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Methylotrophs and Methylotroph Communities

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Lanthanides in Methylotrophy

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Abstract

Lanthanides were previously thought to be biologically inert owing to their low solubility; however, they have recently been shown to strongly impact the metabolism of methylotrophic bacteria. Leading efforts in this emergent field have demonstrated far-reaching impacts of lanthanide metabolism in biology; from the identification of novel roles of enzymes and pathways dependent on lanthanide-chemistry to the control of transcriptional regulatory networks to the modification of microbial community interactions. Even further, the recent discovery of lanthanide-dependent enzymes associated with multi-carbon metabolism in both methylotrophs and non-methylotrophs alike suggests that lanthanide biochemistry may be more widespread than initially thought. Current efforts aim to understand how lanthanide chemistry and lanthanide-dependent enzymes affect numerous ecosystems and metabolic functions. These efforts are likely to have a profound impact on biotechnological processes involving methylotrophic communities and the biologically mediated recovery of these critical metals from a variety of waste streams while redefining our understanding of a fundamental set of metals in biology.

Introduction: lanthanides in methylotrophy

In the last few decades, the study of methylotrophy has become an exciting and expanding area of research with applications in carbon capture, chemical production, bioremediation, and biomining (Chistoserdova, 2018). Laboratories across the world are engineering methylotrophic bacteria to produce value-added chemicals from single-carbon compounds, turning atmospheric pollutants like methane and methanol into green chemicals like biofuels, terpenoids, and biodegradable plastics (Höfer *et al.*, 2011; Hwang *et al.*, 2014; Ochsner *et al.*, 2015; Pfeifenschneider *et al.*, 2017). Yet others in the field are investigating the abilities of methylotrophs to promote agricultural growth, degrade toxicants like methylmercury, or recover critical metals for our technologies in environmentally friendly and sustainable ways (Iguchi, 2015; Kumar *et al.*, 2016; Martinez-Gomez, 2016; Lu *et al.*, 2017). It is clear from decades of study that not all methylotrophs operate in the same way in terms of their core central metabolic pathways or their transcriptional regulation. Even among their shared alcohol oxidation enzymes, differences in substrate specificities, cofactor ranges, and catalytic efficiencies are observed. With this complexity comes breadth, flexibility, and challenges in methylotroph bioengineering efforts. By better understanding methylotrophic metabolism and its regulation in a variety of organisms, the applications for methylotrophy can be advanced at a rapid pace. Shared goals may be realized. For example, it may soon become possible to capture methane, produce value-added chemicals, and biomine for critical metals, all in a single process.

A key step in methylotrophy is the oxidation of methanol to either formaldehyde or formate, which is carried out by different enzymes including methanol dehydrogenase (MDH) and alcohol oxidase depending on the specific methylotroph. In 2011, the first evidence was published that suggested that some types of MDHs use lanthanides (Ln) instead of calcium (Ca) as cofactors (Hibi *et al.*, 2011; Fitriyanto *et al.*, 2011) paving the way for novel discoveries surrounding the biochemical use of these elements and allowing cultivation of organisms previously thought uncultivable (Pol *et al.*, 2014; Skovran and Martinez-Gomez, 2015; Vekeman *et al.*, 2016; Del Rocío Bustillos-Cristales *et al.*, 2017; Howat *et al.*, 2018).

The Ln family consists of 15 elements ranging from lanthanum (La) to lutetium (Lu). Along with scandium (Sc) and yttrium (Y), these metals are commonly referred to as rare earth elements or REE. REE have been called 'the seeds of technology' since they are essential components of many technological devices including smartphones, computers, solar cells, batteries, wind turbines, lasers, and optical glasses due to their super magnetic properties and unique chemistries (Zepf *et al.*, 2014; Martinez-Gomez *et al.*, 2016). The term REE is misleading as these metals can be as abundant as other well-known industrial metals such as copper or zinc (Tyler, 2005). However, they are highly insoluble and scarce in pure form. Because of these properties, their inherent use in biological processes was thought implausible. It has now been demonstrated that some methylotrophs are dependent upon REE for growth as it is a cofactor for XoxF-type MDHs and ExaF-type ethanol dehydrogenases (EtDHs) (Pol *et al.*, 2014; Good *et al.*, 2016; Del Rocío Bustillos-Cristales *et al.*, 2017; Howat *et al.*, 2018). And for characterized methylotrophs that contain both Ca- and La-dependent MDHs, the Ln-dependent XoxF-type MDH is often preferred over its Ca counterpart as transcription of the Ca-dependent enzyme is repressed by Ln in multiple studied systems (Farhan UI Haque *et al.*, 2015; Chu and Lidstrom, 2016; Gu *et al.*, 2016; Vu *et al.*, 2016; Masuda *et al.*, 2018).

In many ecosystems, the concentrations of REE are low; in the pico- to nanomolar range (Byrne and Sholkovitz, 1996; Pol *et al.*, 2014), yet are likely high enough to support methylotrophic growth. For perspective, batch culture growth of Ln-dependent *Methylobacterium extorquens* AM1 strains can reach maximum density ($OD_{600} = 1.6$ in culture tubes) with 100 nM La, though maximum growth rate and density are achieved with 1 μM La (Vu *et al.*, 2016). Some ecosystems like the acidic volcanic mud pots from the Solfatara contain concentrations of soluble REE in the low micromolar range (Pol *et al.*, 2014). Additionally, environments can be artificially induced to contain increased concentrations of REE. For example, REE are found in different types of fertilizers, and plants have been shown to effectively concentrate these elements (Gao *et al.*, 2003; Hu *et al.*, 2004, Nakagawa *et al.*, 2012). The addition of REE to the soil through fertilization may serve to promote the growth of associated Ln-dependent methylotrophs in the phyllosphere.

One question that remains is how do methylotrophic bacteria obtain these insoluble Ln if they are not located in acidic environments such as volcanic mudpots where soluble Ln are abundant? The capacity for solubilization and transport must exist as methylotrophs such as *M. extorquens* AM1 have even been shown to obtain Ln from computer hard drive magnets for growth (Martinez-Gomez *et al.*, 2016).

With the challenges that methylotrophs face in acquiring Ln, why would they have evolved enzymes that require these elements? Before the discovery of Ln-containing enzymes, it was hypothesized that Ln would have catalytically superior properties compared with Ca when acting as a Lewis acid, as Ln are more potent Lewis acids than Ca (Lim and Franklin, 2004). It has been corroborated that this is the case for some pyrroloquinoline quinone (PQQ)-dependent MDHs (Pol *et al.*, 2014; Lumpe *et al.*, 2018).

In Gram-negative bacteria, methanol utilization begins with oxidation of methanol in the periplasmic space by PQQ-dependent MDHs that are either Ca-dependent (MxaFI), or Ln-dependent (XoxF) enzymes. XoxF-type MDHs are more abundant in characterized environmental communities and often exist in multiple copies within methylotroph genomes (Vorobev *et al.*, 2013; Keltjens *et al.*, 2014; Beck *et al.*, 2015). Intriguingly, XoxF-type MDHs have also been found in non-methylotrophs though their functions are mostly unknown (Vorobev *et al.*, 2013; Chistoserdova, 2016). XoxF has been phylogenetically classified into five major clades

(XoxF1 to XoxF5), but additional metagenomic sequence analyses indicate that *xoxF*-like genes are more widespread than previously thought suggesting the potential for additional XoxF clades (Chistoserdova, 2011; Keltjens *et al.*, 2014; Taubert *et al.*, 2015; Chistoserdova, 2016; Chistoserdova and Kalyuzhnaya, 2018). Detailed biochemical studies that facilitate comparisons between the different types of XoxF MDHs are limited (Pol *et al.*, 2014, Wu *et al.*, 2015; Good *et al.*, 2018; Huang *et al.*, 2018; Jahn *et al.*, 2018) so it is yet unknown if XoxF MDHs from different clades will exhibit distinct and unique characteristics and roles.

Ln such as La and cerium (Ce) are predicted to have similar bond lengths to PQQ and catalytic amino acids when incorporated into MDHs (Pol *et al.*, 2014). However, the biochemical versatility and promiscuity of substrates and cofactors among the XoxF MDHs is slowly emerging (Bogart *et al.*, 2015; Pol *et al.*, 2014; Good *et al.*, 2018; Huang *et al.*, 2018; Jahn *et al.*, 2018; Lumpe *et al.*, 2018). The possibility of different binding and positioning properties for different Ln may allow production of different products. For example, some XoxF-type MDHs have been suggested to produce formate directly from methanol rather than formaldehyde (Pol *et al.*, 2014). Structural, biochemical, and mechanistic differences of the currently characterized PQQ- and Ln-dependent enzymes reveal metabolic flexibility that could explain the diversity of XoxF-type and ExaF-type alcohol dehydrogenases present in methylotrophic and non-methylotrophic bacteria.

Ln-dependent alcohol dehydrogenases are widespread and have ample metabolic flexibility

Overview of Ln-dependent methanol dehydrogenases

So far, the only known Ln-dependent enzymes are PQQ-dependent alcohol dehydrogenases (Chistoserdova, 2016). Though very divergent when compared with each other, they also share common features such as amino acid sequences and spatial orientation in the active site. Specifically, the amino acids that coordinate PQQ are conserved (Pol *et al.*, 2014; Bogart *et al.*, 2015; Jahn *et al.*, 2018; Lumpe *et al.*, 2018; McSkimming *et al.*, 2018). One feature that has been suggested as structurally important for coordinating Ln instead of Ca is an additional Asp (Asp^{301}, numbering according to XoxF from *Methylacidiphilum fumariolicum*) two positions downstream from a catalytic Asp (Asp^{299}) (Keltjens *et al.*, 2014; Bogart *et al.*, 2015) (Fig. 5.1).

Overall, the protein backbone of Ln-MDHs resembles an eight propeller-like peptide with PQQ buried inside its hydrophobic pocket (Pol *et al.*, 2014; Jahn *et al.*, 2018). In addition to the catalytic Asp, two Cys residues that form a disulfide bond reside next to PQQ and are likely essential for electron transfer to cytochrome c_L (XoxG; Ghosh *et al.*, 1994; Yu *et al.*, 2017; Zheng *et al.*, 2018). From the few crystal structures and DFT calculation studies that have been done, there is a consensus that the Ln atom coordinates with PQQ and functions as a Lewis acid to facilitate electron extraction from methanol by PQQ (Bogart *et al.*, 2015; Deng *et*

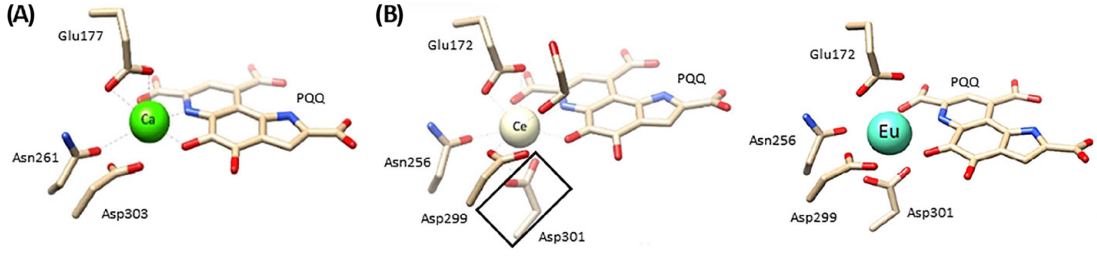


Figure 5.1 Metal coordination for PQQ-dependent alcohol dehydrogenases in the active site. (A) Ca-MxaF from *M. extorquens* AM1 [PDB ID: 1W6S (Williams, *et al.*, 2005)]. (B) Ce-XoxF and Eu-XoxF from the *M. fumariolicum* SolV strain [PDB ID: 4MAE (Pol *et al.*, 2014)]. The additional aspartate that has been suggested to be necessary for Ln coordination is depicted inside a black box. The catalytic aspartate coordinating the Ln is also highlighted (Asp299). In addition, Ce-XoxF shows the catalytic cysteines necessary for electron transfer to the cytochrome.

al., 2018; Lumpe *et al.*, 2018; McSkimming *et al.*, 2018). The mechanism of methanol oxidation also includes a proton abstraction from the alcohol by the catalytic Asp residue where the Ln assists the reaction as a Lewis acid. Some debate exists regarding whether a hydride transfer or an addition/elimination mechanism takes place (Leopoldini *et al.*, 2007; Lumpe *et al.*, 2018; McSkimming *et al.*, 2018). However, two recent studies strongly suggest the hydride transfer mechanism (Lumpe *et al.*, 2018; McSkimming *et al.*, 2018).

Different Ln can be utilized as cofactors for Ln-dependent alcohol dehydrogenases. Owing to Ln contraction throughout the series, there is a decrease in ionic radius that correlates with an increase in Lewis acidity suggesting that smaller, heavier Ln would enhance the catalytic efficiency and oxidation capacity of the enzymes. Currently, only a few kinetic studies of purified MDHs coordinated with different Ln are available, and each demonstrated a correlation of increasing MDH catalytic efficiency with increasing coordination throughout the Ln series up to neodymium (Nd) (Pol *et al.*, 2014; Good *et al.*, 2018; Jahn *et al.*, 2018; Lumpe *et al.*, 2018). However, coordination with Ln heavier than Nd, such as europium (Eu) or Lu, did not provide higher catalytic efficiency (Jahn *et al.*, 2018; Lumpe *et al.*, 2018). These results suggest that the Lewis acidity of the Ln is not the only chemical property influencing activity. It is likely that the Ln size may affect the metal interaction with the catalytic amino acid, PQQ, and/or the substrate (Fig. 5.1).

In *M. extorquens* AM1, incorporation of Ln into XoxF also appears to be less efficient throughout the series. Apo-XoxF (no metal incorporated into XoxF) was not found when purified from cells grown in the presence of La, but in the presence of Nd, ≈ 50% of the recovered XoxF was in the apo-form (Good *et al.*, 2018). Similarly, for *M. fumariolicum*, apo-XoxF is not obtained when grown in the presence of La, but in the presence of Eu, only 70% of XoxF is fully occupied (Jahn *et al.*, 2018). It is yet unknown whether Ln are unequally obtained and transported into the cell, contributing to the decreased occupancy of XoxF with the heavier Ln, or whether decreased occupancy is due to decreased insertion of the Ln into XoxF once inside the cell, or whether this phenomenon is due to a combination of both possibilities.

Numerous reports have shown differences in the distance between the catalytic Asp and metal cation (tested so far with Ca, Ce, and Eu), highlighting the flexibility of this residue (Leopoldini *et al.*, 2007; Jahn *et al.*, 2018; Lumpe *et al.*, 2018). Furthermore, differences in substrate affinities and V_{max} values have also been observed when different Ln were coordinated with the enzyme (Good *et al.*, 2018; Lumpe *et al.*, 2018). It has also been shown that in some cases, Ln can influence the substrate orientation when bound to the protein (McSkimming *et al.*, 2018). The ability of these enzymes to coordinate different Ln provides flexibility in the use of a wide range of alcohols and aldehydes available in the environment for which concentration and availability can fluctuate greatly.

The oxidation capacity of the Ln-dependent MDHs is also relevant. XoxF from *M. fumariolicum* has a higher oxidation capacity than XoxF from *M. extorquens* AM1 such that the final product of methanol oxidation appears to be formate in the former case, while formaldehyde is the product in the latter case (Pol *et al.*, 2014; Good *et al.*, 2018). It has been shown *in vitro* that XoxF from *M. extorquens* AM1 is able to efficiently use formaldehyde as a substrate but *in vivo*, formaldehyde is still being produced and accumulates at similar rates whether Ln are present or not (Good *et al.*, 2018). These findings highlight the fact that the current assays used to define the activities and the kinetic parameters of alcohol/aldehyde dehydrogenases may not reflect physiological realities. *In vivo*, PQQ-dependent MDHs are coupled to natural electron acceptors (typically cytochromes) and other accessory proteins that can influence the oxidation capacities of these enzymes (Huang *et al.*, 2018; Zheng *et al.*, 2018).

Metabolic consequences of differing methanol oxidation capacities

A major consequence of oxidizing methanol directly to formate is the loss of NAD(P)H which is produced to store electrons when oxidizing formaldehyde to formate via either the tetrahydromethanopterin or the glutathione-dependent formaldehyde oxidation pathways (Barber *et al.*, 1996; Vorholt *et al.*, 1998; Hagemeier *et al.*, 2000). This is particularly important for methanotrophs that require reduced quinone equivalents for activation of methane in addition to the requirement for NAD(P)H in biosynthesis (Cook and

Shiemke, 2002; Choi *et al.*, 2003; Shiemke *et al.*, 2004). Methanotrophs may instead need to rely on additional reduced cytochromes or on a reversed electron transport complex (e.g. Complex III bc_1) driven by proton motive force to reduce quinones. Alternatively, redistribution of carbon flux through the pentose phosphate pathway or increasing transhydrogenase activity may serve to balance reducing equivalents.

M. extorquens AM1 Ln-dependent XoxF and ExaF enzymes produce both formaldehyde and formate, respectively, as final products when using methanol as substrate. It is interesting to note that for *M. extorquens* AM1 grown with methanol and exogenous La, the tetrahydromethanopterin pathway is essential, but 2- to 3-fold down-regulation of the genes encoding three of the four formate dehydrogenases occurs, and less CO_2 (around 18%) is released by cultures compared with growth with methanol in the absence of La. This suggests that *M. extorquens* AM1 rewires its metabolic network to reduce formate oxidation when Ln are present and alters NAD(P)H production (Good *et al.*, 2018). Further examples characterizing both final products of different types of XoxF, and how different strains may balance differences in catabolism/anabolism derived from higher methanol oxidation capacities are currently needed.

Ln-dependent ethanol dehydrogenases

Ln dependency for PQQ alcohol dehydrogenases was recently expanded to include EtDHs. The first reported Ln-EtDH was discovered in *M. extorquens* AM1, a homologue 57% identical to ExaA from *Psuedomonas aeruginosa* that has been named ExaF to denote its dependency on Ln as a cofactor for catalytic activity and to distinguish it from Ca-dependent enzymes. (Görisch and Rupp, 1989; Good *et al.*, 2016; Vu *et al.*, 2016). Like XoxF-type MDHs, ExaF has a second Asp residue coordinating the Ln. ExaF is the most efficient PQQ-EtDH described to date; an increase in activity of 1,000-fold was observed when the enzyme was purified with La when compared with Ca-ExaF. Similar to XoxF from *M. fumariolicum*, ExaF oxidizes ethanol and methanol to acetate and formate, respectively (Good *et al.*, 2016).

A second representative of the PQQ Ln-EtDHs, PedH, was discovered in a non-methylotroph, *P. putida* KT2440 (Wehrmann *et al.*, 2017). PedH is 49% identical to ExaA and 63% identical to ExaF. PedH has a relatively high catalytic efficiency for ethanol, but its K_M for ethanol is in the micromolar range, around 200-times higher than that seen for ExaF. Further, when PedH is compared with its Ca-dependent counterpart, PedE, an approximate 19% decrease in catalytic efficiency for ethanol is observed. An even greater reduction in efficiency is observed with acetaldehyde (Wehrmann *et al.*, 2017). Hence, the only two Ln-dependent EtDHs identified as of now show important kinetic differences after Ln coordination. Detailed structural studies and DFT calculations of these two PQQ-dependent EtDHs can highlight the Ln influence on substrate orientation and substrate affinity. Importantly, it has become apparent that Ln-biochemistry and its role in metabolism as a whole is not unique to one-carbon metabolism.

Overview of the lanthanide- or rare earth-switch

In many methylotrophic organisms, the presence of exogenous Ln results in up-regulation of the Ln-dependent MDH, *xoxF*, and repression of the Ca-dependent MDH, *mxaFI* (Farhan UI Haque *et al.*, 2015; Skovran and Martinez Gomez, 2015; Chu and Lidstrom 2016; Gu *et al.*, 2016; Vu *et al.*, 2016). This differential regulation of the Ca- and Ln-dependent MDHs has been termed the Ln- or rare earth-switch. In multiple methylotrophs, Ln-dependent growth and expression appears limited to La, Ce, Nd, and praseodymium (Pr) with severely limited growth and mediation of the Ln switch also facilitated by samarium (Sm) in some cases (Farhan UI Haque *et al.*, 2015; Chu and Lidstrom 2016; Gu *et al.*, 2016; Vu *et al.*, 2016; Huang *et al.*, 2018; Lv and Tani, 2018; Masuda *et al.*, 2018). However, more recent studies show that some organisms such as *Methylotenera mobilis* and *M. fumariolicum* SolV are capable of supporting Ln-dependent methanol oxidation with heavier Ln such as europium (Eu) and gadolinium (Gd) (Huang *et al.*, 2018; Jahn *et al.*, 2018). However, kinetic studies using Eu-XoxF from SolV suggest that the catalytic efficiency of methanol oxidation decreases when heavier Ln than Nd are bound in the active site (Jahn *et al.*, 2018).

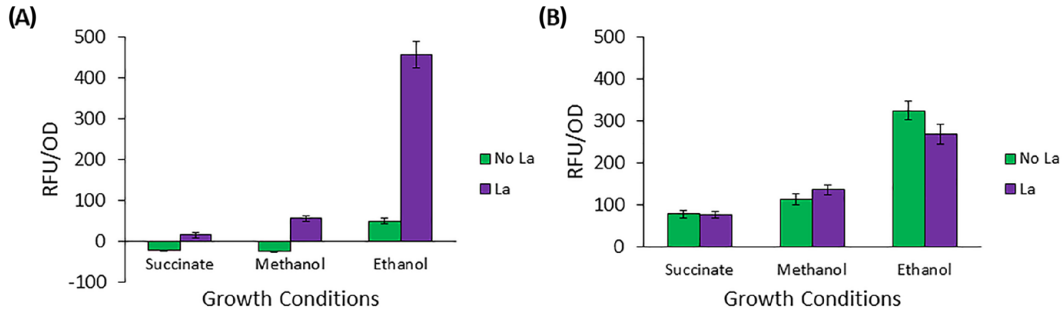
The study of the regulatory network controlling the Ln switch is complicated by a variety of factors. The regulators that control this switch differ in different organisms lending it difficult to transfer knowledge about this regulation across the methylotrophic community. In some methylotrophs, additional signals or nutrients and metabolic processes affect regulation of the Ln-switch, yet how these signals contribute to changes in gene expression is not yet understood. Direct binding to promoters by regulators involved in the switch has not been demonstrated lending it unclear whether their involvement is direct or indirect. Finally, in many methylotrophs, there are multiple copies of the *xoxF* genes, some of which only appear to be expressed *in situ* or in co-culture. It is not yet clear why different versions of XoxF would be required or preferred under different growth conditions. It has even been suggested that microorganisms in communities can excrete signals that affect the Ln switch in their methanotrophic neighbours causing methanol to be released as a carbon source for the methylotrophs present in the same community (Krause *et al.*, 2017). Understanding how the Ln switch is controlled, the regulators involved in the switch, and the signals that control the switch is a nascent area of study.

ExaF: A new member of the lanthanide switch

Good *et al.* (2016) reported that ExaF from *M. extorquens* is a Ln- and PQQ-dependent ethanol dehydrogenase, but that it can also oxidize methanol, albeit with lesser efficiency. Subsequently, RNAseq results from Okayam University in Japan determined that two putative PQQ-dependent non-XoxF MDHs, including c07235 which shares 37% identity with ExaF, are also differentially regulated by Ln in *Methylobacterium aquaticum* Strain 22A (Masuda *et al.*, 2018). In *M. extorquens* AM1, RNAseq work from Michigan State University showed that expression of *exaF* is also upregulated by La adding new members to the Ln switch (Good *et al.*, 2018).

In *M. extorquens* AM1, *exaF* is encoded downstream of the c-type cytochrome *exaG* (*pedF*), and the periplasmic binding protein *exaJ*, which shares sequence similarity with *xoxJ*. A 292 bp gap exists between *exaJ* and *exaF* suggesting the possibility that two independent promoters may be used to control expression of the *exa* genes. To investigate the expression of the *exa* genes, transcriptional reporter fusion assays were used which fused the fluorescent reporter Venus (Nagai *et al.*, 2002) to the predicted *exaF* and *exaGJ* promoter regions (Fig. 5.2C). Expression was measured in the presence and absence of 2 μM La with either succinate, methanol, or ethanol as carbon sources (Fig. 5.2A and B). Expression from the *exaF* promoter was upregulated by exogenous La in both succinate and methanol media and further upregulated in ethanol medium (15.0 ± 6.2 RFU/OD in succinate, 55.3 ± 6.2 RFU/OD in methanol, and 456.0 ± 31.5 RFU/OD in ethanol in the presence of La). However, expression from the *exaF* promoter was below background levels in the absence of Ln when succinate or methanol were the carbon sources, and 9.4-fold reduced expression from the *exaF* promoter was seen in the absence of La when ethanol was the carbon source compared with when La was present (Fig. 5.2A). Of note, background levels of fluorescence are due to expression read-through of the tetracycline resistance gene encoded on the transcriptional reporter fusion plasmid, pAP5 (Skovran *et al.*, 2011; Vu *et al.*, 2016). Depending on the transcriptional reporter fusion construct, a negative value (below background) may indicate that repression of the cloned promoter region is occurring or that the cloned promoter region contains a transcriptional terminator from an upstream gene. Expression from the *exaGJ* promoter was upregulated by ethanol but exogenous Ln did not appear to have a marked effect on expression (Fig. 5.2B).

These studies support the findings by Good *et al* that ExaF is Ln-dependent and that ethanol is the preferred substrate in *M. extorquens* AM1 (Good *et al.*, 2016). These data also suggest that expression of *exaF* is controlled by more than just Ln as providing ethanol as the carbon source increased expression 8.2-fold compared with when methanol was the carbon source in the presence of La. The presence of exogenous La and ethanol appear to have a synergistic effect as expression of *exaF* is highly induced only when both La and ethanol are present. It is intriguing that expression from the *exaGJ* promoter does not appear to be regulated by Ln and that significant expression occurs under all conditions tested. The fact that expression from the



(C)

Plasmid or Primer	Description or Sequence (5'-3')	Reference
pAP5	Promoterless *venus* fusion (from pCM62) (Tc^r)	55
pHV3[a]	pAP5 containing the *exaF* promoter region (Tc^r)	This study
pGS2[b]	pAP5 containing the *exaGJ* promoter region (Tc^r)	This study
p*exaF*_1139aclIbeg	ATCGAACGTTCCGCAACGAGCAGATCTTCCTGA	This study
p*exaF*endEcoRI	GATCGAATTCACCTTGTCCTTGTAGAGGGCGGC	This study
p*exaG*_1137aclIbeg	ACGTAACGTTTCCTCGGCCGGCACATTCAGAAC	This study
p*exaG*endEcoRI	ATGAATTCTAAGGATTCTCCTCGCGCCATGT	This study

Abbreviations: ^{r} = resistant; Tc, tetracycline. [a]Plasmid constructed by Huong N. Vu. [b]Plasmid constructed by Gabriel A. Subuyuj.

Figure 5.2 *exaF* expression is controlled by the Ln switch and expression from the *exaF* and *exaGJ* promoters is upregulated by ethanol. Expression from the *exaF* (A) and *exaGJ* (B) promoters was measured using the modified yellow fluorescence protein, Venus as a fluorescent reporter (Nagai *et al*., 2002). Strains were grown in MP minimal medium with 0.4% succinate, 0.5% methanol, or 0.3% ethanol as the carbon source with (purple bars) and without (green bars) 2 μM lanthanum as previously described (Vu *et al*., 2016). Expression is reported as relative fluorescence units (RFU) per OD at 600 nm. Data are the average of biological triplicates and the error bars represent one standard deviation from the mean. Background expression levels from the promoterless venus fusion vector construct (pAP5) were subtracted from the experimental values for each condition and strain tested. Primers and plasmids used in this study are shown in (C).

exaGJ promoter is upregulated by ethanol suggests that ExaG and ExaJ may work with ExaF during ethanol oxidation but this has not yet been demonstrated.

Master regulators of the lanthanide switch: MxaYB and MxbDM?

Understanding the complexity of factors that control the switch is in its infant stages. Multiple two-component systems have been described that are required for expression of the *mxa* and *xox* genes. In *M. extorquens* AM1, the regulatory network controlling expression of the *mxa* and *xox1* operons involves at least two two-component systems, MxbDM and MxcQE, and an additional orphan response regulator, MxaB. The MxcE, MxaB, and MxbM response regulators are all required for expression of the *mxa* operon while only MxbM is required for the repression of the *xox1* operon (Morris and Lidstrom, 1992; Xu *et al*., 1995; Springer *et al*., 1997, 1998; Skovran *et al*., 2011). In addition, XoxF plays an unknown regulatory role in the network as the *xoxF* genes are required for expression of the *mxa* operon and for repression of the *xox1* operon (Skovran *et al*., 2011; Vu *et al*, 2016). XoxF is also likely to be required for *mxa* expression in *M. aquaticum* Strain 22A as *xoxF* mutants are unable to grown in methanol medium lacking Ln even though *mxaF* is encoded in the genome and is the predominant enzyme in the absence of Ln (Masuda *et al*., 2018).

In the methanotroph *Methylomicrobium buryatense* 5GB1C, a functional Ln switch exists yet homologues of the MxcQE and MxbDM two-component systems are absent. Additionally, growth patterns suggest that XoxF is not required for expression of the *mxa* genes in *M. buryatense,* as growth of a *xoxF* mutant is not impaired in methanol medium lacking Ln (Chu and Lidstrom, 2016). These discrepancies highlight the diversity that may exist in the regulatory networks that control the Ln switch in different methylotrophs, as described in more detail below.

Methylomicrobium buryatense 5GB1C

Work from the University of Washington demonstrated that deletion of the *mxaB* response regulator gene results in a growth defect similar to the loss of *mxaF* or *mxaI* when grown in the absence of Ln, suggesting that MxaB is required for expression of the MxaFI-MDH (Chu and Lidstrom, 2016). qRT-PCR data showed that expression of *mxaFI* was reduced ≈1000-fold in an *mxaB* mutant strain while *xoxF* was upregulated 30-fold, signifying that MxaB plays a major role in the Ln switch (Chu and Lidstrom, 2016). *mxaB* expression is also repressed by Ln similar to the two-component systems that are involved in the switch in *M. extorquens* AM1 (Good *et al.*, 2018). In this same study from the University of Washington, second site suppressors of *xoxF* were isolated which restored growth due to induced expression of *mxaFI* in methanol medium containing La. These suppressor mutations were subsequently mapped to the sensor kinase, MxaY (Chu *et al.*, 2016). By changing a Glu to Gly at position 147, the MxaY sensor could be constitutively activated resulting in expression of the *mxaFI* MDH regardless of Ln presence or absence. Intriguingly, both loss of *mxaY* and constitutive activation of MxaY in the presence of La result in a significant growth defect (doubling time ≈7.5 h compared with 2.4 h in WT), suggesting that MxaY has an important regulatory role during Ln growth beyond regulating MDH gene expression.

Methylobacterium extorquens AM1

In *M. extorquens* AM1, the regulatory network controlling differential expression of the *mxa* and *xox1* genes is complex, involving at least two sensor kinases, MxbD and MxcQ, and three response regulators, MxbM, MxcE, MxaB, though neither the phosphorylation state nor direct binding of these regulators to DNA has been shown (Morris and Lidstrom, 1992, Xu *et al.*, 1995; Springer *et al.*, 1997, 1998; Skovran *et al.*, 2011). It has been suggested that the MxcQE and MxbDM two-component systems controlling *mxa* and *xox1* expression may work in a cascade with MxcQE activating expression of *mxbDM* (Springer *et al.*, 1997). To add an additional layer of complexity, the XoxF-type MeDH is required for expression of the MxaFI-type MeDH and the MxbDM two-component system when cells are grown in the absence of La, and also for repression of itself (Skovran *et al.*, 2011; Vu *et al.*, 2016). Vu *et al.* (2016) have hypothesized that Ln sensing may be done through a sensor kinase such as MxcQ or MxbD using apo-XoxF rather than sensing Ln directly. Similar to work with *M. buryatense* 5GB1C, second site suppressors of *xoxF* were isolated (but in the absence of Ln) which restored wild-type levels of expression from the *mxa* promoter (Skovran *et al.*, 2011). Complementation and sequence analyses mapped these suppressor mutations to the MxbD sensor kinase. Strains containing a suppressor mutation in MxbD no longer require XoxF for expression of the *mxa* genes in the absence of Ln (Bang Luong and Elizabeth Skovran, San Jose State University, unpublished data).

Taken together, work with *M. buryatense* 5GB1C and *M. extorquens* AM1 suggests that MxbD and MxaY may serve analogous roles in the Ln switch as the main Ln sensors. If the hypotheses from these authors are correct, that MxaY senses Ln directly, and MxbD may instead sense apo-XoxF, this may help to explain why divergent regulatory systems have evolved as the signal for Ln presence is different. As these theories have yet to be demonstrated via binding interaction studies, it is only possible to speculate at this point.

Regulation of the lanthanide switch in non-methylotrophs

To date, characterization of a Ln switch has only been reported in one non-methylotroph, *P. putidia* KT2440 (Werhmann *et al.*, 2018). In this organism, the Ln switch controls differential expression of *pedH* and *pedE,* which encode the Ln- and Ca-dependent EtDHs that facilitate oxidation of

a broad range of volatile alcohols and aldehydes (Werhmann *et al*., 2017). In the absence of *pedH*, strains are unable to grow in the presence of Ln with phenylethanol as a carbon source. Using an adaptive evolution approach, Werhmann *et al*. (2018) generated and isolated *pedH* suppressor mutant strains that could grow in the presence of Ln. These strains contained a broken Ln switch which allowed expression of *pedE* which encodes the Ca-dependent EtDH enzyme. The suppressor mutations mapped to *pedS*2 which encodes a sensor histidine kinase with 25% amino acid identity to MxaY. Adjacent to *pedS* is a LuxR-type response regulator, *pedR2*. Further genetic analyses suggested that PedR2 is the response regulator mediating the Ln switch and identified Asp 53 as the site of phosphorylation. The authors propose a model of Ln-dependent regulation where in the absence of Ln, PedS2 phosphorylates PedR2 which functions as both a strong activator of *pedE* expression and a repressor of *pedH* expression. When Ln are present, PedS2 kinase activity decreases such that *pedH* expression occurs while *pedE* expression is repressed (Werhmann *et al*., 2018).

Copper as an override control of the lanthanide switch

Copper is an extremely important metal in methanotrophy as it is required in the active site of the membrane-associated or particulate methane monooxygenase (pMMO) (Lieberman and Rosenzweig, 2005; Semrau *et al*., 2010). Of the methanotrophs that contain pMMO, some contain an additional soluble methane monooxygenase (sMMO) which instead contains a di-iron centre (Sirajuddin and Rosenzweig, 2015; Ross and Rosenzweig, 2017). Organisms that contain both pMMO and sMMO are subject to the Cu-switch which promotes expression of the Cu-containing pMMO over sMMO when Cu is available (Stanley *et al*., 1983; Nielsen *et al*., 1997; Murrell *et al*., 2000).

The effect of Cu on the Ln switch has been studied in Type I and Type II methanotrophs and appears to override the Ln switch in some methanotrophs but not others. In the Type II methanotroph, *M. trichosporium* OB3b, exogenous Cu attenuates the effect of Ln on the switch allowing expression of the Mxa-MDH even when Ln are present (Gu *et al*., 2016; Farhan UI Haque *et al*., 2015). Cu also decreases expression of both *xox* genes encoded in the *M. trichosporium* genome by almost an order of magnitude in the presence of Ln (Farhan Ul Haque *et al*. 2015). This is in stark contrast to the Type I methanotroph, *M. buryatense*, in which exogenous Cu has very little effect on the expression of the *mxa* and *xoxF* genes (Chu and Lidstrom 2016). There are not yet sufficient data to conclude if this difference is generally conserved among the Type I and Type II methanotrophs. Cu control over the Ln switch in some methanotrophs does however lend further support to the idea that the regulatory networks controlling the Ln switch in methylotrophic bacteria are complex and diverse.

The effects of community interactions and substrate availability on the lanthanide switch

Recent work with two species co-cultures as well as with more complex mixed communities suggests that the Ln switch may be controlled by additional factors, among them community interactions, though the nature of those interactions is not yet well understood (Vorobev *et al*., 2013; Krause *et al*., 2017; Yu and Chistoserdova, 2017; Yu *et al*., 2017; Zheng *et al*., 2018). Krause *et al*. (2017) compared expression of *mxaF* and *xoxF* genes in cultures of a methanotroph, *Methylobacter tundripaludum*, grown with and without non-methane-utilizing methylotrophic bacteria of the genus *Methylotenera*. While the Ln switch responded as expected when *M. tundripaludum* was grown in pure culture, in co-cultures, the *M. tundripaludum mxaFI* genes were predominantly expressed even when La was available. This apparently resulted in methanol excretion into the culture medium which could provide carbon to and perhaps spare Ln for the non-methane utilizing methylotrophs (Krause *et al*., 2017). Methanol excretion was not observed when *M. tundripaludum* expressed *xoxF* in pure culture possibly due to the proposed superior catalytic properties of the XoxF enzymes (Lim and Franklin, 2004; Pol *et al*., 2014). Interestingly, in the non-methane-utilizing methylotroph, *Methylotenera mobilis* JLW8 which lacks the *mxaFI* genes but encodes two different XoxF enzymes instead, *xoxF_1770* was highly expressed in pure culture, while *xoxF_2048*

was overexpressed in the co-culture. A similar trend was seen for *M. mobilis* 13, in which one *xoxF* gene was preferentially expressed in pure culture while the other *xoxF* gene was preferentially expressed in co-culture (Krause *et al.*, 2017). It is yet unclear why different XoxF enzymes would be preferred over others under different conditions.

Recently, a large-scale OMICS study was completed that followed population and transcriptomic changes that occurred when 50 pure cultures of bacteria representing different functional guilds were combined to create a synthetic methylotrophic community. This synthetic community was subjected to different nitrogen sources (nitrate versus ammonium) and partial pressures of methane and oxygen, in the absence of Ln (Yu *et al.*, 2017). Community population dynamics were followed over 212 days and transcriptomics were analysed at various time points up to 159 days. This study showed that expression of *mxaF* and *xoxF* could be affected by the nitrogen source as well as oxygen and methane partial pressures, and that these responses could differ among species (Fig. 5.3). With nitrate as the nitrogen source, high methane and high oxygen promoted expression of *mxaF* in *Methylomonas* LW13 and *Methylphilus* Q8, which were the dominant species in the community under these conditions. When methane and oxygen were low, expression of *mxaF* was repressed in *Methylomonas* LW13 and *xoxF* was induced. Perhaps when more methane is available to the methanotrophs in the community, production of MxaF may promote sharing of methanol as 'public goods' as carbon is not as precious/scarce. There was more variability in expression of the *xoxF* and *mxaF* genes among the non-methanotrophic methylotrophs which often seemed to favour *xoxF* expression regardless of the condition tested. Production of XoxF may be a way for organisms that have access to methanol (1) to keep the methanol rather than excrete it in the case of a methanotroph; or (2) to efficiently use methanol, if scarce, in the case of a non-methanotroph. This would only make sense however, if Ln were available. As Ln are not present as in this study, the patterns observed only reflected the transcriptional regulation in response to the different factors and not necessarily enzyme activity. Perhaps, XoxF enzymes could serve as methanol sequestration and storage systems by binding the volatile methanol. Perhaps some of the less-characterized XoxF enzymes may have a wider cofactor range. It is also possible that trace Ln contamination may have allowed for expression of *xoxF* in these experiments. Regardless, these results highlight the importance of community studies and again point to the complex nature of the Ln-switch, further suggesting that the Ln switch is affected by more than simple presence or absence of exogenous Ln.

Conclusions

Ln-dependent methylotrophy and its regulation is an emerging field of study. Very little is known about how organisms that use Ln for growth obtain these insoluble micronutrients and incorporate them into the enzymes that utilize them as cofactors. Likely, the importance of Ln will extend beyond the world of methylotrophs. Recently, PedH, a homologue of ExaF was described from non-methylotrophic *Pseudomonas putida* KT2440, and it was shown to also use light Ln and be transcriptionally regulated by their presence (Wehrmann *et al.*, 2017, 2018). It remains to be seen whether these critical metals will have an inherent role in enzymes other than PQQ-dependent alcohol dehydrogenases.

The fact that many methylotrophs have multiple copies of *xoxF* is intriguing. Once thought by some to be duplicate or remnant copies, variants of *xoxF* that have low expression in pure culture appear to have functional roles in communities based upon their gene expression profiles. Biochemically, different types of XoxF MDHs exhibit diverse kinetic parameters for a wide set of substrates. Parsing out the distinct roles for these variant XoxF MDHs is an exciting area of study that is just beginning. Understanding how expression of these variant *xoxF* genes fits into the poorly understood regulatory networks that control the Ln switch will likely add further complexity to an already complex system.

For organisms that contain both Ca- and Ln-dependent MDHs, regulation of the Ln switch appears to be mainly controlled by exogenous Ln when grown in pure culture with the ability of Cu to override the switch in some methanotrophs. However, as there are multiple regulators that contribute to expression of genes under control of the Ln switch in characterized organisms, this suggests that there are likely additional signals that have yet to be identified. In *M. extorquens* AM1 for example,

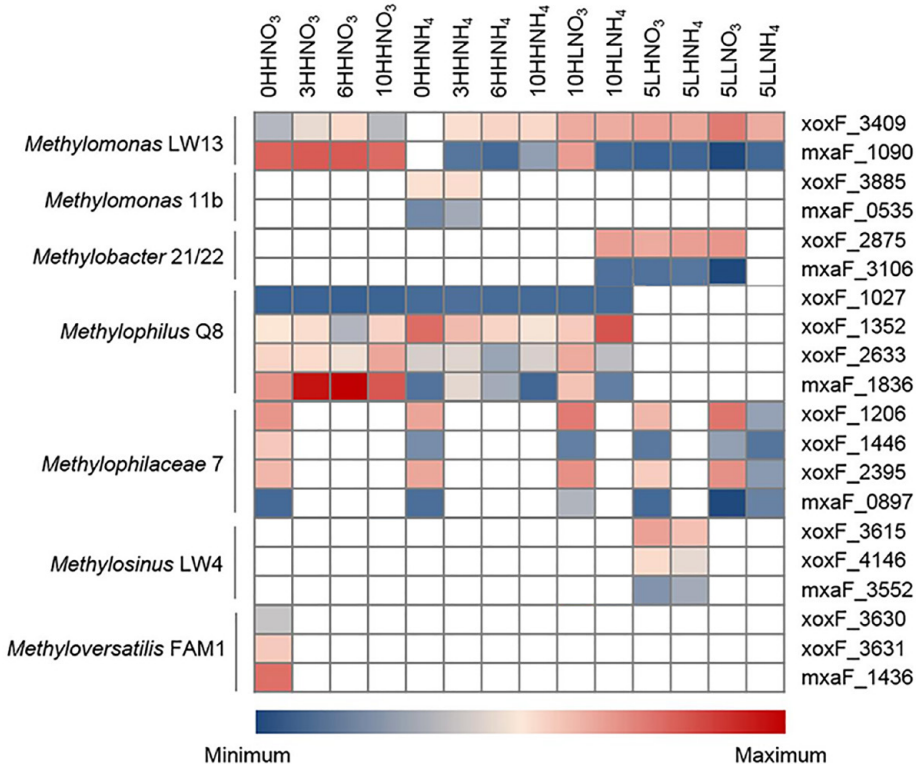


Figure 5.3 Heat map depicting expression of *xoxF* and *mxaF* genes in selected species. Figure and legend are adapted from Yu *et al*. (2017). Sample designations refer to number of transfers (bottom number), followed by methane and O_2 regimens (H for high partial pressure, L for low partial pressure), followed by the nitrogen source. On the left, genus and strain names. On the right, gene name followed by gene number as per genome annotation.

expression of *exaF* is upregulated by Ln, but presence of ethanol rather than methanol or succinate exhibits a larger and synergistic effect on expression (Fig. 5.2A).

Regulation of methanol oxidation is of particular importance as the product of methanol oxidation is either formaldehyde which is highly reactive and toxic, or formate, an acid. It is likely that a variety of signals beyond Ln exert control over the MDHs to help maintain homeostasis in organisms whose central metabolism must balance production and consumption of multiple toxic intermediates while sequestering enough volatile carbon for growth (Skovran *et al*., 2010). These enzymes controlled by the Ln switch require not only metals for activity but PQQ and cytochromes, therefore also iron and haem. A multitude of processes are required for a single metabolic step and may have as of yet unknown effects on expression of the enzymes that carry out this step. As use of XoxF over MxaFI affects production of nucleotide pools in some organisms either by circumventing NADPH production pathways or by decreasing formate oxidation, it is possible that nucleotide homeostasis may also be sensed by the Ln switch which could help maintain balanced nucleotide pools by shifting the MDH that is used. More studies are needed to go beyond speculation to gain important knowledge about how methylotrophs sense and adapt to Ln availability in the presence and absence of other organisms.

There is much left to learn regarding Ln-dependent enzymes and the regulation of the *mxa*, *xox*, and *exa* genes. The mechanisms of Ln acquisition and transport have yet to be explored. A deeper understanding of these processes may facilitate the engineering of methanotrophs and methylotrophs to better function as environmental platforms for carbon capture, production of value added chemicals, and recovery of REEs from electronic

waste and other Ln waste streams. Ln in biology is an emergent and rapidly expanding field full of 'first discoveries.' Researchers have just scratched the surface of novel revelations surrounding Ln in ecology, biochemistry, genetics, bioremediation, biotechnology, and more.

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