growth demonstrates that microorganisms use certain approaches for the acquisition of the REE from the insoluble REE sources in the environments [27]. Just as microorganisms utilize another insoluble but important metal, iron, genes associated with siderophores and TonB-dependent receptors were recently identified in two methylotrophs and confirmed to be involved in REE acquisition [101, 106]. Studies about such insoluble REEs utilization and uptake haven't been reported in methanotrophs. Possessing the potential of a promising way of degrading atmospheric methane, the utilization of REEs in methanotrophs drew attention. In this chapter, insoluble REE utilization in a methanotroph *Methylococcus capsulatus* Bath was investigated, further, the acquisition of REEs from insoluble REEs source was confirmed. From the spent medium of Bath cultured in REEs deficient conditions, REE-chelator was detected, and the genes involved in the biosynthesis of REE-chelator were demonstrated with transcriptome analysis.

2.2 Materials and methods

2.2.1 Cultivation conditions

For cultivation, an inorganic basal medium was used. It included 5 mM $NaNO_3$, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 0.1 mM Na_2SO_4, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10 mL L^{-1} each of a trace element solution ($CaCl_2$ was omitted) and vitamin solution as described previously [137]. pH was adjusted to 7.0 using 6N KOH. Filter sterilized methanol was added as substrate in the concentration of 20 mM after autoclaving. For the growth test under insoluble supplementation, 1 g/L CeO_2 powder was used. Soluble REE supplementation was added using 1 mM $CeCl_3$ stock solution which was filter sterilized and kept at -30 ℃.10 μM of $CuCl_2$ and 20 μM of $CaCl_2$ were added except otherwise specified. *M. capsulatus* Bath was cultured with a volume of 24 ml in vial bottles (125-mL capacity) at 37 ℃ with shaking (180 rpm). Bottles are sealed with butyl rubber stoppers and aluminum seals. Growth was monitored by measuring the optical density at 600 nm (OD_{600}), besides, adding CeO_2 powder may have some effect on the value of OD_{600} thus the concentration of methanol in the culture fluid was measured at the same time by using HPLC. pH was measured during growth using LAQUA Twin (AS one). *M. capsulatus* Bath was regularly kept on agar plates with 10 μM of Cu and 20 μM of Ca supplementation, and was pre-cultured in Cu, Ca omitted inorganic basal medium overnight, culture fluid was centrifuged with 4700 ×g for 10 mins at 15 ℃ and washed with inorganic basal medium for twice and inoculated to each treatment medium with starting OD_{600} of 0.03. Triplicate was used for all cultivations.

2.2.2 Inductively Coupled Plasm (ICP-OES)

The Ce ion concentrations in solution or culture fluid were measured by ICP-OES. 1 g/L CeO_2 powder was suspended in ether uninoculated inorganic basal medium, culture medium of Bath, or 3-days methane applied culture spent medium of *M. capsulatus* Bath at 37 ℃ for 3 days with shaking (180 rpm). The result solutions were filtered with a 0.22 μm filter and stored at 4 ℃. The analysis was performed at the TIA Central Office of the National Institute of Advanced

Industrial Science and Technology. Standard solutions were prepared with a series of concentrations of Ce chlorides dissolved in MilliQ water. This analysis was carried out in triplicate.

2.2.3 Total RNA isolation and purification

After 18 h of incubation to reach the mid-log phase, culture fluid was cooled on ice for 10 min and then transferred to 50 mL centrifuge tubes (Corning) and centrifuged at 4700 ×g for 15 mins at 4 ℃. After decanting the supernatant, centrifugation was performed again, and the aqueous portion was carefully removed with a pipette. The insoluble Ce oxide powder in the culture fluid caused an inefficient RNA extraction. Thus, for the Ce oxide powder treatment condition, before the second time centrifugation, the cell pellet was transferred to a new tube, and the precipitate was discarded. The cell pellet was subsequently frozen using liquid nitrogen and stored at -80 ℃. RNA extraction was carried out by adding 1 mg/ml of lysozyme solution to the cell pellet and incubating for 5 min at RT, subsequent steps followed the previously described bead-beating method [121]. Briefly, a mixture of cell pellets and lysozyme solution was resuspended in 0.9 mL of Isogen II reagent and mixed with 200 µl of chloroform in a 2 mL lysing matrix tube (MP Biomedicals). The slurry was homogenized using FastPrep-24™ Classic Instrument (MP Biomedicals). After centrifugation at 15,000 ×g for 5 min at 4℃, the aqueous layer was collected. RNA was then precipitated in isopropanol and washed with 70% ethanol. RNA purification, as well as DNase treatment, was performed respectively according to the manufacturer's instructions for RNeasy Mini kit (Qiagen) and RNase-free DNase set (Qiagen). The concentration of purified RNA was quantified by Qubit 2.0 fluorometer (Thermo Fisher Scientific), and samples were processed following the manufacturer's instructions of Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific). Isolated RNA was stored at -80℃ for further use.

2.2.4 Quantitative RT-PCR

Quantitative RT-PCR was performed as described in Chapter 1.

2.2.5 RNA-sequencing and statistical analysis

RNA sequencing was performed using DNBSEQ-G400 sequencer by Bioengineering Lab. The raw reads were trimmed by Trimmomatic v0.39 and mapped to the genome of *M. capsulatus* Bath (GCA_000008325) using BWA v0.7.17. The gene expression level was calculated as transcripts per million (TPM) by using StringTie v2.2.1 and then normalized to the average ribosomal protein of each sample [123, 124].

2.2.6 Purification of putative REE chelators

M. capsulatus Bath was cultured with Ce chlorides supplementation at different concentrations from 0~10 μM, after 3 days of incubation, 20 mL of supernatant was collected by 4700 ×g centrifugation for 15 min at 4 ℃ and filter sterilized with 0.22 μm filter. Subsequently, the supernatant was adjusted to 20% (v/v) methanol with 100% methanol. The putative REE chelator was purified by reverse-phase chromatography. Sep-Pak C18 Plus Long Cartridge (Waters) with a hold-up volume of 1.6 mL was used. The cartridge was conditioned with 10 mL of 100% methanol and equilibrated with 10 mL of 20% methanol. Samples were loaded to the cartridge and 1.6 mL of 40% methanol was used for elution. Finally, the cartridge was washed using 1.6 mL of 100% methanol. The elution and wash fractions were collected and dried in the vacuum concentrator (Eppendorf) and then dissolved in 500 μL Milli-Q water. The result solution was stored at 4 ℃ for further assay.

2.2.7 Liquid Chrome Azurol S assay

The REE chelating ability of purified putative chelator was detected with the liquid Chrome Azurol S (CAS) assay described previously [101, 138, 139] with some minor modifications.

Briefly, for the working solution, 25 µL of the mixture of 0.15 mM (final concentration of 18.75 µM) CAS solution and 0.4 mM (final concentration of 50 µM hexadecyltrimethylammonium bromide solution was added into 120 µL of 2.5 mM (final concentration of 1.5 mM) 2-(N-morpholino)ethanesulfonic acid (MES)/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution. Subsequently, 15 µL of 0.25 mM (final concentration of 18.75 µM) Ce chlorides solution was added. 40 µL purified putative REE chelator sample was applied. All assays were carried out in triplicate and elute fraction from an uninoculated medium with the same purification process and were taken as a negative control. The mixture was further incubated at RT with an orbital shake (180 cpm) for 5 mins. An absorbance scan was performed with a range of 350 nm to 750 nm in 1 nm intervals using Tristar 5 Multimode Reader (Berthold Technologies).

2.2.8 Construction of a Deletion Mutant

The deletion mutants of MCA1883 and MCA0779 (*mxaF*) were constructed by the double crossover method by using mutated *pheS* as the counter-selectable marker as previously described method with some modifications [140]. Primers, bacterial strains, and plasmid used for the construction of the mutant strain are listed below in Table 2.2.8A and B. Briefly, the linear pJQY3 was amplified by PCR from SacI to XbaI site with the primer set pJQY3-F and pJQY3-R and 1-kb regions upstream and downstream of those three genes were amplified by PCR using primer sets MCA1883_upstF/MCA1883_upstR and MCA1883_dwstF/MCA1883_dwstR and MCA0779_upstF/MCA0779_upstR and MCA0779_dwstF/MCA0779_dwstR, respectively with Phusion High Fidelity PCR Master Mix with HF Buffer (BioLabs). Fragments were then cloned into the SacI and XbaI sites of pJQY3 by using In-Fusion HD Cloning Kit (TaKaRa), generated pJQYM1 and pJQYN1 were transformed to *E. coli* DH5α (TaKaRa) by electroporation using Gene Pulser Xcell (BioRad).

After checking the insertion by colony PCR, the plasmids pJQYM1 and pJQYN1 were isolated following the manufacturer's instruction of QIAprep Spin Miniprep Kit (QIAGEN) and further transformed to the *E. coli* WM6026 [141]. *M. capsulatus* Bath wild type was mated with *E. coli* WM6026 harboring pJQYM1 and pJQYN1 on an inorganic basal medium agar plate supplied with 600 μM DAP, 20 μM $CaCl_2$, 10 μM $CuCl_2$ and 0.1 μM $CeCl_3$, incubated at 37 ℃ in methane gas vapored AnaeroPack (MITSUBISHI GAS CHEMICAL). After 24 h, the cells were collected in 500 μL of inorganic basal medium and spread onto an agar plate containing 10 μg/mL gentamicin, 20 μM $CaCl_2$, 10 μM $CuCl_2$, and 0.1 μM $CeCl_3$, and then incubated at 37 ℃ in the methane gas vapored AnaeroPack until colonies were generated. The insertion was checked by colony PCR and DNA sequencing. The resultant candidate single crossover mutant was grown in an inorganic basal medium without gentamycin for 3~4 days, after observation of significant growth in the liquid medium, its dilutions were applied on an inorganic basal medium agar plate containing 10 mM p-chloro-phenylalanine, 20 μM $CaCl_2$, 10 μM $CuCl_2$, and 0.1 μM $CeCl_3$ and incubated at 37 ℃ in the methane gas vapored AnaeroPack until colonies generated. Colony PCR was then performed to confirm the generation of double-crossover.

Strain or plasmid	**Description**	**Reference or source**
***M. capsulatus* Bath**		
Wild type	Wild-type strain	[92]
E. coli		
DH5α	strain used for in-fusion cloning	Toyobo
WM6026	Donor strain for conjugation	[141]
Plasmid		
pJQY3	Suicide plasmid produced by substitution of *sacB* in pJQ200sk with *pheS**	[140]

pJQYM1	DNA fragment containing upstream and downstream regions of *mxaF* ligated into SacI and XbaI site of pJQY3	this study
pJQYN1	DNA fragment containing upstream and downstream regions of MCA1883 ligated into SacI and XbaI site of pJQY3	this study

Table 2.2.8A Bacterial strains and plasmid used in this study.

Primer name	**Sequence (5' to 3')**
pJQY3-F	TCTAGAAcTAGTGGATCCCC
pJQY3-R	GAGCTCCAGCTTTTGTTCCC
mxaF-upsF	GAACAAAAGCTGGAGCTCTCCTCTTCCTGAATCGCCAG
mxaF-upsR	CCACCCATCTTGCATGTGTCTCCTCCAAGA
mxaF-dwsF	GACACATGCAAGATGGGTGGATCGGTGTTC
mxaF-dwsR	ATCCACTAGTTCTAGAGCCTTTCCTTCGATCATGGG
MCA1883-upsF	GAACAAAAGCTGGAGCTCCCATGGTCTGGACCCGTT
MCA1883-upsR	CCAGCGTGCGACAGGGTCGCCGAGAGCATG
MCA1883-dwsF	GCGACCCTGTCGCACGCTGGCCGACGAACT
MCA1883-dwsR	ATCCACTAGTTCTAGAGCGAAAGGGCCGAGTTCC

Table 2.2.8B Primers used in this study.

2.3 Results and discussion

2.3.1 Growth of *M. capsulatus* Bath in different forms of Cerium

In the previous chapter 1, it was found that *M. capsulatus* Bath can sense extremely low concentrations of REE (10 nM~) and induce the “REE-switch”. Low concentrations of REE supplementation did not affect the growth rate and growth yield of *M. capsulatus* Bath. Here

the growth in insoluble CeO_2 powder was monitored and compared to the growth under various concentrations of $CeCl_3$. The results showed the forms (soluble $CeCl_3$ or insoluble CeO_2) of REE supplementation only brought modest alterations in the growth rate and growth yield of *M. capsulatus* Bath as shown in Figures 2.3.1A and B. This result demonstrated that Bath could utilize the insoluble REE source in the natural environment.

So far, only a few studies have used insoluble REE sources to elaborate on the utilization of natural forms of REE and related mechanisms of methanotrophic and methylotrophic bacteria. In *Alphaproteobacterial* methanotroph, *Methylosinus* sp. strain Ce-a6, which harbors the REE-MDH but shows a large-scale deletion around the *mxa* gene cluster, 1 g/L of insoluble CeO_2 particles brought competitive growth rate and growth yield compared to soluble $CeCl_3$ [27]. While in Δ*mxaF* of the *Alphaproteobacterial* methylotrophic bacterium, *Methylorubrum extorquens* AM1, poorly soluble Nd_2O_3 led to attenuated growth rate and growth yield than in soluble $NdCl_3$ [101]. It can be inferred that wild type methanotrophs and methylotrophs can utilize the insoluble REE source and elute the REE with yet unclear mechanisms.

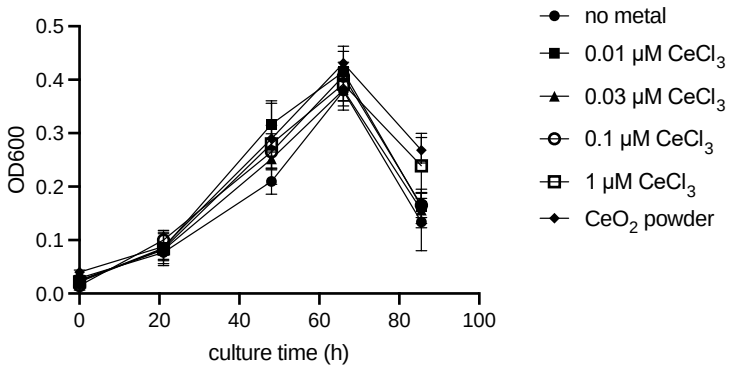


Figure 2.3.1A Growth f *M. capsulatus* Bath in poorly soluble CeO_2 and different concentrations of $CeCl_3$. Error bars are showing the standard deviations, triplicate was used in this culture experiment.

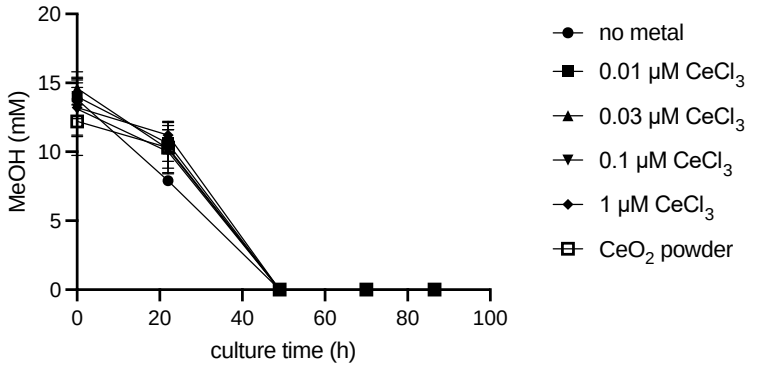


Figure 2.3.1B Methanol consumption during the Bath growth. Error bars are showing the standard deviations, triplicate was used in this culture experiment.

2.3.2 Medium pH kept stable during the growth of *M. capsulatus* Bath

Metal oxides in low solubility can achieve higher solubility in low pH conditions, it was considered as a possible strategy of REE utilization of methanotrophs and methylotrophs in the natural environment [53, 142-144]. Here the pH values were measured during the whole growth in both CeO_2 and $CeCl_3$ supplied conditions. The pH value of culture fluid kept stable during the growth of *M. capsulatus* Bath regardless of the forms of REE source and concentrations as shown in Figure 2.3.2. Results here confirmed that the *M. capsulatus* Bath utilization of insoluble CeO_2 is supported by mechanisms other than depending on the change of pH.

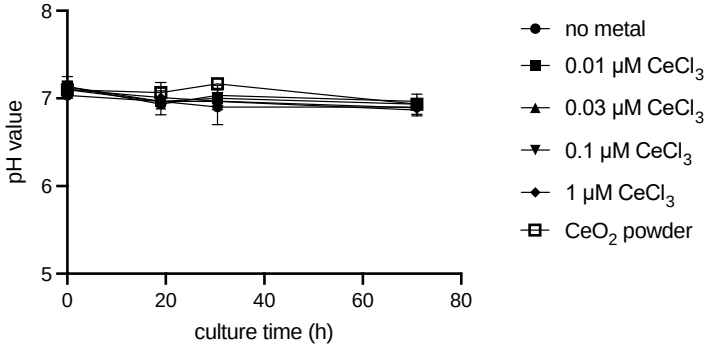


Figure 2.3.2 Changes of pH during Bath growth in different forms of Ce. Error bars are showing the standard deviations, triplicate was used in this culture experiment.

2.3.3 Insoluble CeO_2 powder supplementation induced the "REE-switch"

To demonstrate that *M. capsulatus* bath is indeed utilizing insoluble REEs and the insoluble REE triggers the "REE-switch" in Bath as the soluble REE, qRT-PCR was performed to determine the regulation of the expression of two MDHs genes with the addition of 1 g/L CeO_2 powder. As shown in Figure 2.3.3, the expression of Ca-MDH was significantly down-regulated and that of REE-MDH was significantly up-regulated by the addition of CeO_2 powder. From this result, the direct addition of CeO_2 powder brought more significant regulation, which indicated that Bath could elute Ce ions from CeO_2. In comparison with other treatment groups with various $CeCl_3$ concentrations, it can be found that 0.1 g/L of CeO_2 powder can regulate both MDHs to a degree comparable to 0.03~0.1 μM of $CeCl_3$. Thus, we confirmed that Bath can utilize poorly soluble REE in the environment and trigger their REE switch of regulating the expression of both two types of MDHs.

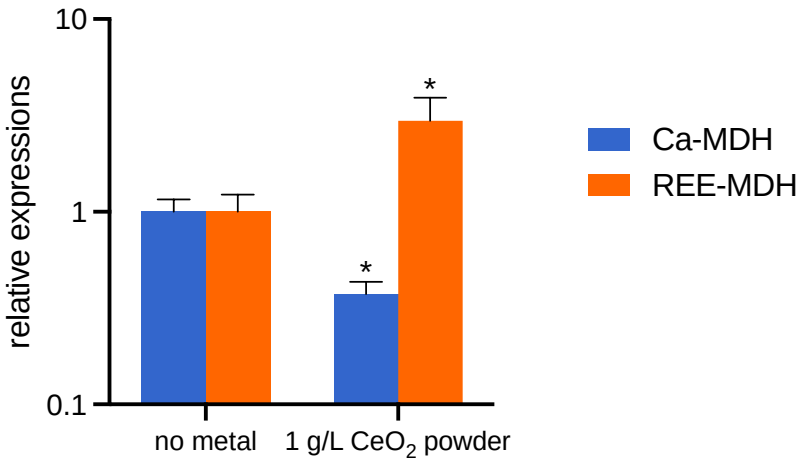


Figure 2.3.3 Expressions of two MDHs regulated by 1 g/L insoluble CeO_2 powder. Error bars are showing the standard deviations, triplicate was used in this analysis. Asterisks represent a significant difference (> 2-fold changes, $p < 0.05$) from the no metal control cultures.

2.3.4 Spent medium of *M. capsulatus* Bath promoted the solubility of CeO_2 powder

To further demonstrate the utilization of insoluble REE (CeO_2 used in this study), the solubility of CeO_2 in *M. capsulatus* Bath culture fluid, spent medium or uninoculated medium was measured by ICP-OES. As shown in Figure 2.3.4, in an inorganic basal medium, eluted Ce ion from 1 g/L CeO_2 powder was lower than the detection limit, which is 10 nM in the present experiment condition. With the cultivation of *M. capsulatus* Bath, the solubility of CeO_2 powder was improved up to around 14 nM. In the spent medium of *M. capsulatus* Bath cultured in REE-deficient condition, more than 20 nM of Ce ion was detected. The promotion of CeO_2 solubility in *M. capsulatus* Bath spent medium indicated the production of extracellular REE chelators [145, 146]. In *Methylosinus* sp. strain Ce-a6, at least 30 nM $CeCl_3$ is required to achieve optimal growth due to the lack of *mxa* cluster. Although CeO_2 has less than 10 nM solubility in the medium, it is sufficient for the Ce-a6 to achieve comparable growth rates and cell yields to those under $CeCl_3$ supplementation. It can be speculated that even though CeO_2 has only a small solubility in the uninoculated medium, Ce ions are continuously eluted from CeO_2 to supply the growth as the eluted ions are consumed or bound by the putative-produced REE chelator [27, 147].

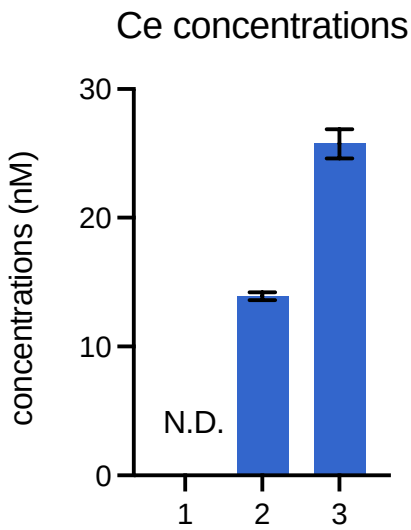


Figure 2.3.4 Changes in solubility of CeO_2 powder in different conditions. Condition 1 indicated 1 g/L CeO_2 powder in an uninoculated inorganic basal medium. Condition 2 indicated *M. capsulatus* Bath culture under the supplementation of 1 g/L of CeO_2. Conditions 3 indicated 1 g/L CeO_2 powder in *M. capsulatus* Bath spent medium cultured under no REE supplementations. Error bars are showing the standard deviations, the duplicate was used in this culture experiment.

2.3.5 Isolation and Ce chelating ability detection of putative REE chelator produced by *M. capsulatus* Bath

Previous results of CeO_2 solubility measurement suggested to some extent the possibility of extracellular Ce chelator production by *M. capsulatus* Bath. The putative REE chelator produced by *M. capsulatus* Bath was isolated from the supernatant of culture fluid under various $CeCl_3$ concentrations (0~10 μM) with a method of reverse chromatography which is commonly used for hydrophobic siderophore isolation. After the vacuum drying at 45 ℃, the isolated substances from the culture supernatant of < 0.1 μM $CeCl_3$ supplementation conditions resulted in a light-yellow color, while after redissolved in Milli-Q water, the color was more invisible. Further, the Ce chelating ability of those putative chelators was detected by Chrome Azurol S (CAS) assay. Results are shown in Figure 2.3.5. The same REE chelator isolation process was performed using an uninoculated culture medium, and the resulting solution was taken as the control. In the CAS assay, the control showed an absorbance peak at 630 nm. For the treatments, Ce chelating ability was detected in 0~0.03 μM $CeCl_3$ supplied conditions, showing a loss of absorbance peak at 630 nm. No chelating ability was detected from 0.1~10 μM $CeCl_3$ treatment culture supernatant. Combined with the results of RT-PCR in Chapter 1, which revealed that *M. capsultatus* Bath requires 0.1 μM of $CeCl_3$ to fully regulate the expressions of REE-MDH and Ca-MDH. Therefore, when the Ce was added in less than the concentration of 0.1 μM, the REE chelator was produced to sequester more Ce ions.

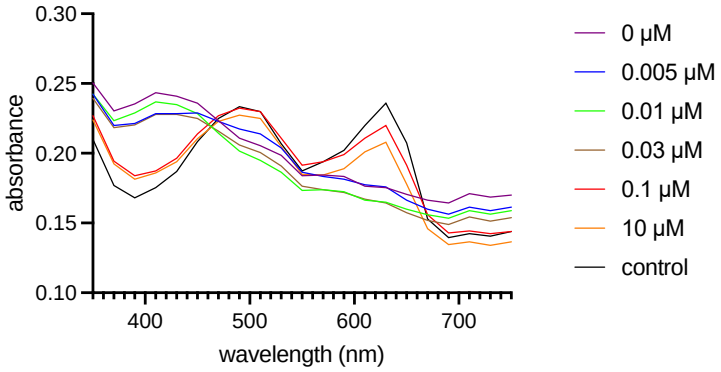


Figure 2.3.5 Liquid CAS assay. Loss of the absorbance peak at 630 nm is suggesting the presence of a Ce-chelator.

2.3.6 Comparative transcriptome analysis

To further reveal the mechanism of insoluble REE utilization in *M. capsulatus* Bath, RNA-seq was performed to analyze differentially regulated genes or clusters under the presence and absence of CeO_2. 1 g/L of CeO_2 in the medium could only dissolve a bit and resulted in precipitates in the bottom of the medium, which largely affected the concentration of extracted RNA. However, the detectable Ce concentration in 1 g/L CeO_2 and 0.1 g/L CeO_2 supplemented medium was tested to be the same level. Which were both under 10 nM. Thus, for the transcriptome analysis, to achieve a higher concentration of extracted RNA, 0.1 g/L of CeO_2 was added as the treatment. As a crucial metal for *M. capsulatus* Bath metabolism, the effect of Cu was considered here as well.

Consistent with the regulation brought about by 1 g/L CeO_2 on the expressions of two MDHs, as shown in Figures 2.3.6A and B, the 0.1 g/L CeO_2 also readily induced the "REE-switch" regardless of the presence or absence of Cu. The "REE-switch" represented the up-regulated expressions of the XoxF-MDH gene cluster (MCA299-300), on the other hand, the down-regulated expressions of the MxaF-MDH gene cluster (MCA776-791). Besides, in the comparison of gene expressions in the presence or absence of CeO_2 and in the presence of Cu,

the MCA1883 gene which encodes a non-ribosomal peptide synthase was downregulated by the presence of CeO_2, as well as a gene encoding a P-loop NTPase family protein. The secondary metabolite gene clusters predicting tool antiSMASH suggested that an NRPS cluster comprising MCA1883 as the core biosynthetic gene, NRPS-dependent pathways are commonly found in bacteria and fungi for siderophore biosynthesis [148]. In addition, genes MCA1870, 1872, 1879, 1880, and 1899 were also predicted to be involved in this NRPS cluster, their product and putative function are listed in Table 2.3.6 [149]. Furthermore, protein structure and function predicting tool Phyre2 predicted the MCA1883 has 27% amino acid sequence similarity with an enterobactin synthase component f from *E. coli* [150]. Gene MCA1886 was predicted to be a type I restriction-modification system methyltransferase with may be involved in NRPS assemblies [151]. Combining these results, the NRPS cluster especially the MCA1883 gene is a highly possible function in insoluble REE utilizations in *M. capsulatus* Bath, and synthesis an enterobactin-like REE-chelator when M. capsulatus Bath was cultured under the deficiency of REEs.

In the presence of Cu, different forms of Ce didn't bring significant regulation of transcriptome. The treatment applied here was 0.1 g/L CeO_2 powder or 0.1 μM $CeCl_3$, both supplementations can support the growth of *M. capsulatus* Bath and induce the "REE-switch" to regulate expressions of two MDHs.

Recently, Zytnick and her colleagues reported a novel lanthanide chelator in *Methylorubrum extorquens* AM1, synthesized by a siderophore synthetase component sharing 99.8% identity with the aerobactin synthase lucA. This cluster was named LCC for the "Lanthanide Chelation Cluster". This cluster also comprises other genes encoding proteins like TonB dependent receptor, transmembrane sensor, and putative NRPS [101]. While in *M. capsulatus* Bath, the putative REE chelator synthesis cluster was partially truncated, and no TonB-dependent

transporters were found to express differentially under the presence or absence of REEs.

The genes up- or down-regulated (>2-fold expression change and $p<0.05$) in the Ce-supplemented culture (supplemented with 0.1 g/L CeO_2) compared to the control culture (supplemented with 0.1 μM $CaCl_2$) with the presence of 10 μM of Cu were listed in Appendix 3.

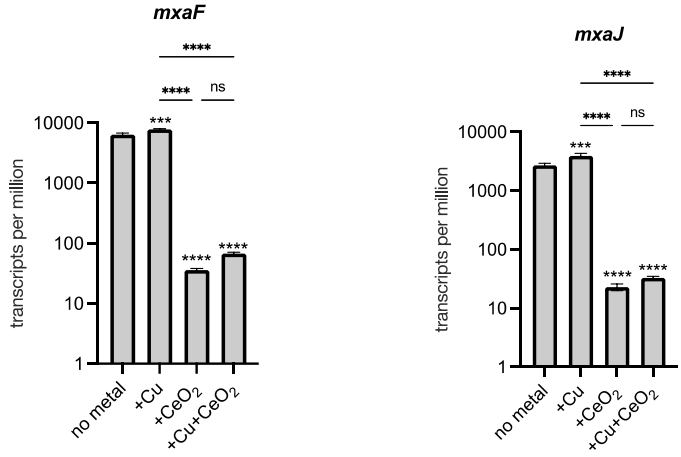


Figure 2.3.6A Expression of *mxaF* and *mxaJ* in each metal supplementation. Error bars indicate standard deviations from triplicate. Indicated P values are from a one-way analysis of variance (ANOVA).

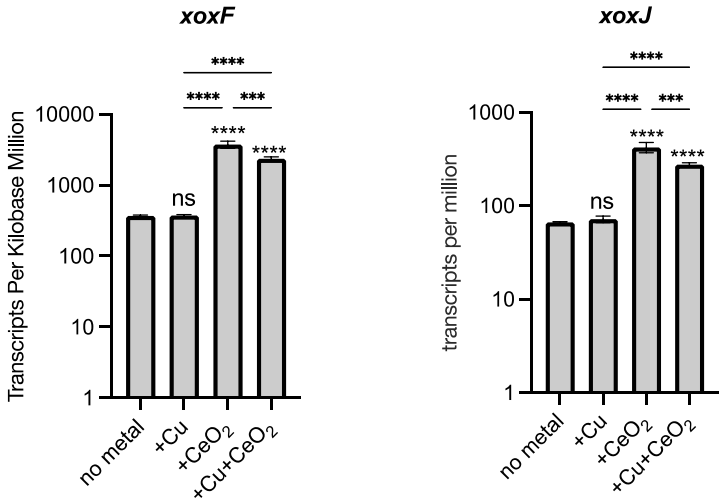


Figure 2.3.6B Expression of *xoxF* and *xoxJ* in each metal supplementation. Error bars indicate standard deviations from triplicate. Indicated P values are from a one-way analysis of variance (ANOVA).

Gene ID	Product	Function
MCA1870	ribD C-terminal domain protein	biosynthetic-additional
MCA1872	C4-dicarboxylate transporter	transport
MCA1879	nicotinate (nicotinamide) nucleotide adenylyltransferase	biosynthetic-additional
MCA1880	gamma-glutamyl phosphate reductase	biosynthetic-additional
MCA1883	non-ribosomal peptide synthetase	biosynthetic
MCA1899	cysteine synthase B	biosynthetic-additional

Table 2.3.6 NRPS cluster in *M. capsulatus* Bath predicted by antiSMASH

2.3.7 Deletion mutant construction

Based on transcriptome comparative analysis, single mutant ΔMCA0779 (*mxaF*), ΔMCA1883, and double mutant ΔMCA0779&MCA1883 are being constructed to verify the gene function of MCA1883. The plasmid containing the upstream and downstream fragments of MCA0779 and MCA1883 named pJQYM1 and pJQYN1 respectively has been constructed and successfully transformed into *E. coli* WM6096. Though the double-crossovers are still under construction. The yield of single-crossovers after conjugation of *E. coli* WM6096 and *M. capsulatus* Bath wild type showed better growth on the REE-applied agar plates, which suggested the genes knocked out here were needed for growth under the deficiency of REEs.

2.4 Conclusion

In this chapter, insoluble REE utilization was confirmed in *M. capsulatus* Bath, represented by the regulation of two MDHs in the presence of Ce oxide powder, and the solubility of Ce oxide powder was promoted in Bath spent medium. When Bath was cultured under REE-sufficient conditions (less than 100 nM of $CeCl_3$), the REE-chelator was detected from the supernatant of the spent medium of Bath. Transcriptome comparison between REE-sufficient and deficient

conditions suggested a gene encoding a non-ribosomal peptide synthase may be involved in the REE-chelator production. Expression of this gene was found to be downregulated when Bath was cultured under an REE-sufficient condition. Further, this gene was found to be a homolog with a gene encoding an enterobactin (siderophore from *E. coli*) synthase, thus an enterobactin-like REE-chelator may be involved in the acquisition of REEs in *M. capsulatus* Bath.

CHAPTER 3: REE effect on protease-producing bacteria

3.1 Introduction

Rare earth elements (REEs) were thought to be inert in biological activities for a long time until the findings were stressed in the first two chapters. While the specific effects of REEs on other biology haven't been clearly uncovered yet, there is a gradual upward trend in recent relevant studies and was extended to not only the microbes. La was demonstrated to show the "homesis effect" in *Salvia miltiorrhiza*, certain concentration ranges of La^{3+} solution displays the promotion impact on germination rate and germination potential with one of the effects in improvement of the antioxidant enzyme system, though high concentrations beyond the range can reversely inhibit the growth [16]. Many similar studies have explored the close interaction between plants and rare earth elements in the environment [17, 19]. It is therefore inevitable that rare earth elements will enter the human body through the food chain, and although their effects on humans have not been clearly investigated, some studies have shown that some short- to medium-term observations (mostly 1-3 months) provided multiple evidence for adverse effects of REEs on animals [36, 152]. Therefore, it is very important to deeply investigate the REEs effects on the microbes in the natural environment, especially those rhizosphere microbes. Limited studies have shown that rare earth elements are toxic to some microorganisms. The growth of *E. coli* (BW25113 strain) was found to be inhibited by REEs, especially by the 6~36 μM of HREEs and 4 μM of non-lanthanides Sc [28]. Similar antimicrobial properties have been demonstrated in other studies, and rare earth elements have antimicrobial properties that are weaker than those of Cu but are seen as potentially capable of replacing Cu as a safer antimicrobial metal. Besides, REEs were confirmed to have antifungal activity against common fungi like *A. niger*, *P. citrinum* [29].

Considering some of the properties of rare earth elements, it is not difficult to explain why it is biologically active. Rare earth elements especially the light ones are chemically similar to calcium. Such as Ce^{3+} and Ca^{2+} exhibit similarities in ionic radius, bonding, and preferences for donor atoms. And it is known that cerium can replace calcium in biomolecules or act as an antagonist, and as such is used in pharmacology [107]. In addition to MDH, there are other enzymes, such as amylase and protease, which also contain Ca. Recent studies have shown that the thermal stability of amylase and proteinase was significantly promoted by calcium substitution with rare earth elements in an artificial approach [109, 153]. The presence of both types of MDHs also implies the possibility of other Ca-dependent enzymes such as proteases having the possibility of REE-utilizations. Therefore, in this chapter, the protease-producing bacteria resistant to REEs were enriched and isolated from a natural sample, the microbial community has been analyzed, and suggested the REEs supplement has effect on microbial communities. The isolated protease-producing bacteria were observed to grow in various REEs and the produced proteases were detected by SDS-PAGE. Further, the produced proteases were concentrated and REEs effect on the protease activities was measured.

3.2 Materials and methods

3.2.1 Bacteria source and enrichment culture

Water samples collected from the Ono Pond at the Hokkaido University campus were used as bacteria sources. The same inorganic basal medium described earlier in Chapter 1 was used for the enrichment culture here. Besides, the final concentration of 2 g/L of skim milk as substrate and 0.1 g/L of yeast extract was added to the medium. For metals supplement, 10 µM $CuCl_2$ was added for every enrichment batch, and Ca, Ce, Sc, and Dy were added in 20 µM as chlorides for different enrichment treatments. Each treatment was enriched with the duplicate. The

enrichment culture was applied in test tubes with 26-mL capacity, 3 mL medium was added in each tube and a 10% (v/v) bacteria source was applied. Samples were cultured at 30 ℃ with 180 rpm shaking. After observation of significant growth of bacteria, 10% (v/v) culture fluid was applied to a fresh medium.

3.2.2 DNA extraction and PCR amplification

Enriched culture fluid was introduced to centrifuge at 15,000 ×g for 10 min to collect the cell pellet. DNA was extracted from each sample according to the manufacturer's instruction of MP Bio FastDNA Spin Kit for soil. The bacterial 16S rRNA gene was PCR-based amplified with the primers of 515f (5-GTGCCAGCMGCCGCGGTAA-3) and 907r (5-CCGTCAATCMTTTRAGTTT-3). The 16S rRNA gene library was sequenced on an Illumina MiSeq platform using 300-bp reads and MiSeq v3 reagents at Hokkaido System Science (Sapporo, Japan).

3.2.3 Isolation and identification of protease-producing strains

After 5 times of transferring, culture fluid was diluted in a gradient and applied to the agar plate with the addition of skim milk and different metals. Plates were cultured at 30 ℃ until colonies were shown. Strains with clear zone were picked and transferred to new plates for further use and regular culture. Colony PCR was performed with each isolated strain. Sequencing reactions were performed according to the manufacturer's instructions for BigDye Terminator v3.1 Cycle Sequencing Kit. The amplified DNA was purified and precipitated with ethanol.

3.2.4 Culture and protease production of bacterial strain

Isolated strains with high protease production were selected for further investigation. Those strains were regularly cultured on casein-containing agar plates. For protease characterization, strains were transferred from agar plates to LB medium with a starting OD600 of 0.01 and pre-

cultured at 30 ℃ with 180 rpm shaking. After 16 h growth, 10% (v/v) culture fluid was inoculated to a protease-producing medium comprising inorganic basal medium described in Chapter 1, 1 g/L casein as substrate, and 20 μM REE chlorides supplementation. After 70 h growth at 30 ℃ with 180 rpm shaking, the supernatant was collected by centrifugation at 10,000 rpm for 15 min at 4 ℃ [154]. The collected supernatant was 10 times concentrated using Amicon Ultra 15 mL devices with 10 kDa regenerated cellulose membranes (Merck Millipore). The concentrated supernatant was subsequently desalted with a PD-10 column (Cytiva) to remove the ions, fractions were eluted with Milli-Q water. Eluted fractions were collected and dripped onto a casein-containing agar plate and incubated at 37 ℃ for overnight. The protease activity was confirmed by the generation of clear zones, the eluted fractions were used as crude enzymes for further characterization. The crude enzyme was stocked at 4 ℃ and used within one week.

3.2.5 Zymogram

Zymogram was performed according to the method described previously with minor modifications [154, 155]. The crude enzyme was mixed with 2× loading dye buffer and subjected to SDS-PAGE with 5% stacking gel and 12% separating gel containing 0.8 mg/mL of casein as substrate. After electrophoresis at a constant voltage of 220v, the gel was washed twice with 2.5% (v/v) Triton X-100 for 30 min at RT and three times with distilled water for 5 min. The gel was then incubated in the protease reaction buffer (50 mM Tris-HCl pH 8.3, 50 mM $CaCl_2$) at 37 ℃ for overnight. Gel was stained with Quick-CBB PLUS (Wako) and detained following the manufacturer's instruction.

3.2.6 Protease activity assay

Protease activity assay was performed with Sigma's non-specific protease activity assay with

some modifications [156]. Briefly, 0.65% casein was dissolved in 50 mM Tris-HCl pH8.3 buffer. 400 µL of the casein solution was used as a substrate, pre-heat at 50 ℃ for 3 min. 10 µL of 10 mM metal solutions were added (for the final concentration of 0.2 mM) when needed. After pre-heating, 100 µL of crude enzyme was added except for the blank and incubate at 50 ℃ for 2 h. After incubation, the same amount of crude enzyme was added to the blank and immediately, 500 µL of 10% TCA was added to precipitate the undecomposed substrate. The resulting solutions were centrifuged at 130,000 ×rpm for 1 min at RT. 200 µL of the supernatant was mixed with 500 µL of 0.5 M Na_2O_3, then 0.5 M of Folin's Phenol reagent was added and incubated at RT for 30 min. The OD660 was measured to determine the activity of the protease. A standard curve was established with L-tyrosine solutions. Each sample was analyzed in triplicate.

3.3 Results and discussion

3.3.1 Microbial community analysis

Figure 3.3.1 showed the results of microbial community analysis from skim milk added mediums enrichment, where the individual dominant strains varied greatly under different metal supplement conditions. Especially the Ce brought distinctive microbial communities with other REEs. Some batches with the same REE supplementation showed different communities, which suggested diverse microbes can survive under 20 µM of Dy or Sc. The selectivity of Ce on microbial communities suggested it as an effective way for the enrichment and isolation of microbes that prefer Ce.

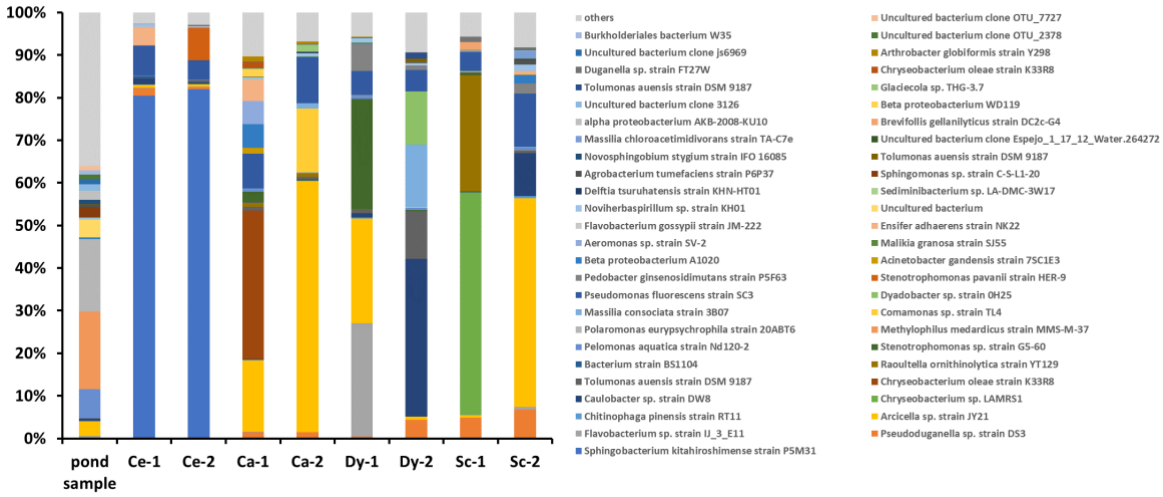


Figure 3.3.1 Microbial communities of microbes enriched from different metal-supplemented skim milk media.

3.3.2 Isolated protease-producing strains

Bacteria with the production of extracellular protease that showed the clear zones on the skim milk powder-applied agar plates were further isolated. Five strains with high protease production were isolated from Dy, Ce, Sc, and Ca-supplemented batches as listed in Table 3.3.2. Gene blast results showed these strains were belonging to *Stenotrophomonas* and *Chryseobacterium*.

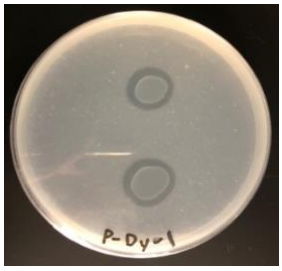
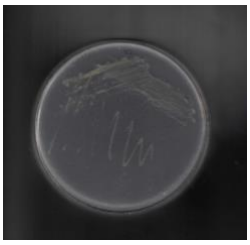
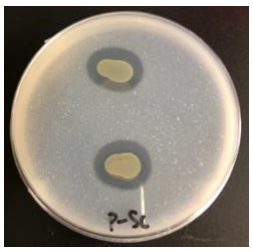
Isolates	Metal supplement	Closest related strains	Identity	Colonies
PDy-1	Dy	*Stenotrophomonas sp.* strain G5-60	100%	 
PCe-1	Ce	*Stenotrophomonas maltophilia* strain SH4	100%	 
PCa-1	Ca	*Chryseobacterium cucumeris* strain Xs3	100%	
PCa-4	Ca	*Stenotrophomonas sp.* strain G5-60	100%	
PSc-2	Sc	*Chryseobacterium balustinum* strain WK6/7_B	100%	 

Table 3.3.2 Isolated strains with their closest related strains.

3.3.3 Growth of isolated strains in various REEs and concentrations

Growth of isolated strains was observed in various REEs (Ce, Dy) and concentrations (0.001~1 mM), Figures 3.3.3 showed the growth curve of PCe-1 in Ce and Dy. 1 mM of Ce and Dy exhibited inhibition of the growth of PCe-1. Similar growth curves were observed in the other 4 strains (data not shown), that >1 mM of REEs can inhibit the growth.

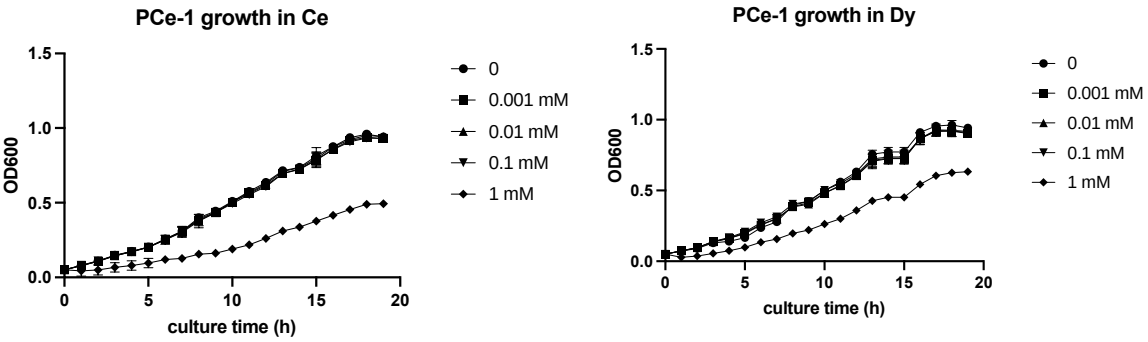


Figure 3.3.3 Growth curve of PCe-1 in various REEs of Ce (left) and Dy (right) and concentrations.

3.3.4 Proteases produced in isolated strain PCe-1 and PSc-2

A Zymogram was performed to observe protease production by isolated strains PCe-1 and PSc-2. PCe-1 was cultured with 20 μM cerium supplement and PSc-2 was cultured with 20 μM of scandium. As shown in middle Figure 3.3.4, the clear bands represent protease production in PCe-1. Four bands are shown in both culture conditions, representing protease with the size of 75, 65, 45, and 35 kDa were produced. The right Figure 3.3.4 is showing the protease production in PSc-2, two proteases with the size around 65 kDa and 45 kDa were detected.

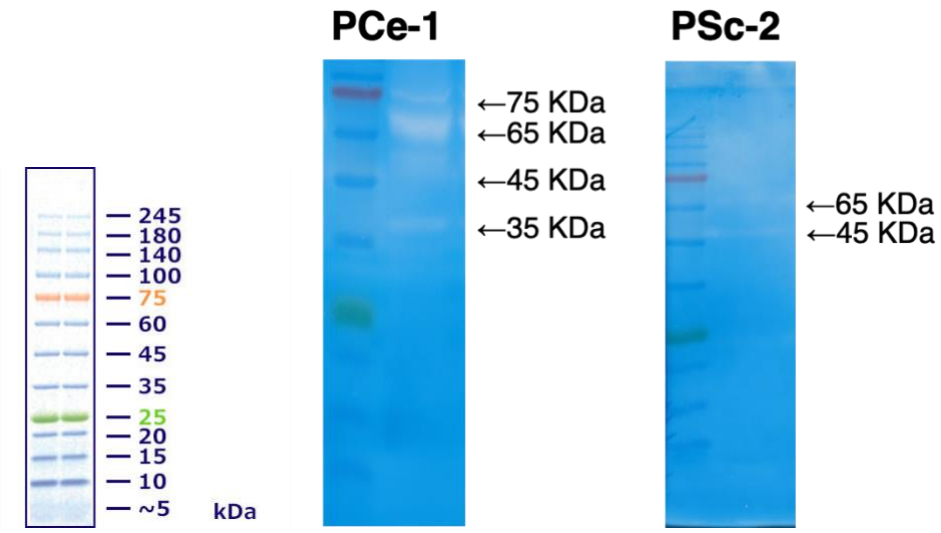


Figure 3.3.4 Zymogram gel. of PCe-1. Clear bands are showing the activity of the protease.

3.3.4 Effect of REE on protease activity produced in isolated strain PCe-1 and PSc-2

The protease activity was analyzed with casein as the substrate. The crude enzyme was prepared from Ce-free, and Ce-added culture fluid respectively. The protease activity was tested to investigate the effect of REE. As shown in Figure 3.3.4A, the activity of proteases produced by PCe-1 was elevated by 0.2 µM of Ca, Ce, Dy, and Sc. Ca showed the most positive effect on the activity of proteases, light REE Ce and Dy can also promote the activities but are not effective as Ca. Sc showed the weakest promotion. And Figure 3.3.4B showed the REEs effect on the activities of protease produced by PSc-2. Ca readily promoted the activity of the proteases, and Sc also brought comparative promotion as Ca. However, Dy only has a slight positive effect on the activities, Ce here showed a suppression of the activities of proteases.

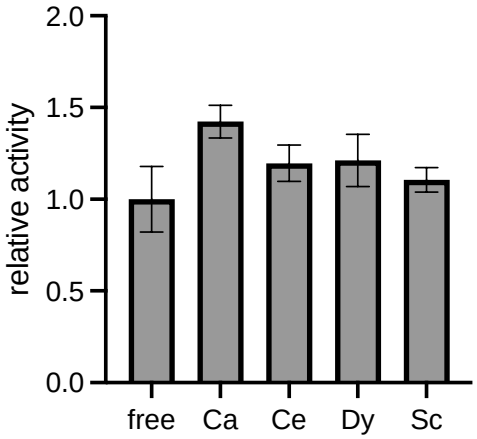


Figure 3.3.4A Effect of REEs on the activity of protease produced by PCe-1. Activity with no metal treatment was taken as the control, and activities under metal treatment were shown as a relative activity. Error bars indicate the standard deviations, triplicate was used in this analysis.

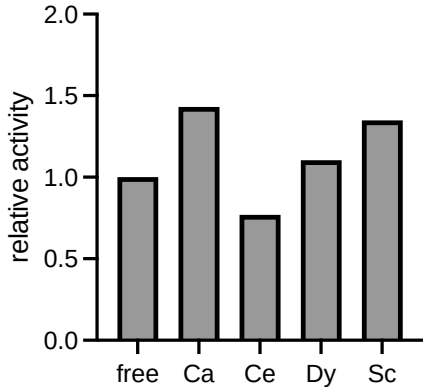


Figure 3.3.4B Effect of REEs on the activity of protease produced by PSc-1. Activity with no metal treatment was taken as the control, and activities under metal treatment were shown as a relative activity. Error bars indicate the standard deviations, triplicate was used in this analysis.

3.4 Conclusion

The effect of various REEs on the microbial communities was demonstrated in this chapter. The microbial communities varied when enriched with different metal supplements. Five strains were isolated with an exhibition of protease-producing ability. The proteases produced in two isolates were detected and the sizes were confirmed with the casein-substrate SDS-PAGE. Further, the detected proteases were concentrated and desalted, subsequent protease

activity assay showed the effect of REEs ions on the protease activity. Though the effect was weak than Ca ions, REEs ions especially the light REEs showed a positive effect on protease activities.

General discussion

Rare earth elements, as a “new life element”, have renewed our thought about their biological inactivity. In this booming phase of REE-related research, its definitive utilization is currently only found in certain microbes and enzymes. The growing acquirement of REEs for advanced industry and their potential bio-availability called more and more attention to expanded studies [87].

Both methylotrophs and methanotrophs play a crucial role in the global carbon cycle, their utilization of REEs has been intensively researched in the latest decade. With the diversity of methylotrophs and methanotrophs, there are some strains that haven’t been demonstrated about their REE utilizations. Besides, the mechanisms of environmental REE sources utilization by methylotrophs and methanotrophs were barely studied [157].

Further, the toxicity presented in REEs on common microbes such as *E. coli* and fungi suggested they are more commonly involved in biology, either with a positive or a negative effect [28, 29].

This present study first filled the empty of REE utilization by one of the model strains of *Gammaproteobacterial* methanotrophs *Methylococcus capsulatus* Bath. “REE-switch” in Bath was first time confirmed to be triggered by both soluble and insoluble REE sources. The light REE preference of Bath proved the selective utilization of REEs in methylotrophs and methanotrophs. Compared to other reported strains, Bath responses to extremely low $CeCl_3$ concentrations suggested it presents a highly sensitive REE sensing ability. Moreover, the independent “copper switch” and “REE-switch” operations provided evidence of different responses to multiple metal supplementations among methylotroph and methanotroph strains, that is “REE-switch” in *Alphaproteobacterial* methylotrophs or methanotrophs was reported to be overridden by “copper-switch”, while not in the *Gammaproteobacterial* methylotrophs or

methanotrophs [84, 158].

As for the copper utilization, the copper chelating MopE was found to be encoded for copper uptake when Bath grows under copper pressure. However, the transporter for such chelating compounds hasn't been investigated in Bath. A gene cluster encoding the ABC-transporter was found to be differentially expressed in the absence and presence of copper in this study, its actual function remains to be verified.

The solubilization of insoluble REEs source and detected REE-chelators in Bath provided insight into insoluble REE utilization by methanotrophs. The mechanism of REEs uptake from the environment was only partially studied in methylotrophs before. This study suggested an REE-chelator-mediated REE utilization mechanism in methanotroph Bath, which is a siderophore-like REE utilization mechanism similar to the finding in the other two reported cases of methylotrophs [101, 106]. However, compared to methylotrophs, less is known about the more comprehensive REE utilization mechanisms in methanotrophs. Besides the siderophore-like REE chelators, other genes involved in REE recognition, transport, and uptake were demonstrated in other strains, such as the Lanthanides-binding proteins, electron acceptors, and two-component systems [103, 127, 159]. The transcriptome analysis in this study revealed some genes encoding yet unknown functions expressed differentially under the presence or absence of REEs, providing a reference for further study about REE utilization in Bath.

The enrichment culture collected bacteria that were resistant to REEs, and the change in microbial communities under different REE supplementation batches revealed the close interaction of REEs and microbes. Though not much research has been done about the REE utilization of other enzymes than methanol dehydrogenases, the chemical similarities make it possible for REE to replace Ca and perform even better biological roles [3, 157]. Strains isolated from REE-supplemented media in this study were previously reported to be heavy metal

resistant, the promoted protease activities by REEs suggested that REEs are more closely involved in the metabolism of those bacteria. Intriguingly, some methylotrophs and methanotrophs were reported to be able to degrade the harmful chemical such as methylmercury through a methanobactin (copper chelator) way, which confers the metal resistance a more multi-functional possibility. To confirm the utilization of REEs in isolated strains, detailed studies on those detected proteases are needed, such as the structures, and the metal binding abilities. In conclusion, the finding of this study extended the exploration of REE utilization by less-studied methanotroph and microbes other than methylotrophs and methanotrophs, suggesting a border interaction between REEs and microbes. Considering the REE effect as fertilizers, these findings can be helpful for more agricultural applications of REEs. On the other hand, the insoluble REE utilization of microbes facilitates the recycling of REEs from used devices or other recyclable REE sources. At last, the superior enzymic properties of REEs suggested a more widespread and effective role of REEs in enzymes.

Appendix

Appendix 1 Differentially expressed genes in *M. capsulatus* Bath when grown in 10 μM copper + 0.1 μM cerium chlorides vs. 10 μM copper + 0 μM cerium chlorides.

Gene ID	Gene Name retrieved from cDNA information	Log_2 fold change	*p* value
MCA0299	XoxF, methanol dehydrogenase	2.84	2.63E-03
MCA0300	MxaJ protein	1.96	1.39E-03
MCA0898	conserved domain protein	1.33	2.96E-05
MCA0899	sulfate starvation-induced protein 2	1.46	3.84E-03
MCA0900	hypothetical protein	1.38	9.20E-03
MCA1111	putative membrane protein	1.03	1.04E-02
MCA1181	cysA,sulfate ABC transporter	1.06	1.20E-02
MCA1183	cysT,sulfate ABC transporter, permease protein	1.21	4.47E-04
MCA1886	conserved hypothetical protein	1.52	2.13E-02
MCA1887	conserved hypothetical protein	1.03	7.46E-04
MCA2279	cpxR,DNA-binding response regulator	1.42	1.50E-03
MCA2280	hypothetical protein	1.91	3.75E-03
MCA2507	cyanobacterial globin family protein	1.24	1.03E-02
MCA0715	hemerythrin family protein	-2.18	3.76E-03
MCA0776	hypothetical protein	-1.55	5.13E-03
MCA0777	DNA binding response regulator, LuxR family	-2.14	1.88E-03
MCA0778	putative methanol utilization control sensor protein moxY	-2.91	2.19E-03
MCA0779	mxaF,methanol dehydrogenase protein, large subunit	-7.52	3.53E-04
MCA0780	MxaJ protein	-7.47	3.32E-03
MCA0781	cytochrome c family protein	-7.16	2.01E-03
MCA0782	mxaI,methanol dehydrogenase, small subunit	-6.93	7.80E-04
MCA0783	moxR,moxR protein	-6.46	2.45E-03
MCA0784	conserved hypothetical protein	-6.53	8.50E-03
MCA0785	MxaA protein	-5.32	4.06E-03
MCA0786	MxaC protein	-5.85	6.97E-03
MCA0787	MxaK protein	-4.86	5.75E-03
MCA0788	MxaL protein	-4.80	8.99E-03
MCA0789	MxaD protein	-4.82	8.46E-03
MCA0791	putative transporter	-1.31	1.02E-02
MCA1187	cytochrome c family protein	-1.20	4.73E-04
MCA1446	pqqB,coenzyme PQQ synthesis protein B	-1.00	3.94E-03

MCA1883	non-ribosomal peptide synthetase	-1.29	3.90E-06
MCA1884	hypothetical protein	-1.14	1.16E-02
MCA2575	conserved domain protein	-1.66	4.04E-03
MCA2576	formate dehydrogenase, alpha subunit	-2.07	7.17E-03
MCA2577	putative dehydrogenase subunit	-2.75	4.95E-03
MCA2578	molybdenum ABC transporter	-3.10	6.57E-03
MCA2579	molybdenum ABC transporter, permease protein	-1.79	1.70E-05
MCA2580	molybdenum ABC transporter, periplasmic molybdenum-binding protein	-2.87	1.36E-03
MCA2618	cytochrome c-555	-1.24	3.51E-04

Appendix 2 Differentially expressed genes in *M. capsulatus* Bath when grown in 10 μM copper + 0 μM cerium chlorides vs. 0 μM copper + 0 μM cerium chlorides.

Gene ID	**Gene Name retrieved from cDNA information**	**Log_2 fold change**	***p* value**
MCA0590	nasA,nitrate reductase	1.29	1.73E-02
MCA0591	nirD,nitrite reductase [NAD(P)H], small subunit	1.35	1.54E-02
MCA0592	nirB,nitrite reductase [NAD(P)H], large subunit	1.52	1.36E-02
MCA0593	protein phosphatase/kinase family protein	1.28	1.51E-02
MCA0594	putative nitrate transporter	1.21	9.64E-03
MCA1111	putative membrane protein	1.71	3.44E-02
MCA1310	rpsO,ribosomal protein S15	1.05	6.39E-05
MCA1316	nusA,N utilization substance protein A	1.02	3.53E-05
MCA1796	pmoB,methane monooxygenase, B subunit	2.31	3.14E-03
MCA1797	pmoA,methane monooxygenase, A subunit	2.51	5.30E-03
MCA1798	pmoC1,methane monooxygenase, C subunit	1.74	3.83E-03
MCA1886	conserved hypothetical protein	1.12	1.15E-03
MCA1906	cytochrome c peroxidase family protein	1.18	2.21E-02
MCA2002	fabD,malonyl CoA-acyl carrier protein transacylase	1.05	5.18E-04
MCA2030	hypothetical protein	1.02	1.63E-02
MCA2080	atpD-2,ornithine carbamoyltransferase	1.07	2.20E-04
MCA2141	hypothetical protein	1.01	1.97E-04
MCA2245	hypothetical protein	1.12	6.71E-03
MCA2318	conserved hypothetical protein	1.04	2.06E-03
MCA2319	putative formylmethanofurane dehydrogenase, subunit	1.64	5.90E-04
MCA2320	conserved hypothetical protein	2.64	7.08E-04

MCA2321	putative TonB-dependent receptor	2.53	4.14E-03
MCA2853	pmoB2,methane monooxygenase, B subunit	2.26	6.00E-03
MCA2854	pmoA2,methane monooxygenase, A subunit	2.43	6.22E-03
MCA2855	methane monooxygenase, subunit C family protein	1.80	7.64E-03
MCA3040	tkt-1,transketolase	1.11	3.17E-03
MCA3043	hexulose-6-phosphate synthase	1.07	4.57E-04
MCA3044	SIS domain protein	1.02	1.10E-02
MCA3045	tal,transaldolase	1.48	1.72E-02
MCA3046	tkt-2,transketolase	1.16	6.38E-03
MCA3049	hexulose-6-phosphate synthase	1.06	7.19E-04
MCA0075	lipase family protein	-1.01	6.72E-03
MCA0347	hypothetical protein	-1.87	3.40E-04
MCA0348	glycine cleavage system P protein, subunit 2	-1.38	3.57E-03
MCA0421	cytochrome c5530 family protein	-3.86	6.10E-04
MCA0423	cytochrome c5530	-4.89	6.83E-04
MCA0424	cytochrome c family protein	-3.52	7.61E-04
MCA0425	hypothetical protein	-4.32	2.06E-06
MCA0426	cytochrome c family protein	-3.43	1.09E-03
MCA0427	hypothetical protein	-2.92	8.67E-04
MCA0428	hypothetical protein	-2.98	9.84E-05
MCA0429	hypothetical protein	-2.71	2.36E-03
MCA0430	4Fe-4S binding domain protein	-2.66	6.88E-04
MCA0431	flavodoxin domain protein	-2.73	1.11E-03
MCA0432	putative lipoprotein	-2.50	3.06E-03
MCA0433	NHL domain/cytochrome c family protein	-1.99	7.57E-04
MCA0434	dsbE-2,thiol	-1.08	2.17E-04
MCA0436	MotA/TolQ/ExbB proton channel family protein	-1.00	1.46E-03
MCA0437	exbB2,MotA/TolQ/ExbB proton channel family domain protein	-1.09	1.42E-02
MCA0438	biopolymer transport protein, ExbD/TolR family	-1.63	3.76E-03
MCA0440	putative TonB-dependent receptor	-3.28	2.67E-04
MCA0441	TonB domain protein	-2.68	2.37E-05
MCA0442	MotA/TolQ/ExbB proton channel family protein	-3.01	1.81E-04
MCA0443	biopolymer transport protein, ExbD/TolR family	-3.15	1.28E-04
MCA0444	hypothetical protein	-1.67	3.76E-04
MCA0445	hypothetical protein	-3.78	2.18E-03

MCA0446	hypothetical protein	-4.06	1.70E-03
MCA0447	sigma-54 dependent DNA-binding response regulator	-3.07	1.25E-03
MCA1000	conserved hypothetical protein	-1.13	1.26E-03
MCA1001	conserved hypothetical protein	-1.29	1.69E-04
MCA1002	glutathione S-transferase domain protein	-1.49	1.36E-03
MCA1101	multicopper oxidase family protein	-1.75	2.68E-06
MCA1184	conserved hypothetical protein	-1.64	6.30E-04
MCA1185	cytochrome c family protein	-3.73	2.27E-03
MCA1186	putative membrane protein	-4.00	3.78E-03
MCA1187	cytochrome c family protein	-4.71	1.30E-03
MCA1188	inorganic H(+)-translocating pyrophosphatase	-4.75	1.78E-03
MCA1189	conserved hypothetical protein	-4.40	1.03E-03
MCA1190	hypothetical protein	-4.57	1.55E-03
MCA1191	SCO1/SenC family protein	-4.27	1.92E-03
MCA1192	conserved domain protein	-3.99	2.15E-03
MCA1193	conserved hypothetical protein	-4.14	4.37E-04
MCA1194	mmoX,methane monooxygenase, A subunit, alpha chain	-8.65	1.06E-03
MCA1195	mmoY,methane monooxygenase, A subunit, beta chain	-7.42	1.53E-03
MCA1196	mmoB,methane monooxygenase, B subunit	-7.28	2.54E-03
MCA1198	mmoZ,methane monooxygenase, A subunit, gamma chain	-7.01	4.18E-04
MCA1199	mmoD,methane monooxygenase, D subunit	-5.46	1.32E-03
MCA1200	mmoC,methane monooxygenase, C subunit	-5.25	2.07E-03
MCA1201	hypothetical protein	-4.89	1.37E-03
MCA1202	groEL-2,chaperonin, 60 kDa subunit	-3.34	1.01E-03
MCA1203	response regulator	-1.62	1.57E-03
MCA1205	sigma-54 dependent transcriptional regulator	-3.05	2.85E-04
MCA1216	hypothetical protein	-1.23	1.29E-03
MCA1350	nuoK,NADH dehydrogenase I, K subunit	-1.19	1.11E-02
MCA1354	nuoG,NADH dehydrogenase I, G subunit	-1.09	1.83E-03
MCA1355	nuoF,NADH dehydrogenase I, F subunit	-1.03	1.09E-03
MCA1357	nuoCD,NADH dehydrogenase I, C/D subunits	-1.01	4.62E-04
MCA2160	cytochrome c5530 family protein	-1.79	1.23E-05
MCA2163	hypothetical protein	-1.56	3.29E-06

MCA2169	BNR repeat domain protein	-4.42	3.57E-04
MCA2170	putative copper resistance protein	-2.29	5.09E-04
MCA2171	hypothetical protein	-3.25	1.83E-04
MCA2172	ABC transporter, ATP-binding family protein	-3.19	5.55E-04
MCA2173	conserved hypothetical protein	-2.11	1.05E-05
MCA2174	adhesion lipoprotein	-2.55	5.52E-04
MCA2176	hypothetical protein	-5.64	9.62E-04
MCA2177	hypothetical protein	-6.32	1.24E-03
MCA2179	putative lipoprotein	-1.18	3.07E-05
MCA2259	cytochrome c5530 family protein	-1.36	2.77E-05
MCA2457	conserved domain protein	-1.01	1.64E-03
MCA2589	mopE,surface-associated protein precursor	-3.85	9.10E-04
MCA2590	putative cytochrome c peroxidase family protein	-4.59	1.60E-04
MCA2957	hypothetical protein	-1.06	4.77E-03
MCA2978	putative lipoprotein	-1.05	9.67E-04

Appendix 3 Differentially expressed genes in *M. capsulatus* Bath when grown in 10 μM copper + 0.1 g/L cerium oxides vs. 10 μM copper + 0 g/L cerium oxides.

Gene ID	**Gene Name retrieved from cDNA information**	**Log_2 fold change**	***p* value**
MCA0204	nitrogenase cofactor biosynthesis protein NifB	1.07	2.17E-02
MCA0233	nifE,nitrogenase iron-molybdenum cofactor biosynthesis protein nifE	1.25	3.63E-02
MCA0247	iscS,cysteine desulfurase	1.54	1.70E-02
MCA0248	NifU family protein	1.06	5.54E-03
MCA0249	HesB/YadR/YfhF family protein	1.13	7.27E-03
MCA0299	XoxF, methanol dehydrogenase	2.66	1.53E-03
MCA0300	MxaJ protein, MoxJ2	1.94	3.09E-04
MCA0898	conserved domain protein	1.70	1.13E-02
MCA0899	sulfate starvation-induced protein 2	1.67	1.54E-02
MCA0900	hypothetical protein	1.49	1.28E-02
MCA1183	cysT,sulfate ABC transporter, permease protein	1.37	2.03E-02
MCA1886	conserved hypothetical protein, Methylase_S domain-containing protein	1.19	3.97E-02
MCA2279	cpxR,DNA-binding response regulator	1.29	2.48E-02
MCA2280	hypothetical protein	1.59	1.85E-02
MCA0715	hemerythrin family protein	-2.03	3.73E-03
MCA0776	hypothetical protein	-1.31	3.14E-03

MCA0777	DNA binding response regulator, LuxR family	-1.70	1.17E-03
MCA0778	putative methanol utilization control sensor protein moxY	-2.33	2.30E-03
MCA0779	mxaF,methanol dehydrogenase protein, large subunit	-6.85	3.56E-04
MCA0780	MxaJ protein, MoxJ	-6.90	3.34E-03
MCA0781	cytochrome c family protein	-6.74	2.02E-03
MCA0782	mxaI,methanol dehydrogenase, small subunit	-6.52	7.85E-04
MCA0783	moxR,moxR protein	-6.19	2.46E-03
MCA0784	conserved hypothetical protein	-6.24	8.55E-03
MCA0785	MxaA protein	-5.20	4.09E-03
MCA0786	MxaC protein	-5.55	7.02E-03
MCA0787	MxaK protein	-4.66	5.80E-03
MCA0788	MxaL protein	-4.48	9.18E-03
MCA0789	MxaD protein	-4.73	8.50E-03
MCA0791	putative transporter	-1.11	6.24E-03
MCA1563	conserved hypothetical protein	-1.04	4.68E-03
MCA1883	non-ribosomal peptide synthetase	-1.26	3.84E-07
MCA1884	hypothetical protein, P-loop NTPase family protein	-1.13	1.12E-02
MCA2575	conserved domain protein	-1.64	3.93E-03
MCA2576	formate dehydrogenase, alpha subunit	-2.19	7.32E-03
MCA2577	putative dehydrogenase subunit	-2.87	4.87E-03
MCA2578	molybdenum ABC transporter, ATP-binding protein	-3.13	6.18E-03
MCA2579	molybdenum ABC transporter, permease protein	-1.77	2.04E-06
MCA2580	molybdenum ABC transporter, periplasmic molybdenum-binding protein	-2.84	1.35E-03

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