WIDESPREAD BACTERIAL USE OF LANTHANIDES FOR METHYLOTROPHY ACROSS ECOSYSTEMS

A Dissertation
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Master of Science in the
School of Earth and Atmospheric Sciences

Georgia Institute of Technology
December 2024

WIDESPREAD BACTERIAL USE OF LANTHANIDES FOR METHYLOTROPHY ACROSS ECOSYSTEMS

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Date Approved: November 11, 2024

ACKNOWLEDGEMENTS

I would first like to thank my advisor Dr. Jennifer B. Glass for sharing her time and knowledge with me. This thesis would not have been possible without her continuous support. I would also like to thank my committee members Dr. Yuanzhi Tang and Dr. Martial Taillefert for providing insight and instruction through this thesis. I would also like to thank Dr. Frank Rosenzweig for allowing me to use his lab's plate reader and Emily A. Cook for her instruction in operating it. I would like to thank PhD candidate Claire E. Elbon for her training in lab operations and always being available for my many questions. I would also like to thank undergrad Kylee D. Graham for her assistance in cell growth experiments and undergrads Abdulaziz M. Alajlan and Shahd Bargouthi for their work in creating Genesweeper. I would lastly like to thank my family for their unwavering support even from far away.

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LIST OF SYMBOLS AND ABBREVIATIONS

CadM	Calmodulin
IMG	Integrated Microbial Genome
JGI	Joint Genome Institute
LanM	Lanmodulin
Ln	Lanthanide
MDH	Methanol dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide
PIPES	1,4-piperazinediethanesulfonic acid
PQQ	Pyrroloquinoline quinone
REE	Rare earth element
RPKM	Reads per kilobase per million

SUMMARY

Methylotrophs are aerobic bacteria that consume one-carbon compounds as carbon and energy sources. Methylotrophs use the enzyme methanol dehydrogenase (MDH) to oxidize methanol to formaldehyde. MDH requires either calcium or lanthanides as a cofactor. Despite being classified as light rare earth elements, lanthanides are relatively common in the Earth's crust but poorly soluble in most environments. Accumulating evidence suggests that lanthanide-dependent MDHs encoded by the gene *xoxF*, are more widespread than calcium-dependent MDHs encoded by the gene *mxaF*. In this thesis, I first studied the prevalence of lanthanide utilization in laboratory experiments using a model methylotroph alphaproteobacterium, *Methylorubrum extorquens* AM1. At 1 μM, lanthanides (La, Ce, or Nd) supported fastest growth and highest cell density of *M. extorquens*. At 100 μM, *M. extorquens* grew optimally with Ca, while Ce, Nd, and Eu suppressed growth. Second, I studied the environmental abundance and relevance of lanthanide-dependent MDH using environmental metatranscriptomes in the Joint Genome Institute's (JGI) Integrated Microbial Genome (IMG) database. XoxF amino acid sequences can be distinguished from MxaF sequences by an extra aspartate in XoxF sequences. I found that *xoxF* genes were transcribed in all ecosystems (soils, freshwaters, sediments, salt marshes, seawater, and hot springs), whereas *mxaF* was only transcribed in one freshwater ecosystem and one soil ecosystem. The majority of transcribed *xoxF* genes in marine environments belonged to the classes Alphaproteobacteria and Gammaproteobacteria (including Burkholderiales). Transcribed *xoxF* genes in terrestrial environments showed more taxonomic diversity, with many belonging to the phyla

Nitrososphaerota, Actinomycetota, Chloroflexota, Myxococcota, and Gemmatimonadota. This thesis highlights the importance of lanthanide-dependent methylotrophy in global biogeochemical cycles and suggests that previously unidentified methylotrophs are active in global environments.

CHAPTER 1. INTRODUCTION

1.1 Motivation

Methylotrophs are microorganisms that utilize one-carbon compounds, such as methane, methanol, or methylamine, as both a carbon and energy source [1]. Methylotrophs are most prominent in terrestrial soils, particularly in association with plants. As plants are a source of methanol, plant phyllospheres are an important habitat for many methylotrophs. In return, methylotrophs promote plant growth by increasing hormone production and nutrient uptake efficiency [2, 3]. Methylotrophs may also provide health benefits by limiting pathogen growth and inducing systemic resistance [4]. Methylotrophs also have biotechnological applications, especially in the production of polyhydroxyalkanoates, amino acids, dicarboxylate acids, alcohols, and proteins from methanol [5]. Methylotrophs' role in the carbon cycle makes them important in understanding climate change.

Methanol dehydrogenase (MDH) is an enzyme important to the metabolisms of methylotrophic bacteria. This enzyme facilitates the oxidation of methanol to formaldehyde [6]. Formaldehyde can then be further oxidized to CO_2 or assimilated into carbon for cellular growth. [7]. Several types of MDHs exist, differing in cofactors and cellular locations. In Gram-positive bacteria, MDH is nicotinamide adenine dinucleotide (NAD^+) -dependent and cytoplasmic [6, 7]. In Gram-negative bacteria, MDH is pyrrolo-quinoline quinone (PQQ)- dependent and periplasmic [6, 7]. It was long believed that only calcium (Ca) could be used as the catalytic center in PQQ-MDHs. More recent studies have found that light lanthanides (Lns), or rare earth elements (REEs), can also act as the catalytic center within PQQ-MDH [8].

Before this discovery, Lns were long thought to be unessential in biology [9], but their use in methylotrophy proves a link between the carbon and lanthanide biogeochemical cycles. Ln-dependent methylotrophs also offer a potential alternative method of Ln extraction. Current extraction and separation practices for Lns requires highly acidic chemical leaching, which releases toxic products into the environment [10]. The potential of applying methylotrophs to the bioleaching and recycling of Lns is currently being studied [11, 12].

1.2 Lanthanide geochemistry and distribution in the environment

Lanthanides (Lns) are a group of elements ranging from lanthanum (La) to lutetium (Lu). Lns, with the addition of scandium and yttrium, are also referred to as rare earth elements (REEs) [13]. REEs have similar physical and chemical properties due to their electronic configuration forming a stable oxidation state (3+). The exceptions are Ce and Eu, which may also occur as Ce^{4+} and Eu^{2+} [14]. Their unique properties make Lns important to many industries and high-tech products such as cell phone and car batteries, permanent magnets, X-ray imaging, fluorescent lights, and wind turbines [10, 15, 16]. Lns were long thought to be biologically inert. While it was proposed that Lns could substitute for calcium ions because of their similar size and stronger Lewis acidity, it was assumed that no organisms had evolved to depend on them because of their poor solubility [9]. Lns have been used in some farming practices as additives to fertilizers and some animal feeds, but they have also been shown to be toxic to some plants and animals [17].

Despite their name, REEs are relatively abundant (0.015% by weight) in the Earth's crust [18]. REE concentrations in the environment are affected by several factors, such as pH, weathering, redox conditions, and the types of minerals [16]. Lns are lithophilic and can substitute for other cations of similar radius and charge in minerals. In most terrestrial ecosystems, they are found in silicates, carbonates, oxides, phosphates, and oxyhydroxysalts [18]. REEs often occur as inclusions in major and minor mineral phases and are highly resistant to weathering, making Ln mobilization complex. In acidic environments, Ln remobilization is often caused by the oxidation of pyrite. Acid mine drainage waters often contain concentrations of Lns in the hundreds of µg/L, mainly as sulfate complexes ($LnSO_4^+$ and $Ln(SO_4)^{2-}$) or free Ln^{3+} ions [18].

In aquatic environments, Ln speciation is dependent on pressure of the water column and the atomic weight of the Ln atom. In surface seawaters, Lns primarily occur as carbonate complexes: ~85% of La and >99% of Lu is present as carbonate complexes [19]. In the deep sea, carbonate complexation is suppressed, (40% of La and only 17% for Lu), free ions of La increase to >25% and La-sulfate, chloride, silicate, and hydroxide species dominate. For Lu, silicate species dominate (80%) [19]. The pH has a strong effect on aqueous Ln concentrations. Generally, acidic environments produce the highest concentrations of dissolved Ln [20], with acidic mud pots having dissolved Lns in the micromolar range [21]. In freshwater, the highest Ln concentrations are found in groundwaters, with an average of 35 nmol/kg [20] , whereas lakes and rivers have average Ln concentrations of 3.8 and 3.3 nmol/kg, respectively [20]. In turn, seawater has the lowest average Ln concentrations (19 pmol/kg) [20].

1.3 Lanthanide-dependent MDH and distribution in the environment

Two major forms of the PQQ-MDH protein exist. MxaF-MDH, encoded by the gene *mxaF*, uses Ca^{2+} as the catalytic center [7]. MxaF was previously assumed to be the only type of PQQ-MDH. Genomic studies, however, suggested the occurrence of a homologue to MxaF with less than 50% sequence identity [22]. XoxF, encoded by the gene *xoxF*, was suspected to have MDH catalytic activity [23]. Some cultured methylotrophic bacteria contain *xoxF* and *mxaF* genes, but many methylotrophs contain only the *xoxF* gene [24]. Phylogenies showed that *xoxF* is older than *mxaF* [7, 24, 25]. The role and mechanism of the XoxF proteins remained elusive for some time, until it was found to be a part of an alternative MDH pathway utilizing Lns [24].

Methylorubrum is a genus of aerobic, facultative methylotrophs and is one of the most abundant genera in plant phyllospheres [1]. In studies comparing MDH specific activity in *Methylobacterium spp*. grown on equal concentrations of La^{3+} or Ca^{2+}, specific activity was consistently higher in cultures grown with La^{3+} and Ce^{3+} [8]. *Methylorubrum extorquens* AM1, a facultative methylotroph that contains both *mxaF* and *xoxF* genes, had similar growth rates and growth yields when grown with La^{3+} or Ca^{2+} [1]. However, experiments measuring the specific activity of MDH corroborated earlier studies, showing significantly higher specific activity in MDH grown with La^{3+} than Ca^{2+} [1]. In a mutant strain without *mxaF*, *M. extorquens* could not grow on methanol when supplied with Ca^{2+}, but regained growth when supplied with La^{3+}. These findings highlight an alternative methanol oxidation pathway using La^{3+} as a cofactor in MDH [1].

Methylacidiphilum fumariolicum SolV is an acidophilic methylotroph of the Verrucomicrobia phylum found in volcanic mud pots. *M. fumariolicum* was found not to grow without the addition of mud pot water from its original habitat. Analysis of the highly acidic mud pot water showed that it contained high amounts of dissolved Lns. Ce^{3+} was the most abundant Ln in the waters, accounting for 50% of the total Lns. When supplied with Ln salts, *M. fumariolicum* continued to grow. Heavier Lns were less supportive of growth, as growth rate decreased from Sm^{3+} to Gd^{3+}. Cultures supplied with Ca^{2+} showed no growth. *M. fumariolicum* contains only the *xoxF* form of MDH [21]. This study showed that Lns were essential to the function of many methylotrophs.

Despite limited Ln bioavailability, the occurrence of Ln-dependent enzymes has been shown to be widespread in many environments. Metagenomic sequencing has indicated that the *xoxF* gene is highly diverse [26]. During the Deepwater Horizon Blowout of 2010 in the Gulf of Mexico, there was a bloom of bacteria in response to the increased hydrocarbons in the ocean [27]. Surface waters during that time also showed a depletion of light Ln concentrations associated with the bloom of methanotrophs [28]. In weathered granite communities, the *xoxF* gene is widely abundant, further supporting methylotrophs' importance to biogeochemical cycles, particularly in the weathering of poorly soluble minerals [29]. Methylotrophs are shown to reach their maximum growth rates when supplied with micromolar concentrations of Lns [30]. The only natural environments with those concentrations would be extremely acidic environments such as volcanic hot springs, suggesting that methylotroph metabolisms may be limited by the Ln concentrations [30].

1.4 Adaptations for lanthanide uptake and storage

Given the poor bioavailability of Lns in most natural environments, how methylotrophs acquire enough Lns for their processes remains an open question. Methylotrophs evolved several mechanisms to improve sensitivity to Lns and acquire acceptable amounts. Lanmodulin (LanM) is a high-affinity, Ln-binding protein. LanM is similar to the Ca^{2+} binding protein calmodulin (CaM), but more selective towards Lns. At picomolar Ln concentrations, LanM undergoes a conformational change into a more ordered state, whereas near millimolar Ca^{2+} concentrations were required to trigger a response [31]. LanM may also be involved in the regulation of *mxaF* and *xoxF* expression. In bacteria that use both proteins, Lns were preferably chosen over Ca^{2+}, even when Ca^{2+} was up to 10 times more abundant. In the presence of at least nanomolar amounts of Lns, expression of the *xoxF* gene is upregulated, while *mxaF* expression is downregulated [32, 33]. This change from the MxaF pathway to the XoxF pathway, controlled by Ln concentration, has been termed the "lanthanide switch" [13, 33, 34].

Metallophores are low molecular weight organic molecules secreted from many microorganisms to bind essential metals from the environment, then transport them back to the cell. Metallophores for iron, zinc, nickel, molybdenum, and copper have been identified and characterized [35]. Evidence also suggests the existence of an organic chelator specific for lanthanides, the "lanthanophore" [34]. In bacteria that utilize siderophores, a TonB-ABC transport system is often required. Transposon metagenesis studies suggest the existence of genes encoding homologs of the TonB-ABC receptor system in the Ln utilization and transport (*lut*) cluster of *M. extorquens* [36, 37].

A putative Ln chelator, methylolanthanin, was isolated from *M. extorquens* and is structurally similar to the known siderophore rhodopectrobactin. It is hypothesized that the methylolanthanin pathway entered *Methylorubrum* as an iron-binding pathway through horizontal gene transfer and eventually evolved to chelate Lns instead [35].

In addition to adaptations to acquire Lns, methylotrophs also store Lns. After transport into the cytoplasm, Lns are stored as inorganic phosphate crystals in *M. extorquens* [35, 36]. Ln uptake and storage systems differ between methylotrophic species. In *Beijerinckiaceae* bacterium RH AL1, a methylotrophic species found in early industrial soft coal slags, Lns are accumulated intracellularly in outer membrane vesicles and there is minimal periplasmic Ln and undetectable cytoplasmic Ln. There was also no evidence of lanthanophore productions from RH AL1 [38].

It is possible that mesophilic methylotrophs evolved lanthanophores to scavenge Lns from environments with very low concentrations, such as soil and the phyllosphere. Genes encoding for this system have been found in many mesophilic, methylotrophic, Ln-utilizing bacteria such as *M. extorquens*, but extremophiles like *M. fumariolicum* lack it. *M. fumariolicum* would not require the use of lanthanophores as it grows in acidic mudpots, which have relatively high levels of dissolved Lns. *M. extorquens* is shown to repress the lanthanophore system when excess Lns are available [35]. *M. extorquens* also bioaccumulates more Lns in the cytoplasm than needed for MDH function and continued growth [35]. This bioaccumulation may be another adaptation to account for periods where Lns are not readily available. The presence of the Ca^{2+}-dependent MxaF pathway in mesophilic methylotrophs may also be an adaptation for times when Lns are scarce [30].

1.5 Lanthanide selectivity

Ln-dependent MDHs are highly selective towards light Lns. Whereas Lns from La to Gd (with the exception of Pm) promote growth in most Ln-dependent methylotrophs, , there is no evidence of utilization of the heaviest Lns (Tb to Lu) to date [15, 21, 32, 39, 40] **(Figure 1)**. In studies with *M. fumariolicum*, the specific activity of MDH increased with increasing Ln atomic mass from La^{3+} to Nd^{3+}. Specific activity then decreased with increasing atomic mass from Sm^{3+} to Gd^{3+}. The heaviest Lns (Tb-Lu) yielded weaker MDH response than the control [41]. Similarly, LanM shows a lower conformational change *in vitro* in the presence of Lns heavier than Sm [31].

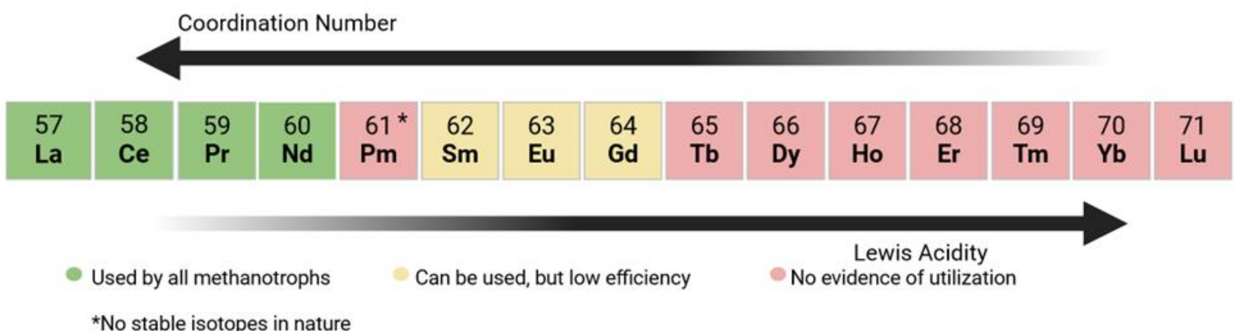

Figure 1: Lanthanide utilization trends by Lewis acidity and coordination number

"Lanthanide contraction" describes the phenomenon in which the Ln series decreases in ionic radius with increasing atomic mass from La to Lu [41]. It is hypothesized that the effects of Ln contraction may influence the selectivity of Ln-dependent MDH. As Ln ionic radius decreases due to Ln contraction, Lewis acidity increases from La to Lu. High Lewis acidity is favored for the PQQ cofactor, which explains the increase in specific activity of MDH seen from La^{3+} to Nd^{3+} (excluding Pm^{3+} as no stable isotopes are found in nature) [9]. The drop in specific activity for Lns heavier than Nd^{3+} is likely due to a decrease in coordination number from Nd^{3+} to Sm^{3+} [41]. The high Lewis acidity and relatively high coordination number of light Lns makes them the best fit for MDH function and bacteria growth, with Nd^{3+} or Pr^{3+} being the optimal Lns for MDH function. It is also suggested that the use of only light Lns may prevent the heaviest Ln from distorting the PQQ-MDH machinery [38]. Sm^{3+} to Gd^{3+} can promote MDH function and bacteria growth, but to a lesser extent than the lightest Ln [15].

1.6 Experimental Goals

The overall motivation of this thesis is to determine the importance of Ln-dependent methylotrophy to global biogeochemical cycling through culture experiments and bioinformatic analyses. This thesis had two major objectives. Two major objectives were reached in this thesis. The first objective was to determine growth rates of the model methylotroph *M. extorquens* AM1 with different concentrations of dissolved Lns. *M. extorquens* was grown in minimal media with 0.1-100μM of the Ln salts $LaCl_3$, $CeCl_3$, $NdCl_3$, or $EuCl_3$, and compared to 0.1-100 μM of $CaCl_2$ as the control. Previous studies have shown that Nd^{3+} promotes the highest methylotrophic growth rates and MDH activity [32, 41]. Thus, I hypothesized that Nd^{3+} would yield the highest growth rate and yield in my experiments.

The second objective of this thesis was to quantify the occurrence of Ln-dependent MDH and compare it to the occurrence of Ca-dependent MDH across ecosystems and microbial phyla. Previous metagenomic, metatranscriptomic, and metaproteomic studies have been used to identify *xoxF* genes and XoxF and identify community dynamics in environments such as laboratory cultures, freshwater sediments, plant phyllospheres, ocean coasts, hydrothermal vent plumes, sea sponges, and marine, estuarine, and freshwaters [42-

50]. Even before being identified as an Ln-dependent methylotrophic gene, *xoxF* was detected in many omics studies on methylotroph communities [43, 51]. This objective was completed by mining every instance of PQQ-MDH in from the Joint Genome Institute's (JGI) Integrated Microbial Genome (IMG) RNASeq Expression Studies database of environmental metatranscriptomes. XoxF and MxaF are highly conserved proteins, but contain amino acid differences at specific sites. Both enzymes contain a catalytic aspartate (Asp^{303}, single letter amino acid abbreviation "D", based on *M. extorquens* AM1 numbering) residue in their sequences, but a second aspartate (Asp^{301}, single letter amino acid abbreviation "D") is found two positions downstream in Ln-dependent MDH (XoxF). In MxaF sequences, an alanine (Ala, single letter amino acid abbreviation, "A") is present instead of the second aspartate [52] **(Figure 2).** The data were further sorted based on ecosystem and *xoxF* gene transcription was quantified. Based on the occurrence of *xoxF* in microbial genomes, it was expected that *xoxF* would be expressed across a wider range of environments than *mxaF*, and the strongest expression would occur in extremely acidic environments rich in dissolved Lns.

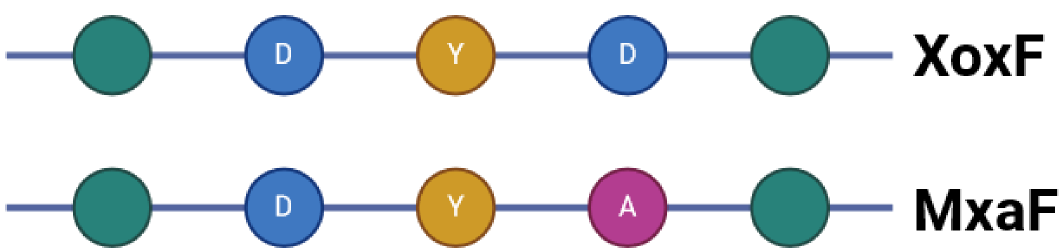

Figure 2: Diagram of MDH amino acid sequences, depicting the DYD (XoxF) and DYA (MxaF) motifs used to differentiate between Ln-pathway (XoxF) and Ca-pathway (MxaF).

CHAPTER 2. METHODOLOGY

2.1 Methylorubrum extorquens cultivation and growth conditions

Methylorubrum extorquens AM1, a terrestrial alphaproteobacterium, is one of the best studied species of methylotrophic bacteria. The bacterial strain was ordered from the American Type Culture Collection (item number 14718; batch number 70047882). PIPES (1,4-piperazinediethanesulfonic acid) minimal medium with 125 mM methanol was used for all experiments [53]. The Ln salts $LaCl_3$, $CeCl_3$, $NdCl_3$, and $EuCl_3$ were used as Ln sources. $CaCl_2 \cdot 2\,H_2O$ was used as the calcium source. Stock cultures of *M. extorquens* were grown in 20 mL of medium at 30°C and 150 rpm shaking.

2.2 Growth rate determinations

For experiments, *M. extorquens* AM1 was grown in a sterile 96-well plate with 200 µL of PIPES media per well. To determine growth rates, OD_{600} was continuously measured in a shaking plate reader (Biotek Synergy HTX) at 30°C with continuous shaking (282 rpm) for 72 hours. *M. extorquens* AM1 was grown with 0.1, 1, 10, or 100 µM of Ca^{2+} or Ln^{3+} salt solution (n=3 for each treatment), or without added metal (n=12).

To determine the doubling times of each treatment, the natural log of each growth curve was taken. The growth rate of the exponential phase of each graph was then taken and the doubling time for each treatment was determined with Equation 1:

$$Doubling\ Time = \frac{\ln(2)}{growth\ rate} \tag{1}$$

2.3 Introduction to GeneSweeper

GeneSweeper is an open-source python program developed by Abulaziz Alajlan (Georgia Tech Computer Science) for filtering large CSV files for a specific product name. The protein product name of interest is input to retrieve lines from the CSV file containing the transcribed genes matching the product name used. The python code follows the Joint Genome Institutes (JGI) CSV data structures. To help build FASTA files from amino acid sequences extracted from each data set, the program also builds a CSV file that includes URLs to the JGI website's page on that specific Gene and Scaffold ID, which has the amino acid sequences. The program is available through a Github repository (https://github.com/GlassLabGT/Python-scripts) and can be used to search for any protein.

2.4 Usage of GeneSweeper

All files were downloaded (as of February 2024) from the JGI's IMG database. The metatranscripts were sourced from the RNASeq Expression Studies (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi?section=RNAStudies&page=rnastudies), processed and filtered, then appended to one large output file. GeneSweeper was used to filter the RNA sequence data sets. The filtration process was split into 8 runs to divide file sizes. As IMG JGI is a public database, the target protein PQQ-MDH may be found under different names, so several different product names were used in the search. The multi-product search feature was performed for the following product names: "PQQ-dependent dehydrogenase (methanol/ethanol family)", "methanol dehydrogenase (cytochrome c) subunit 1", "methanol dehydrogenase (cytochrome)", "alcohol dehydrogenase (cytochrome c)”, “glucose dehydrogenase”, “quinohemoprotein ethanol dehydrogenase”, and “quinoprotein glucose dehydrogenase” were used as search terms.

2.5 Data Processing and classification of XoxF and MxaF

All instances of the target protein were collected. From those files, studies of lab enrichments, experimental treatments, or artificial microbial communities were filtered out to leave only those from natural environments. Sequences with a coverage of less than1 x 10^{-8} and/or gene sequence lengths less than 1,770 base pairs were removed. NCBI’s BLAST program was used to run the remaining transcript sequences against the NCBI nr database (April-May 2024) and classify each transcribed gene to the closest phylum, class, and order. By this system, many enzymes were classified as belonging to the Betaproteobacteria class of Pseudomonadota. This classification has changed in recent years, as Betaproteobacteria are now classified within the order Burkholderiales under the class Gammaproteobacteria. In this thesis, all transcribed genes initially classified as Betaproteobacteria will be referred to as “Burkholderiales” and treated separately from Gammaproteobacteria. Transcribed genes were further sorted by the type of ecosystem from which the sample was collected (freshwaters, freshwater sediment, seawater, marine sediment, coastal waters and estuaries, groundwaters, soils, hot springs, and salt marshes).

Expression of each transcribed gene was quantified as reads per kilobase per million mapped reads (RPKM). To calculate RPKM, the total reads count of the metatranscriptome was required. To find this value, the IMG Genome ID (Taxon Object ID) for the transcript was searched in the IMG-JGI website. The Project ID (Gold ID) for the transcript was then identified from the microbiome details page. We then returned to the RNASeq Expression Studies page, navigated to the study that the transcript was from, and used the Gold ID for the match for the metatranscriptome, to obtain the total reads count. RPKM was calculated from the formula:

$$RPKM = \frac{\text{Reads Count}}{((\text{Gene Seq Length}/1000) * (\text{Total Reads}/1000000))} \qquad (2)$$

CHAPTER 3. RESULTS AND DISCUSSION

3.1 *Methylorubrum extorquens* growth in the presence of calcium or lanthanides

3.1.1 Methylorubrum extorquens growth in Ca

M. extorquens grown in Ca^{2+} had a doubling time of 3.7-5.2 hr. The fastest growth (doubling time: 3.7 hr) was in cultures with 10 µM Ca^{2+} (**Figure 4)**. The highest cell density (max OD_{600}: 0.68) was in cultures with 100 µM Ca^{2+} **(Figure 3a)**.

3.1.2 Methylorubrum extorquens growth in La

The highest growth rate (doubling time: 4.9 hr) and final cell density (max OD_{600}: 0.57) occurred in cultures containing 1 µM La^{3+}. Growth rates were slightly lower in cultures with 10 µM La^{3+} (doubling time: 5.8 hr), while maximum OD_{600} was similar (0.56). Lowest growth rate (doubling time: 9.4 hr) and cell density (max OD_{600}: 0.48) occurred in cultures with 100 µM La^{3+} **(Figure 3b and Figure 4)**.

3.1.3 Methylorubrum extorquens growth in Ce

The highest growth rates was 2.9 hr doubling time in cultures with 1 µM Ce^{3+} **(Figure 4)**. The highest cell density (max OD_{600}: 0.58) was in cultures with 10 µM Ce^{3+} **(Figure 3c)**. Growth rate (12.4 hr doubling time) and cell density (max OD_{600}: 0.36) were lower in cultures containing 100 µM Ce^{3+} **(Figure 3c and Figure 4)**.

3.1.4 Methylorubrum extorquens growth in Nd

Highest growth rate (doubling time: 3.6 hr) was observed in cultures containing 1 μM Nd^{3+} **(Figure 4)**. Highest cell density (max OD_{600}: 0.57) was observed in cultures containing 10 μM Nd^{3+}. Growth rate decreased with increasing Nd^{3+} concentration above 10 μM, reaching a doubling time of 13.7 hr in cultures with 100 μM Nd^{3+} **(Figure 3d)**.

3.1.5 Methylorubrum extorquens growth in Eu

M. extorquens grew the slowest overall with Eu^{3+}. The highest growth rate of *M. extorquens* in Eu^{3+} (doubling time: 8.4 hr) was in cultures containing 1 μM Eu^{3+} **(Figure 4)**. In concentrations of Eu^{3+} up to 100 μM, *M. extorquens* growth was almost completely suppressed (doubling time: 51 hr; max OD_{600}: 0.02; **Figure 3e)**.

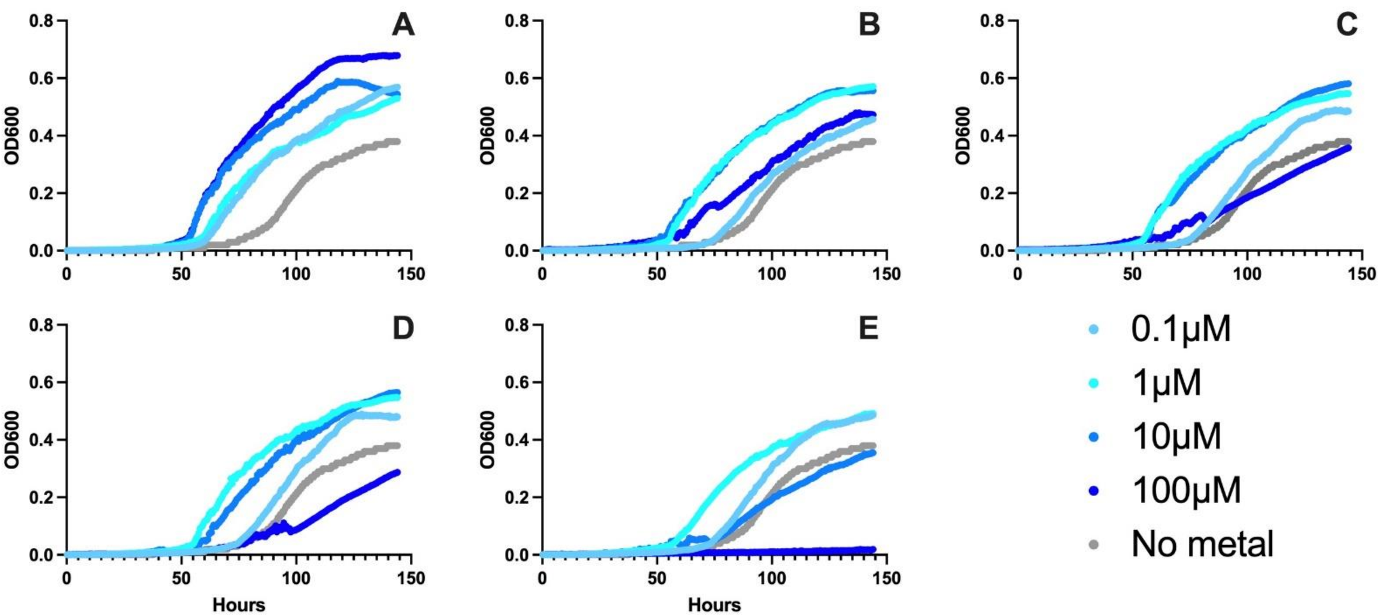

Figure 3: *Methylorubrum extorquens* growth curves under metal concentrations ranging from 0 to 100μM. All samples were cultivated in minimal media on a shaking plate reader over 150 hours. (A) calcium (B) lanthanum (C) cerium (D) neodymium, and (E) europium

3.1.6 *Overall Methylorubrum extorquens growth trends*

Results of growth experiments showed that *M. extorquens* growth was highest in treatments with 1µM Ce^{3+} **(Figure 4)**. Cultures grown with 100 µM Ca^{2+} had the highest cell density in stationary phase **(Figure 3a)**. At concentrations of 100 µM, Lns had a negative effect on *M. extorquens* growth, as growth rate (doubling time = 10.4 hr) and cell density (max OD_{600}: 0.38) of treatments grown with Ce^{3+}, Nd^{3+}, and Eu^{3+} was lower than in control treatments without added metals. This effect was more pronounced for the heaviest Ln tested, Eu^{3+}, as growth began to fall below the control at 10 µM and halted at 100 µM. Treatments with Ca^{2+} showed no negative effects with increasing concentrations. The lag phase of *M. extorquens* grown on 0.1-1 µM Ln was ~53 hours. In treatments grown on 10-100 µM Ln, the lag phase rose to ~70 hours. Treatments with Ca^{2+} did not have the same increase in lag phase time with increasing concentrations **(Figure 3)**.

Previous studies have shown that in many methylotrophs, Nd^{3+} or Pr^{3+} will yield the highest activity in Ln-dependent enzymes [40, 41]. Growth rates at the same concentration are often similar for light Lns and Ca^{2+} [1, 21, 32]. This finding held true in the present experiments when cultures were supplied with 1 µM of the respective metal **(Figure 4)**. More deviation in growth among metals was observed in cultures supplied with 10 µM of the respective metal. Deviation was most severe in cultures grown with Eu^{3+}. Similar behavior was noted with *M. extorquens* strains grown on 10-30 µM Ln^{3+} [54].

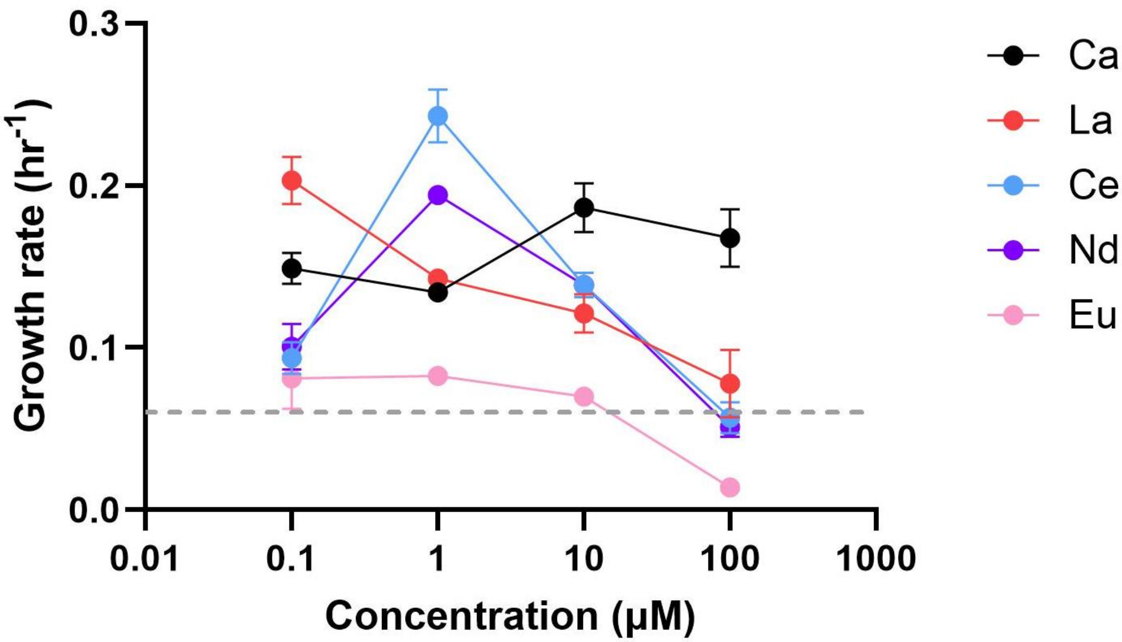

Figure 4: ***Methylorubrum extorquens*** **growth rates at each concentration of the given metal. The gray dashed line represents the average growth rate of *M. extorquens* with no added metal (0.06 hr^{-1}).**

The results presented in this thesis suggest that high concentrations of Lns induce heavy metal toxicity in Ln-dependent methylotrophs. *M. extorquens* is a mesophilic species found in soils containing Ln concentrations in the low nanomolar range. *M. extorquens* displayed maximum growth in conditions of 1-10 µM of light Lns. However, higher concentrations had a negative effect on growth, with the effect most pronounced in heavier Lns. These results suggest that despite their dependence on Lns, high concentrations toxic to mesophilic methylotrophic bacteria. The low bioavailability of Lns in most ecosystems may be beneficial in controlling growth of methylotrophs. The use of LanM and lanthanophores may also help control the amount and type of Lns taken up by the cells to prevent heavy metal toxicity.

3.2 Results of GeneSweeper analysis

3.2.1 Differences in mxaF and xoxF transcription across ecosystems

Results of mining JGI-IMG metatranscriptomic data produced 1,158 total PQQ-MDH hits with a gene sequence length over 1,700 base pairs. Of those, the vast majority contained the DYD sequence, indicating that the XoxF pathway is widely used by methylotrophs over the MxaF pathway **(Figure 5)**. Only two *mxaF* transcripts were found. The first was found in freshwater from Pennsylvania, USA, while the second was found in Tule elk dung pellets from California, USA. Both transcripts belonged to the Alphaproteobacteria class. A previous metagenomic study concluded that *mxaF* genes were restricted to lake ecosystems, while *xoxF* genes were broadly distributed across freshwater and marine ecosystems [55]. This finding is consistent with the present findings as *mxaF* was found in lake ecosystems, but the present results also demonstrate that *mxaF* transcription can be found in soils and animal fecal matter.

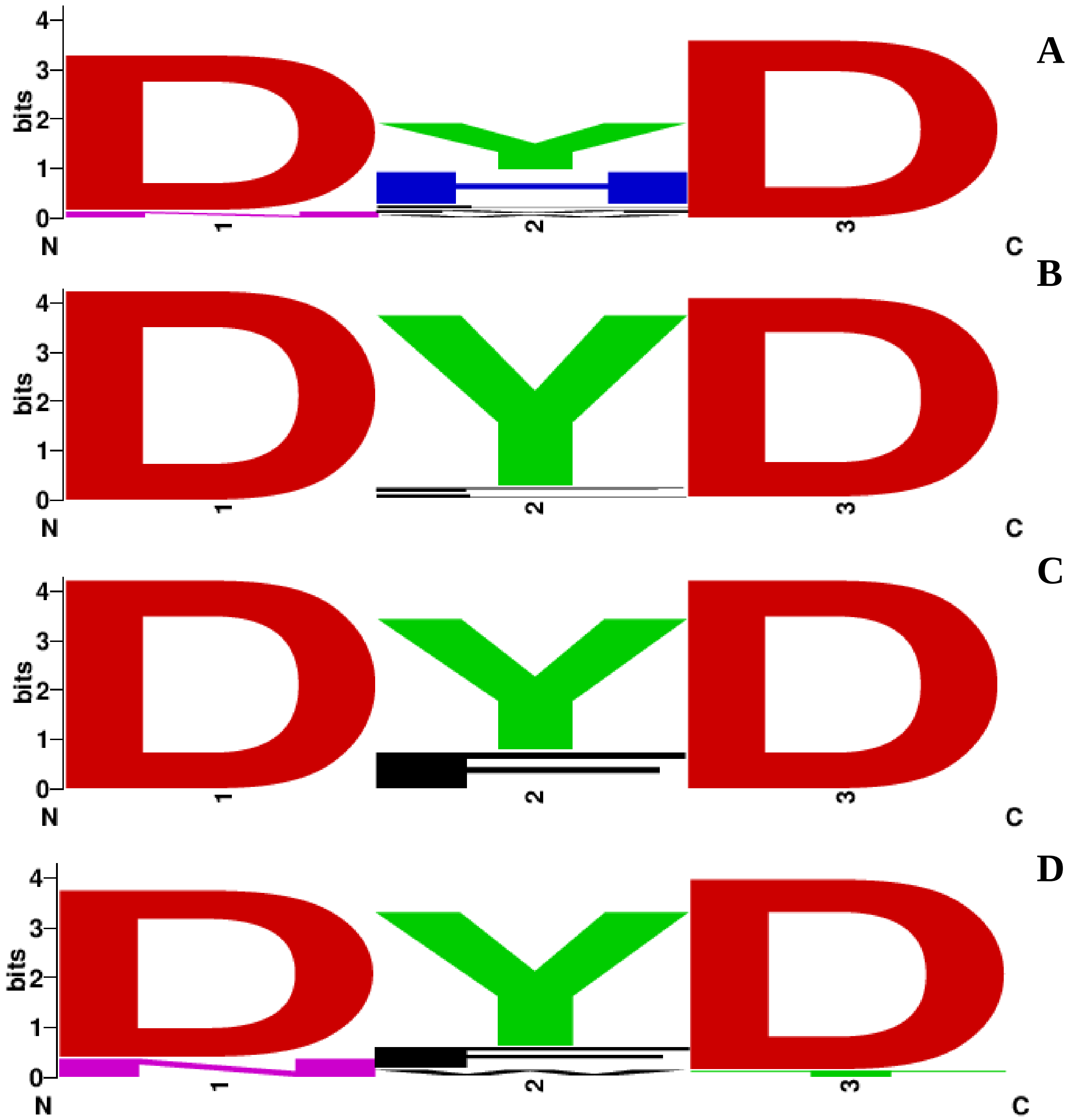

Figure 5: Methanol dehydrogenase amino acid motifs depicted through sequence logos. (A) Acidobacteriota (B) Alphaproteobacteria (C) Burkholderiales (D) Gammaproteobacteria. Sequences were taken from all environments.

The results suggest that the Ln-dependent XoxF pathway is widely utilized among methylotrophs over the MxaF pathway. Previous studies have used metagenomics to probe for methylotrophs and methanotrophs in environments using the *mxaF* and the *xoxF* genes as functional markers [26, 56]. Metagenomic studies have indicated that methylotrophy is ubiquitous across ecosystems and that the *xoxF* gene is found in methylotrophs containing PQQ-MDHs [26]. As many methylotrophs containing both the *mxaF* and *xoxF* genes can switch between the two genes based on environmental Ln concentrations, it was important to consider metatranscriptomic data to determine which gene is most transcribed in ecosystems. Experimental studies have suggested that Ln concentrations in the low nanomolar ranges were needed to trigger the switch in gene expression from *mxaF* to *xoxF* [32, 33]. It is assumed that in most aquatic ecosystems, Ln concentrations would be in the picomolar range, and in soils, Lns are confined to poorly soluble minerals [18, 20]. The results of this study suggest that in most environments, picomolar concentrations or solid sources of lanthanides are sufficient to induce gene expression of *xoxF* and suppress the *mxaF* gene. The prevalence of *xoxF* transcription across ecosystems could also suggest that most methylotrophs in nature only carry the *xoxF* gene and are thus completely reliant on it. The use of metallophores may help sequester Lns from minerals in terrestrial environments [35].

Sequences that did not match the DYD or DYA motifs were also found. Several transcripts showed a DYT motif, with threonine (Thr301, single letter amino acid abbreviation “T”) **(Figure 5)**. Of those from the Alphaproteobacteria class, two transcripts were from soil fungal communities in Rollainville, France, one from water of the Columbia River estuary in Oregon, USA, and one from seawater from the Southern Atlantic Ocean.

The DYT motif was also found in two Gammaproteobacteria transcripts, both from seawater of the Southern Atlantic Ocean **(Figure 5d)**. One Alphaproteobacteria transcript from the Southern Atlantic also showed a DYG motif, with glycine (Gly^{301}, single letter amino acid abbreviation “G”). In the class Gammaproteobacteria, motifs with asparagine (Asn^{303}, single letter amino acid abbreviation “N”) accounted for almost 12% of total Gammaproteobacteria transcripts, forming an NYD or NFD motif **(Figure 5d)**. Gammaproteobacteria transcripts with these motifs were found in marine ecosystems, such as Lake Mendota in Wisconsin, USA, Groves Creek march in Georgia, USA, Monterey Bay in California, USA, and the Southern Atlantic. One Acidobacteriota transcript from Lake Simoncouche, Canada also shared the NYD motif **(Figure 5a)**. Whether these alternative motifs encode for the *xoxF* or *mxaF* genes is still unknown.

3.2.2 Lanthanide-dependent enzyme expression across microbial taxa and ecosystems

Pseudomonadota was the most prominent phylum (>80% of transcripts) detected across all ecosystems. Within Pseudomonadota, Alphaproteobacteria comprised 35%, Gammaproteobacteria comprised 25%, and Burkholderiales formed 22% of transcribed genes. These data are consistent with previous studies, which indicate that XoxF proteins are widely distributed among Proteobacteria, particularly the classes Gammaproteobacteria and Alphaproteobacteria and the order Burkholderiales [26, 57]. The remaining *xoxF* transcripts found belonged to the phyla Gemmatimonadota, Actinomycetota, Acidobacteriota, Nitrososphaerota, Myxococcota, Aquificota, Chloroflexota, Methylomirabilota, Bacteroidota, Verrucomicrobia, and Firmicutes.

Generally, non-saline ecosystems (soils, hot springs, groundwaters, freshwater sediment, and freshwaters) showed higher taxonomic diversity of *xoxF* sequences than saline (seawater, coasts, estuaries, salt marshes, and marine sediments) ecosystems. In saline ecosystems, Pseudomonadota (Alphaproteobacteria, Gammaproteobacteria, and Burkholderiales) dominated, with very low hits for Chloroflexota and Nitrosphaerota found in open seawaters and only one hit for Myxococcota found in salt marshes. Soil ecosystems showed the most diversity of microbial taxa transcribing *xoxF* sequences **(Figure 6).**

Metatranscriptomic data also revealed expression of *xoxF* genes in phyla not generally associated with aerobic methylotrophy, such as Acidobacteriota and some species of Aquificota. Acidobacteriota is a diverse phylum found in ecosystems worldwide, usually associated with plant rhizospheres [58]. This phylum is typically associated with anaerobic methylotrophy and is not known for aerobic methylotrophy facilitated by PQQ-MDH [59]. Aquificota are an extremophilic phylum. The species most closely matched to the present protein sequences, *Hydrogenobacter thermophilus*, utilizes molecular hydrogen, elemental sulfur, or thiosulfate as its energy source [60]. Overall the present findings are consistent with previous metagenomic studies that found that Acidobacteriota and Aquificota contained *xoxF* genes, despite not being known methylotrophs [24, 26, 61]. This metatranscriptomic study shows that *xoxF* genes are being expressed in these phyla, indicating that some species may be capable of methylotrophy or use *xoxF* for another function. For example, an XoxF-MDH homolog, the PQQ-binding thiol dehydrotransferase, has been implicated in sulfur oxidation within the Burkholderiales bacteria *Advenella kashmirensis* [62].

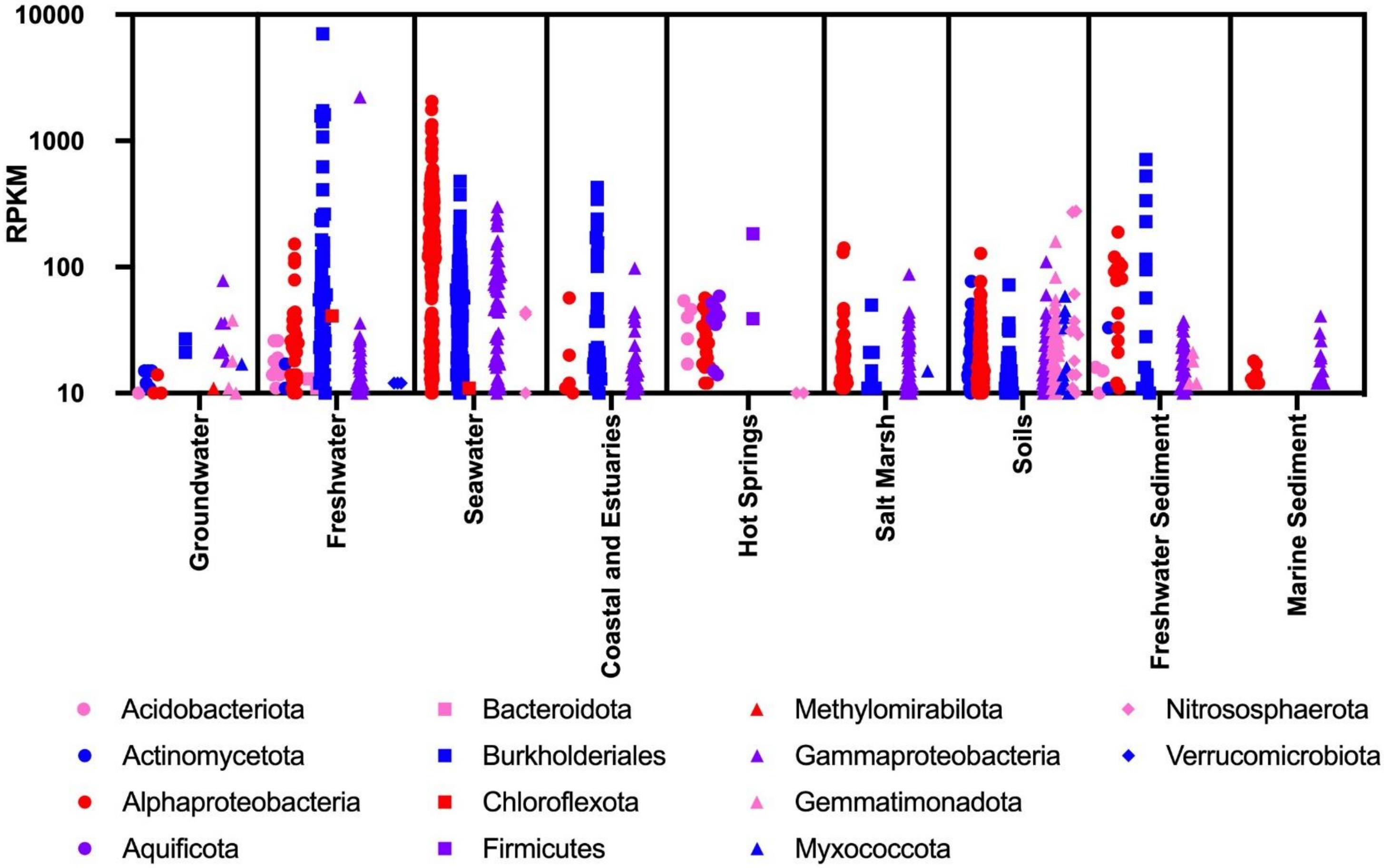

Figure 6: ***xoxF*** **transcript expression by reads per kilobase per million mapped reads (RPKM) across microbial phyla and ecosystem type. The Pseudomonadota phylum is separated into the Alphaproteobacteria and Gammaproteobacteria classes and the order Burkholderiales.**

3.2.3 Ln-dependent MDH enzyme expression by RPKM across ecosystems

In most ecosystem types, the most transcribed (highest RPKM values) MDH genes belonged to members of phylum Pseudomonadota. The highest *xoxF* expression was found for Burkholderiales in freshwater ecosystems, with (up to 7,027 RPKM). In freshwater, freshwater sediment, marine sediment, and coastal and estuarine ecosystems, the order Burkholderiales was responsible for the most *xoxF* expression (314-7,027 RPKM; **Figure 6)**. Previous studies have implicated the Gammaproteobacteria order Methylococcales and genus *Methylobacter* as the most prominent methanotrophs at the Old Woman Creek National Estuarine Research Reserve [63]. Metatranscriptomic data from this site indicate that the order Burkholderiales and genus *Methylophilales* are the most prominent methylotrophs in the ecosystem (11-19 RPKM). This discrepancy could be caused by differences in locations sampled between studies, seasonality, or sampling biases.

The Alphaproteobacteria class was responsible for the highest *xoxF* transcriptions in salt marsh and open sea water ecosystems (142-2,056 RPKM; **Figure 6)**. The Gammaproteobacteria class was responsible for the highest *xoxF* expression in groundwater ecosystems (78 RPKM; **Figure 6)**. In soil ecosystems, the highest *xoxF* expression (277 RPKM) belonged to members of the archaeal phylum Nitrososphaerota **(Figure 6)**, which are chemolithoautotrophic nitrifiers [64]. The prominence of Nitrososphaerota in MDH metatranscriptomic reads also implicates them as important aerobic methylotrophs in soil ecosystems. The phylum Gemmatimonadota had the second highest RPKM measurements at 160 **(Figure 6)**. Nitrososphaerota and Gemmatimonadota represented 3% and 20% of MDH transcripts in the data, respectively.

Hot spring ecosystems from Yellowstone National Park in Wyoming, USA, showed that the phylum Firmicutes was the most prominent methylotroph, with the highest RPKM reading of 183, despite only accounting for 5% of the transcript readings in that ecosystem. The next highest RPKM readings were from the Aquificota phylum at 59% and the Alphaproteobacteria class at 57% **(Figure 6)**. In the hot spring ecosystem, Aquificota represented 24% of transcripts while Alphaproteobacteria represented 53%.

These results further support the importance of Ln-dependent methylotrophy across ecosystems and microbial phyla. The Pseudomonadota phylum was prominent in all ecosystems and was responsible for most of the MDH transcripts across ecosystems. In thermophilic environments such as the hot springs of Yellowstone National Park, extremophiles such as the Firmicutes and Aquificota phyla were the dominant methylotrophs, despite their small presence in comparison to Pseudomonadota. This discrepancy can also be due to differences in the number of samples taken from hot spring water, hot spring runoff, or hot spring sediment in the Yellowstone ecosystem. In soil ecosystems, the archaea Nitrososphaerota phylum and the Gemmatimonadota phylum are the most responsible for methylotrophy. These results imply that methylotrophic archaea play an important role in Ln-dependent carbon cycling.

CHAPTER 4. CONCLUSIONS

4.1 Conclusions

This thesis showed that low amounts of light Lns are important to PQQ-dependent aerobic methylotrophy. *M. extorquens* grew the fastest when supplied with 1 µM Ce^{3+}, in contrast to the expectation that Nd^{3+} would promote the fastest growth based on previous MDH activity studies. *M. extorquens* growth was suppressed when supplied with Ln concentrations of 100 µM, with the effect most pronounced in the heaviest Ln, Eu^{3+}. These results showed that *M. extorquens* in susceptible to heavy metal poisoning by Lns at high concentrations. Metatranscriptomic data showed high transcription of the Ln-dependent MDH across all ecosystems, while Ca-dependent MDH was only transcribed in one freshwater ecosystem. Transcription of *xoxF* was also found in archaea in many bacteria phyla not associated with aerobic methylotrophy. These results further illustrate the importance of Ln-dependent methylotrophy to many ecosystems and bacteria.

4.2 Future Work

Future work is needed for growth studies to better understand the highest concentration of Lns *M. extorquens* can grow with before growth begins to decline. Growth studies will also be performed with different methylotrophs such as *M. fumariolicum* to determine if methylotrophs from environments with high amounts of dissolved Lns experience toxicity to high concentrations of Ln to the same degree as *M. extorquens*.

Future studies will also be performed to better understand if the amino acid sequences that did not match the DYD or DYA motifs encode for *xoxF* or *mxaF* genes.

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