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Review

The biochemistry of lanthanide acquisition, trafficking, and utilization[☆]

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ABSTRACT

Lanthanides are relative newcomers to the field of cell biology of metals; their specific incorporation into enzymes was only demonstrated in 2011, with the isolation of a bacterial lanthanide- and pyrroloquinoline quinone-dependent methanol dehydrogenase. Since that discovery, the efforts of many investigators have revealed that lanthanide utilization is widespread in environmentally important bacteria, and parallel efforts have focused on elucidating the molecular details involved in selective recognition and utilization of these metals. In this review, we discuss the particular chemical challenges and advantages associated with biology's use of lanthanides, as well as the currently known lanthano-enzymes and -proteins (the lanthanome). We also review the emerging understanding of the coordination chemistry and biology of lanthanide acquisition, trafficking, and regulatory pathways. These studies have revealed significant parallels with pathways for utilization of other metals in biology. Finally, we discuss some of the many unresolved questions in this burgeoning field and their potentially far-reaching applications.

1. Introduction

Over the past decade, lanthanide ions have been established as essential metals in the biology of widely distributed and environmentally important bacteria, especially the methylotrophs. Methylotrophic bacteria are able to use reduced one-carbon compounds (including methanol, methylamine, and, in some cases, methane) as their sole carbon source for growth [1,2]. Contrary to prior assumptions that they do not play any specific biological role, lanthanides have been shown to be integrally involved in catalysis of certain pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenases (ADHs), including methanol dehydrogenase (MDH), in methylotrophs and even non-methylotrophs [3–5]. Beyond these essential catalytic functions, the requirement that these metal ions be selectively acquired, trafficked, and inserted into enzymes has led to the discovery of a rich bioinorganic chemistry of these metals with the identification of some of the dedicated machinery for these processes [6,7]. The proteins and other biomolecules involved in this biochemistry have been dubbed the “lanthanome” [7,8].

Methylotrophs are found broadly in the environment, in soil, in aquatic systems, and associated with plants [9–11]. Aerobic methylotrophic metabolism of methanol begins with its oxidation to formaldehyde by MDH followed by oxidation to formate, and then to carbon dioxide or assimilation into biomass, thus playing a key role in the global carbon cycle [1,2]. Some methylotrophs (methanotrophs) are also able to utilize methane as a carbon source through a first step of methane oxidation to methanol by particulate or soluble methane monooxygenase enzymes [12]. Traditionally, PQQ-dependent methanol oxidation was associated with the calcium-dependent, MxaFI-type MDHs (Ca-MDH), but it is now clear that lanthanide-dependent, XoxF-type enzymes (Ln-MDHs) are far more broadly distributed than the Ca-MDHs [4,13,14]. Therefore, recent advances regarding the essential utilization of lanthanides have greatly expanded the understanding of methylotrophic metabolism, as well as biochemistry more generally. Since the discovery and characterization of first MDH lanthanoenzymes, in 2011 and 2012 [15–17], the appreciation for the roles of lanthanides in the microbial world has steadily advanced. These seminal early studies were followed in 2014 with the characterization of the first organism that not just *can* use but *requires* lanthanides for growth (*Methylacidiphilum fumariolicum* SolV) [18]; in 2016, expansion of lanthanoenzymes to PQQ-dependent ADHs with multi-carbon substrates [19]; in 2017, extension to non-methylotrophs [20]; in 2018, characterization of the first protein (lanmodulin) known to selectively bind and handle lanthanides within the cell [21], followed by the first reports and characterization of a genetic locus for chelator-mediated uptake of trivalent lanthanide (Ln^{III}) ions in 2019 [8,22]; the recent observation of lanthanide storage [23]; and the discoveries continue.

As catalogued in this Special Issue, the research activities devoted to understanding how transition metals are selectively recognized and

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utilized in biology are vast. There are several chemical properties of the transition metal ions for biology to exploit in order to discriminate between them, many of which stem from the involvement of the d electrons in metal-ligand interactions [24]: the trend in metal-ligand affinities across the first row (Irving-Williams series) and its consequences for buffered cellular concentrations of metal ions [25,26], hard-soft acid-base properties of ions [27], different preferences for coordination geometry [28], and redox activity [29]. By contrast, biological utilization of the elements of the lanthanide series presents a different and intriguing challenge for biology, in that the chemistry of these elements is largely very similar. All exist predominantly in the +III oxidation state, their bonding with ligands is largely ionic, and they differ only subtly in ionic radius, coordination number, and Lewis acidity across the series [30,31]. However, all 15 elements are not fully interchangeable: biology has shown itself able to use some, but not all, lanthanides (as far as is currently known); the catalytic [32,33] and coordination chemistry [18,21,34] reasons behind this observation are only beginning to be elucidated. A deeper understanding of the basic biochemistry of lanthanides and the bioinorganic principles to be extracted from it promises to not only enrich our understanding of the global carbon cycle but also potentially allow for transformative technologies in metallurgy, agriculture, and medicine [7,35].

Here we will restrict our discussion to lanthanides in bacterial systems, which are best understood at present. (Although lanthanides have long been known to promote plant growth [36], the study of the underlying mechanisms is in its infancy [7,37]). Recently, the general picture of biological lanthanide utilization has rapidly expanded, but many important questions regarding transport, recognition, and utilization remain to be elucidated. In presenting the state of the field as of this writing (May 2020), we emphasize the biochemical functions of lanthanides, the fundamental principles of their selective recognition, and how they relate to the bioinorganic chemistry of other metal ions.

2. Why did biology choose lanthanides?

Being the most recent metals to be added to the bioinorganic periodic table, as well as metals that are understudied relative to s-, d-, and p-block metal ions, the idea that lanthanides are used by biology is often treated as being surprising and exotic. However, rather than asking why these metals were chosen, the more appropriate question is why *wouldn't* they be chosen.

The lanthanide series includes the 15 elements La-Lu, which (along with Sc and Y) belong to the group known as rare earth elements (REEs) (Fig. 1). The lanthanides are divided into two primary groups, light (La-Eu, LREEs) and heavy (Gd-Lu, HREEs). All lanthanides, and particularly the LREEs, are relatively abundant in the crust, as compared to other biologically relevant metals; La-Nd are found with crustal elemental abundances similar to those of Cu and Zn [39,40]. Furthermore, although their aqueous solubility at near-neutral pH is low [41], it is in fact significantly higher than that of Fe^{III}, which is commonly accessed by bacteria, plants, and fungi *via* small-molecule siderophores [42]. These properties make the LREEs potentially biologically accessible.

The lanthanides have relatively similar physicochemical properties; most notably, all lanthanides commonly exist in the +III oxidation state, with only Ce and Eu able to access other oxidation states under biologically accessible conditions (+IV and +II, respectively) [30,31]. Being buried below 6s and 5p orbitals, the 4f electrons of the Ln^{III} ions do not effectively shield the outer orbitals from increasing nuclear charge and they have little impact on bonding. The result of these facts is 1) ionic radii decrease from left to right across the series (the "lanthanide contraction") [43], 2) lanthanide complexes are largely ionic in character, and 3) the strong Lewis acidity of the Ln^{III} ions increases across the series and favors hard ligands such as carboxylates [30]. Although the ionic radii across the entire lanthanide series only vary by ~0.18 Å (coordination number of 9), the size differences between adjacent Ln^{III} ions are most pronounced on an absolute and relative basis for the LREEs (Fig. 1). These larger differences might provide biology an opportunity for more selective uptake and utilization. In addition, the relatively large ionic radii of the LREEs and sterics-driven coordination allows lanthanides, and especially the larger LREEs, to accommodate high coordination numbers of 8–12, which is uncommon with other metal ions. The decrease in preferred coordination number between LREEs and HREEs may also facilitate selective recognition of LREEs [7].

Therefore, there are several advantages for biology in using lanthanides: their high oxidation states and Lewis acidity make them useful catalysts, while their reasonable abundances, relatively large sizes, and uniquely high coordination number make it possible to selectively acquire them from the environment. From a chemical perspective, then, biological utilization of lanthanides, and the lighter lanthanides in particular, is understandable.

From an evolutionary perspective, it has been suggested based on sequencing data that a lanthanide-dependent XoxF-type enzyme is the primordial MDH, likely to have appeared in *Bacteria* following the separation from *Archaea* [13]. This hypothesis is supported by the observation that the XoxF-type enzymes are more diverse than their calcium-dependent equivalents and are found across many methylotrophic and non-methylotrophic organisms [4,14,44]. To date, five phylogenetically distinct clades of XoxF have been identified [13], although other XoxF-like proteins may also exist [44], in addition to the ExaF/PedH enzymes which encode lanthanide- and PQQ-dependent ethanol DHs [19,20]. The *xoxFGJ* operon encoding the core Ln-MDH genes is widespread, with its presence determined in all of the > 200 currently characterized methylotroph species, with most belonging to Alpha-, Beta- and Gammaproteobacteria [45,46]. These genes are also present in organisms not currently characterized as methylotrophs, most notably, *Rhizobiales*, *Burkholderiales*, *Nitrosococcales* [5,13]. Homologs of XoxF have even been revealed in the genomes of anaerobic bacteria, suggesting that during its early evolutionary stages, XoxF may have functioned anaerobically [13]. Lanthanide-dependent ADHs have also been identified in a non-methylotrophic bacterium associated with plants, *Pseudomonas putida* KT2440 [20,47]. We refer the reader to several excellent reviews [4,5,13,44] and references cited therein, which discuss evolution and distribution of XoxF-type enzymes and

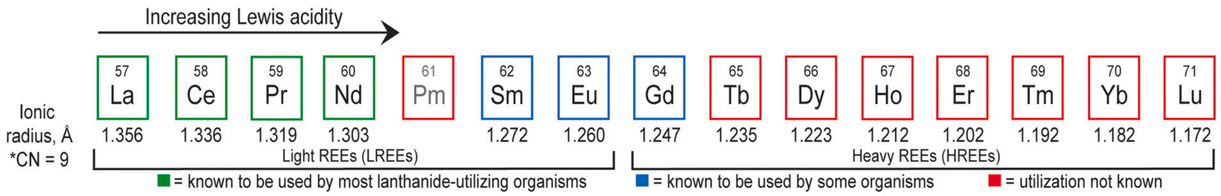

Fig. 1. Properties of the 15 elements of the lanthanide series. Lewis acidity increases across the series from left to right in conjunction with decreasing ionic radius. Ionic radii are listed for a coordination number of 9 [38]. Pm has no stable isotopes. One or more of the lighter lanthanides (boxed in green) have been shown to be used by all currently known lanthanide-utilizing organisms, while the heavier, later lanthanides (boxed in blue and red) have been shown to be less favored by some lanthanide-utilizing organisms, or have no known biological utilization. The rare earth elements also include scandium and yttrium, but these have not been found to be used biologically to date and have been omitted here.

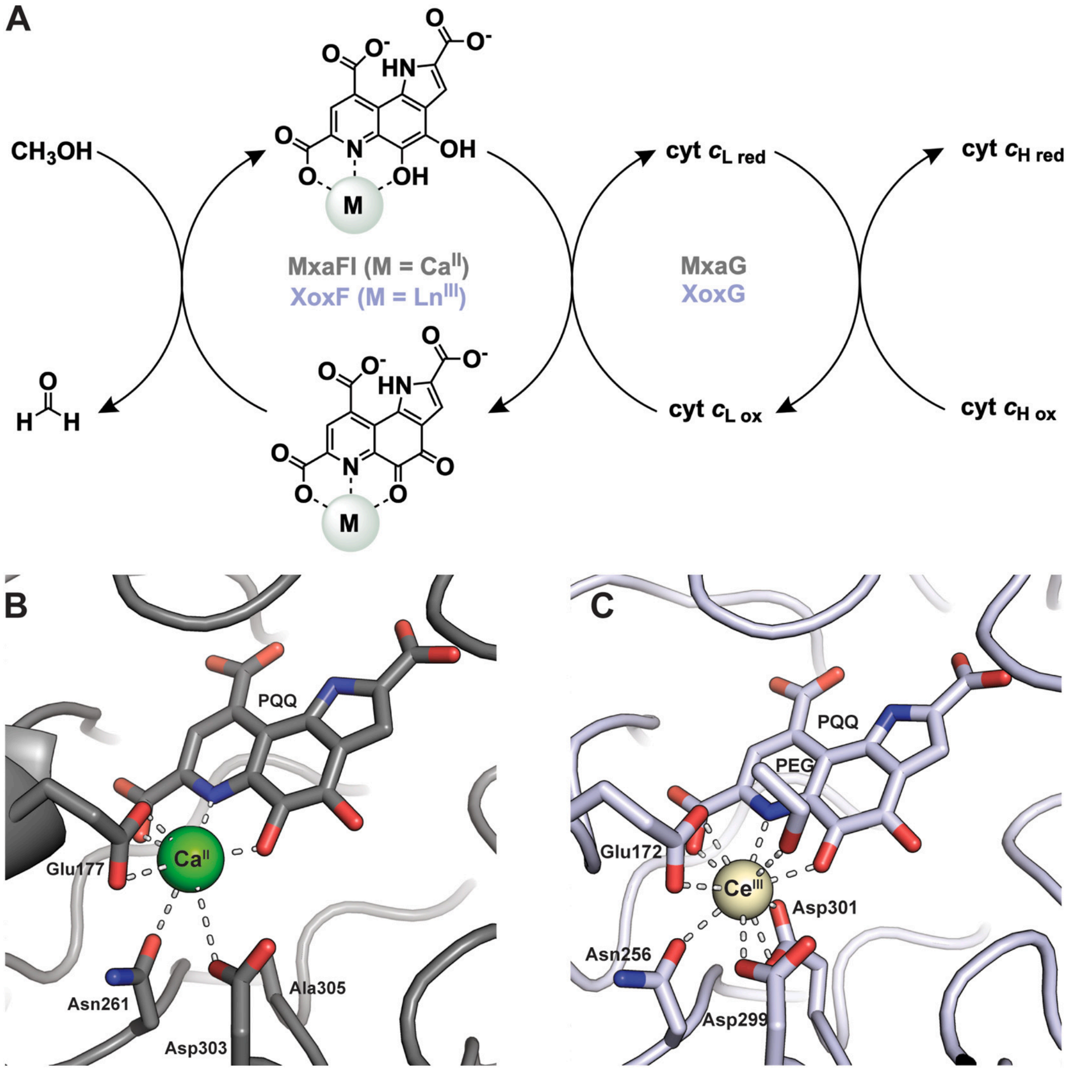

Fig. 2. A) Physiological reaction catalyzed by calcium- and lanthanide-dependent MDHs showing electron flow from methanol to cytochrome c_H. The reduced PQQ cofactor following methanol oxidation passes electrons to a *c*-type cytochrome denoted "cytochrome c_L" (MxaG for Ca-MDH, XoxG for Ln-MDH). In the Ca-MDH system, these electrons are then transferred to cytochrome c_H. Electron transfer from XoxG to cytochrome c_H is hypothesized based on analogy to MxaG but has not been demonstrated. Scheme adapted from [33]. B) Active site of *Methylorubrum extorquens* AM1 MxaFI modeled with Ca (PDB ID: 1W6S). C) Active site of *Methylacidiphilum fumariolicum* SolV XoxF modeled with Ce (PDB code: 4MAE). The modeled PEG is a result of the crystallization process. The additional aspartate residue, Asp301, is shown, with the corresponding Ala305 in Ca-MDH shown for comparison.

lanthanide-utilizing organisms in greater detail.

3. Lanthanide-dependent alcohol dehydrogenases and associated proteins

Before the characterization of XoxF as an Ln-MDH, it was believed that methanol oxidation within methylotrophs was solely catalyzed by the PQQ- and Ca-dependent MDH, MxaFI. MDHs found in Gram-negative methylotrophic bacteria are soluble, periplasmic enzymes that catalyze the oxidation of methanol to formaldehyde (Fig. 2A) [48]. The active sites of these enzymes contain a non-covalently bound PQQ cofactor ligated to a metal ion, Ca^{II} in MxaFI or Ln^{III} in XoxF (Fig. 2B, C). The reaction is formally a hydride transfer from the substrate to the C5 carbonyl of the PQQ cofactor, facilitated by coordination of the metal ion. However, the mechanism of this reaction is the subject of debate, and computational work has outpaced experimental work in this area [32,49–52].

When the *xoxF1* gene was discovered in *Methylorubrum extorquens* AM1, a plant epiphyte that is a model aerobic facultative methylotroph, it was predicted to encode a second PQQ-dependent methanol dehydrogenase [53]. Metaproteogenomic analyses showed XoxFs to be abundant proteins in the plant phyllosphere [9], but when *M. extorquens* AM1 XoxF1 was isolated from growth conditions lacking Ln^{III} and characterized *in vitro* it exhibited a very low rate of methanol oxidation activity [54], making its function cryptic. XoxF1 is encoded from an operon alongside genes encoding a *c*-type cytochrome (XoxG), which is the electron acceptor from the reduced PQQ cofactor by analogy to the Ca-MDH system (Fig. 2A) [48], as well as a periplasmic solute binding protein (SBP) of unknown function (XoxJ). In this section, we briefly review the key proteins involved in lanthanide-dependent alcohol oxidation.

3.1. XoxF

In seminal work, Kawai [15,16], Nakagawa [17] and co-workers demonstrated that XoxF is a methanol dehydrogenase that is in fact induced and activated by La^{III} and Ce^{III} in several methylotrophs, including *M. extorquens* AM1. These results constituted the first recognition of a specific role of lanthanides in biology. Although *M. extorquens* is able to utilize methanol as a carbon source in the absence of Ln^{III} ions using its Ca-MDH, subsequent work by Op den Camp, Jetten, and co-workers discovered that the thermoacidophile *M. fumariolicum* SolV requires lanthanides, and LREEs specifically, for growth, an observation that was linked to XoxF activity [18]. Crystallographic characterization of the latter protein isolated from its native source revealed a similar active site to the Ca-MDH, but with an additional aspartate residue that was proposed to be a hallmark of lanthanide-dependent ADHs (Fig. 2C) [4,18], also observed in crystallographic studies of other XoxFs [55,56]. The incorporation of a Ln^{III} ion instead of Ca^{II} in the active site of XoxF is of chemical interest due to the similar ionic radii of Ca^{II} and the lighter, biologically relevant Ln^{III} ions, as well as the similar metal coordination environments between the two MDHs [18,48,57]. Recent analysis of an Asp→Ala variant of *M. extorquens* XoxF1 shows that the additional Asp residue is indeed critical for proper enzyme metalation *in vivo*, and that the resulting incorporation of Ca^{II} yields inactive protein [56]. This result suggests to us that one function of the extra carboxylate may be to outcompete a presumptive Ln^{III} chaperone to ensure directional metal transfer during XoxF activation.

The lanthanide-dependent ADHs are unusual among metalloenzymes in their ability to be activated by multiple metal ions. Although the Ln^{III} ions are chemically similar, the effects of the lanthanide contraction mean that they are not mere substitutes of one another, because ionic radius, coordination number, and Lewis acidity differences may differentially impact various steps in the reaction, which has been the subject of theoretical study [32,50–52]. The dependence of XoxF activity on the identity of Ln^{III} incorporated and its contribution to growth rates of lanthanide-utilizing bacteria is complex. Systematic activity studies of the *M. fumariolicum* [18,32] and *M. extorquens* [33,58] enzymes (as well as the PedH ADH described below [20]), undertaken using an artificial dye-based assay, reveal different lanthanide dependences of the enzymes, which roughly correlate with the lanthanide-dependent growth rates of each organism (Fig. 3A) [18,33,59]. For example, in line with the activity data, in *M. extorquens* only La-Nd, and to a lesser extent, Sm, activate the *xox1* promoter and support lanthanide-dependent growth on methanol [59], whereas in *M. fumariolicum* lanthanides up to Gd support growth, although La-Nd are most and similarly efficient [18]. However, assays of *M. extorquens* XoxF with different Ln^{III} ions incorporated, using physiological electron acceptor XoxG, reveal similar V_{max} but different XoxF-XoxG K_m values (Fig. 3B), suggesting that, *in vivo*, methanol oxidation chemistry may not be rate limiting. Therefore, the lanthanide-dependence of growth rates may require an additional explanation, such as electron transfer efficiency between XoxF and XoxG (see below) [33] or subtle differences in XoxF stability with different Ln^{III} ions incorporated [60]. *M. extorquens* preferentially acquires and incorporates La over Nd into XoxF [60], suggesting that lanthanide-dependent differences in efficiency of cellular metal uptake and even cofactor assembly likely contribute as well.

3.2. XoxG

XoxG is a *c*-type cytochrome and is the physiological electron acceptor for XoxF. Chistoserdova and co-workers first purified a XoxG protein and showed that it could serve as electron acceptor for XoxF from the methanotroph, *Methylomonas* sp. LW13 [61]. XoxG proteins have also been characterized from *M. fumariolicum* [62,63] and *M. extorquens* AM1 [33]. In the latter case, the protein was crystallographically characterized and its ability to serve as the electron acceptor was assessed with La-, Ce-, and Nd-XoxFs. Our interpretation of the results, that the metal in XoxF did not affect overall XoxF activity but it did affect the K_m of XoxF for XoxG (Fig. 3B), was that an increased reduction potential of the Ln^{III}-PQQ cofactor as Lewis acidity increases from La^{III} to Nd^{III} might result in a less efficient electron transfer to XoxG [33]. This proposal was supported by the low reduction potential of XoxG (+172 mV) relative to other *c*-type cytochromes. In *M. fumariolicum* SolV, XoxG is fused with XoxJ (see below), into a protein dubbed cytochrome c_{GJ} [62]. The reduction potential was determined as +240 mV at pH 7, a relatively similar value to that of other cytochrome *c* proteins, intriguing in that *M. fumariolicum* XoxF is active *in vivo* with later lanthanides than is the *M. extorquens* enzyme. These studies together [33,61,62] suggest that the redox properties of XoxG may be tuned to the lanthanide preferences of its respective XoxF, although this proposal remains to be rigorously tested.

3.3. XoxJ

The third conserved gene in *xox* operons is *xoxJ*, encoding a periplasmic SBP homologous to MxaJ, which is associated with the Ca-dependent MDH. SBPs are commonly associated with ATP-binding cassette (ABC) transporter systems, but there is not an obvious such system associated with *xoxJ*. Many potential functions of MxaJ and XoxJ have been proposed, including binding methanol or formaldehyde [4], or facilitating interaction between the MDH and its electron acceptor [64,65]. Because previous studies suggested that MxaJ is required for activation of MxaFI [66], we suggested that XoxJ that could play a role in XoxF activation. The x-ray crystal structure of *M. extorquens* XoxJ reveals that XoxJ, like other SBPs, consists of two globular domains flanking a putative ligand-binding cavity [33]. However, XoxJ's cavity is especially large and, like MxaJ's [64], contains a beta sheet missing several strands. This cavity contains a large number of

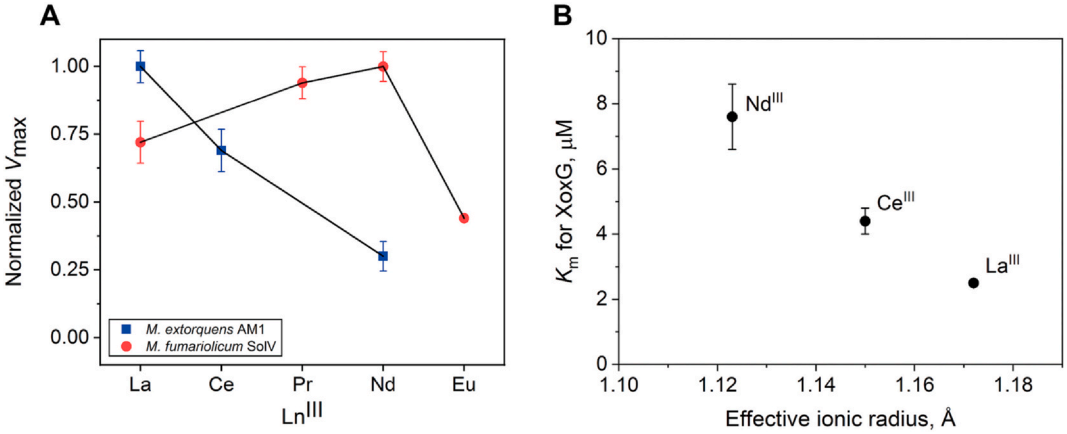

Fig. 3. Lanthanide dependence of XoxF activity varies between organisms. A) Comparison of the V_{max} values for *M. extorquens* AM1 [33] (blue) and *M. fumariolicum* SolV [32] (red) XoxFs, metalated with different lanthanides, as determined by an artificial dye-linked assay. For each enzyme, the metalated enzyme with highest activity was normalized to 1. B) In *M. extorquens* AM1, the K_m of XoxF for its physiological electron acceptor XoxG increases significantly from La-XoxF to Nd-XoxF. Reproduced from Featherston et al. [33].

hydrophobic residues and two loops located on the periphery. It is hypothesized that this large hydrophobic cavity may be optimized to bind a large, hydrophobic protein or peptide target, such as a partially folded form of its operon-mate, XoxF. Such an interaction may make apo-XoxF a suitable receptor for metal ion and PQQ (separately or together), which is otherwise difficult to envision based on the structure of the activated protein [33]. It is also possible that XoxJ's substrate is a different protein, such as a lanthanide and/or PQQ chaperone.

3.4. Other Ln- and PQQ-dependent ADHs

Lanthanide-dependent processes are not limited to methanol oxidation. Skovran and Martinez-Gomez noted that an *M. extorquens* strain lacking all known MDHs was still able to grow, albeit poorly, with methanol as a carbon source in the presence of LREEs [59]. Characterization of the other predicted PQQ-dependent ADH in the genome, named ExaF, showed that it accounted for this growth. ExaF is a homodimer that uses a PQQ cofactor and preferentially binds La^{III} over Ca^{II} in the active site [19]. Kinetic analyses showed ExaF oxidizes ethanol and methanol at similar rates (V_{max}) but with a much lower K_m for ethanol. Although it also has secondary activities *in vitro* in formaldehyde and acetaldehyde oxidation, its primary physiological function appears to be in ethanol oxidation, demonstrating for the first time that lanthanides also play a role in metabolism of multi-carbon substrates [19], a characteristic that may also be shared by certain XoxF-type proteins [67]. Intriguingly, Klebensberger and coworkers reported that *Pseudomonas putida* KT2440 harbors the first example of a lanthanide- and PQQ-dependent ADH in a non-methylotroph, the ethanol dehydrogenase PedH [20]. This enzyme exhibits a strong preference for Pr and Nd in catalysis, although it can also use other REEs up to Tb *in vitro*; *in vivo*, only La-Sm appear to support growth of the deletion mutant of PedE, the calcium-dependent counterpart to lanthanide-dependent PedH. Whereas in *M. fumariolicum* XoxF, *M. extorquens* ExaF, and *P. putida* PedH, the metal cofactors can be exchanged relatively easily to facilitate studies with non-native lanthanides, the cofactors cannot be reversibly removed in *M. extorquens* XoxF1 [33,58], suggesting differences in sophistication of cofactor assembly pathways in different enzymes.

4. The highly selective lanthanide-responsive protein, lanmodulin

Precedents from numerous other metalloenzyme systems [68] suggested that specific machinery existed to ensure the correct metalation of XoxF and other lanthanoenzymes. Lanmodulin (LanM), an *M. extorquens* protein reported by our group in 2018, was the first natural Ln^{III}-selective chelator to be discovered, and the first lanthanide-binding protein other than an ADH to be isolated and characterized, and it has provided the first biochemical and structural insights into how Ln^{III} ions are selectively recognized and trafficked in biology [21].

M. extorquens LanM is a small, 12-kDa protein that contains four carboxylate-rich metal coordination motifs referred to as EF hands (Fig. 4A). These motifs, consisting of a 12-residue metal binding loop flanked by two α helices, are typically associated with Ca^{II} binding in numerous proteins, including the mammalian calcium sensor, calmodulin (Fig. 4B) [69]. Despite utilizing this canonical metal binding motif, LanM exhibits several unique features as compared to other EF-hand proteins. While LanM retains the Asp, Asn, and Glu residues typically involved in Ca^{II} binding in EF hands (positions 1, 3, 5, and 12 in the loop), it also features an Asp residue at the ninth position, a relatively rare occurrence that has previously been shown to increase the selectivity for Ln^{III} ions over Ca^{II} in other EF-hand-containing proteins, in line with higher coordination numbers preferred by Ln^{III} ions (Fig. 4C, D) [21,70]. This "extra" Asp residue is reminiscent of the additional Asp present in the XoxF active site. Additionally, there are unusual conserved proline residues present at the second position in each EF hand, which is almost never found at that position in canonical EF hands [69,71]. Using a variety of biochemical methods, we showed that in the apo state, LanM is an intrinsically disordered protein, and it undergoes a large, cooperative conformational change to a predominantly helical state upon the addition of Ln^{III} ions (3 equivalents being tight binding). This conformational change is induced at picomolar free concentrations of Ln^{III} ions; interestingly, the lanthanides inducing a conformational change at the lowest concentration are La^{III}-Sm^{III}, which are also the only lanthanides that induce expression from the *xoxF1* promoter and activate *M. extorquens* XoxF *in vivo* [59]. By contrast, LanM's response to metal ions other than REEs was weak [8,21]; for example, nearly millimolar concentrations of Ca^{II} are required. Overall, LanM exhibits a remarkable $\sim 10^8$-fold selectivity in conformational response to Ln^{III} ions over Ca^{II}. The unique proline residues appear to be involved in suppression of response to Ca^{II} and other abundant metal ions, presumably to help ensure metal selectivity within the cell [21].

The first structural analysis of a lanthanide-binding protein other than PQQ-dependent Ln-ADHs was the NMR structure of LanM bound to Y^{III} [34]. This structural analysis revealed a central three-helix bundle, forming a hydrophobic core with the EF hand loops located on the periphery of the structure (Fig. 4A). In this regard, the protein is an inverse of calmodulin, which exposes hydrophobic residues when Ca^{II} is bound [72]; LanM's packing interactions are likely crucial for the protein's exquisite metal selectivity. The collapse of LanM on itself upon Ln^{III} binding is possible because of the fusion of the helices of its adjacent EF hands, unlike in calmodulin and other EF-hand proteins, resulting in a unique overall topology and poising the protein between order and disorder [34]. Y^{III} coordination was modeled in EF hands 1–3, showing metal coordination *via* four or five carboxylate side chains and a backbone carbonyl (Fig. 4C). The coordination numbers are 8–9 depending on the metal binding site, higher than typical coordination numbers for EF hand-bound Ca^{II} ions (Fig. 4D). Because Y^{III} is not one of the biologically utilized REEs, studies with LREEs will be important for more detailed understanding of how coordination number and geometry change across the lanthanide series.

Lanmodulin is one of only a handful of proteins significantly upregulated in response to lanthanides in *M. aquaticum* 22A [73] and in *M. extorquens* PA1 [22]; however, it is not an essential protein [22], nor is it universally conserved in methylotrophs. Whether LanM is a periplasmic chaperone, sensor, or repository for Ln^{III} (or a combination of these) is not yet fully clear, but its solvent-exposed metal sites, Ln^{III}-dependent conformational changes, and rapid equilibration kinetics suggest a role in metal hand-off [21,34]. Nevertheless, the studies to date have highlighted several principles of selective recognition of lanthanides. First, in XoxF and LanM, biology has utilized the preference for higher coordination numbers of the lighter Ln^{III} ions, which is relatively unique in the periodic table, to assist in selective recognition. Second, cooperative metal binding in LanM, as in calmodulin and in some synthetic Ln^{III} ligands [74,75], likely aids selectivity for metal ions that would otherwise be difficult to distinguish (*e.g.*, Ln^{III} ions *versus* Ca^{II}, and LREEs *versus* HREEs) by amplifying small differences in radius and coordination preferences. Third, LanM's conformational response is biased toward lighter Ln^{III} ions rather than the more Lewis acidic, heavier ones, which is an unusual trend among ligands for Ln^{III} ions [40]; this property is shared by other biomolecules involved in lanthanide uptake and trafficking [8]. More recent work [76] has revealed that Ln^{III} binding and conformational change in LanM are closely coupled only for the LREEs, reinforcing that the metal site alone is not controlling selectivity and implying that the relative kinetics of metal binding *versus* of protein folding are critical for selectivity. These may be more general principles for how lanthanide-handling machinery overcomes the typical thermodynamic preferences of traditional small-molecule lanthanide ligands.

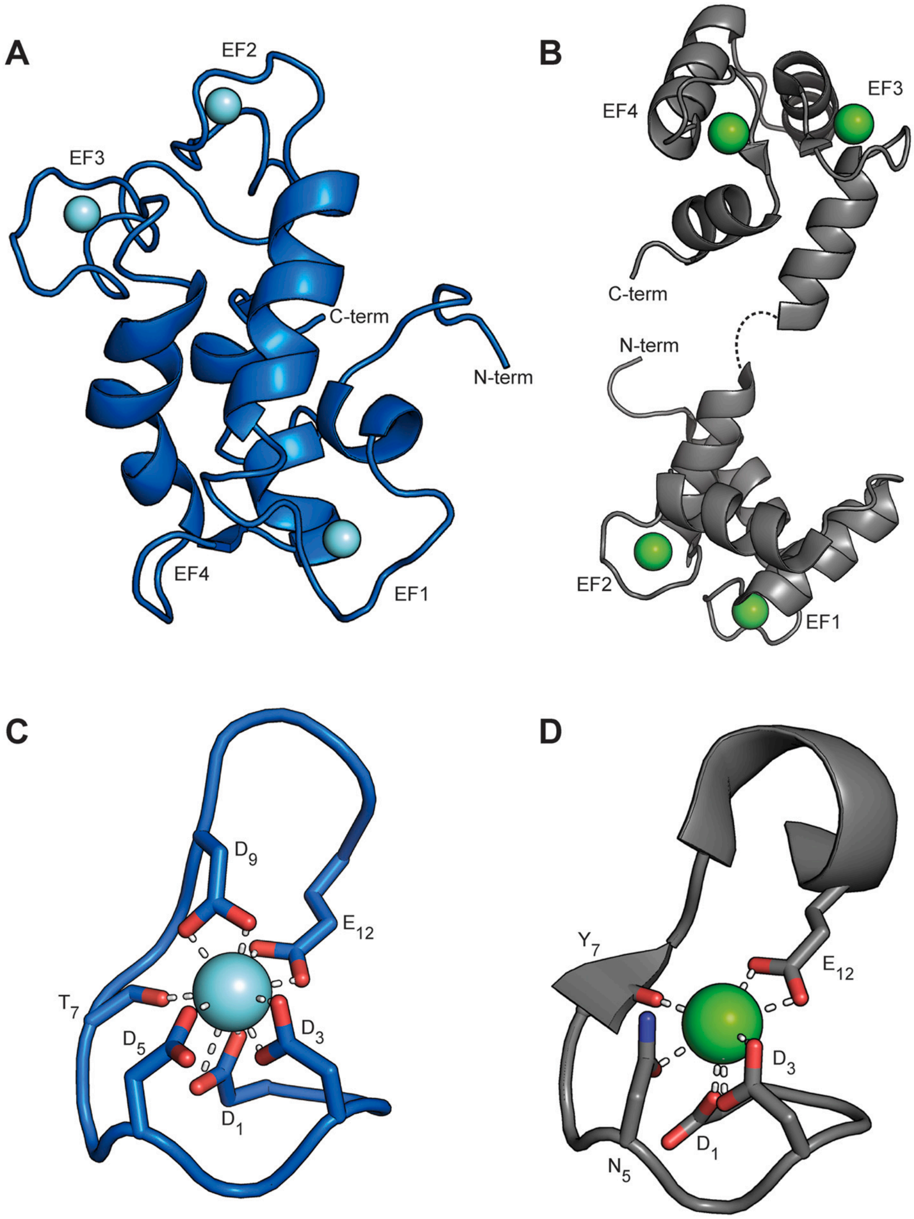

Fig. 4. Comparison of LanM (PDB code: 6MI5) and calmodulin (PDB codes:1J7O and 1J7P). A) Overall solution structure of Y^{III}-LanM showing Y^{III} (teal spheres) bound to first three EF hands. B) Overall solution structures of Ca^{II}-calmodulin showing all four EF-hands bound to Ca^{II} (green spheres). The dashed line represents a flexible linker between the two lobes of the protein. C) and D) Detail of the EF-hand 3 of LanM and calmodulin, respectively, highlighting coordination of the additional aspartate residue (D_9) in LanM, increasing coordination number. Residues are numbered according to their position in the EF hand.

5. Lanthanide uptake and storage

Lanthanide solubilities are rather low [41], and they are often difficult to extract from minerals that contain them. Therefore, several investigators [4,6,12,21] hypothesized that at least some lanthanide-utilizing organisms might secrete a small-molecule lanthanophore for Ln^{III} ion acquisition, analogous to bacterial iron and copper acquisition pathways using siderophores [77,78] and chalkophores [79], respectively. When reporting discovery and characterization of LanM in 2018, we noted that *lanM* was adjacent to a gene encoding a predicted TonB-dependent transporter, a type of import protein commonly linked to uptake of Fe^{III}-siderophore, $Cu^{I/II}$-chalkophore, and other metal complexes [80]. This observation strongly suggested that this genomic region included the uptake machinery for a Ln^{III}-metallophore complex [21]. A few months later in early 2019, a combination of genetic evidence provided by Vorholt and coworkers [22] and biochemical and chemical biology evidence provided by our group [8] confirmed this proposal in rapid succession. In the *M. extorquens* genome, this 10-gene cluster containing *lanM* was shown to contain genes for a Ln^{III}-metallophore uptake system, periplasmic Ln^{III} trafficking, and possibly other functions (Fig. 5). The genetic data were supported by utilization of a protein-based sensor for Ln^{III} ions based on LanM, called LaMP1 [8], to demonstrate for the first time that La^{III}-Nd^{III} were selectively imported into the cytosol – an intriguing result because all currently known lanthanoproteins are periplasmic, but consistent with a metallophore uptake system. While pointing to as yet unidentified roles of cytosolic lanthanides, these results also clarified that the inability to grow on the heavier lanthanides, previously noted [59], resulted from an inability to import them, rather than just an inability to sense and use them. Furthermore, application of LaMP1 to spent media from *M. extorquens* growths indicated the presence of a secreted molecule, presumably a lanthanophore, able to efficiently compete with LaMP1 for

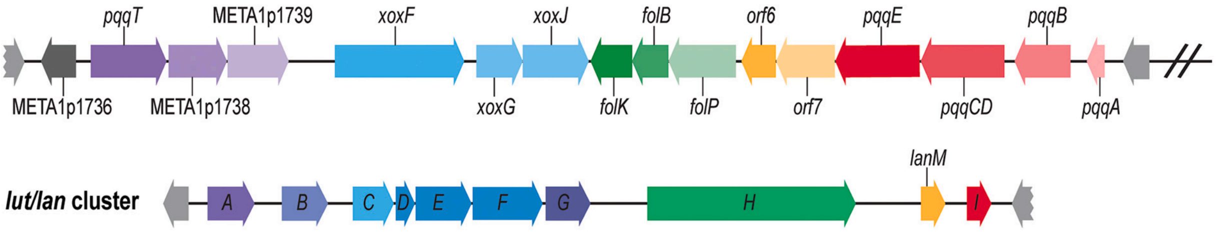

Fig. 5. Gene clusters encoding many of the proteins discussed within the text as being involved in Ln-dependent methylotrophy in *M. extorquens* AM1. Operon predictions were made by BioCyc [83]. *pqqABCDE* are genes required for PQQ biosynthesis [84], immediately adjacent to *orf6* and *orf7*, which encode putative periplasmic proteins. *folP*, *folB*, and *folK* are involved in the biosynthesis of tetrahydrofolate, and META1p1738 and META1p1739 presumably encode an ABC transporter associated with the PQQ-binding SBP, PqqT. The *lut/lan* cluster [8,22,23] is a 10-gene cluster containing necessary genes for $\mathrm{Ln}^{\mathrm{III}}$-metallophore uptake, periplasmic $\mathrm{Ln}^{\mathrm{III}}$ ion trafficking, and potentially other functions.

binding of LREEs but not HREEs. This work was followed by identification of another TonB-dependent transporter for lanthanides in *Methylotuvimicrobium buryatense* 5GB1C, which was named LanA [81]. In *P. putida* KT2440, an ABC transporter for cytosolic lanthanide uptake was identified [82], but no TonB-dependent transporter has been identified to date, potentially suggesting a different uptake mechanism. This work confirmed the idea that at least some Ln-utilizing organisms utilize secreted chelators to mediate lanthanide uptake.

In 2020, Skovran, Martinez-Gomez and co-workers reported results of a clever transposon mutagenesis screen to identify genes involved in lanthanide-dependent processes in *M. extorquens* [23]. Six of the identified 28 genes were from the *lanM* gene cluster (17 others being either core *xox* genes or related to PQQ or heme/cytochrome synthesis/transport), for which they suggested the name *lut*, and assessed the effect of gene knockouts on lanthanide-mediated growth. We suggest that *lan* may be a more appropriate name for this cluster to recognize the fact that *lanM* was the first gene identified (and currently best characterized protein) in handling cellular lanthanides, that it led directly to the first identification of the entire cluster by Vorholt [22] and our group [8], that the functions of all 10 of these genes are almost certainly related, and that the corresponding TonB-dependent receptor in *M. buryatense* was given the name LanA. At a minimum, *lut/lan* is required to recognize that *lanM* is embedded within the cluster.

The functions of four of these genes were assigned with confidence based on homology to known metallophore uptake pathways [8,22] and confirmed by knockouts [22,23]: a putative TonB-dependent outer membrane importer ("*H*" for simplicity here), an ATP-binding cassette (ABC) transporter (*EF*) for cytosolic import, and an SBP (*A*) for the transporter (Fig. 6). The functions of the remaining genes are not yet clear. The genes encoding D and G overlap with the genes for the ABC transporter, so they are presumably closely related to function of that system. We showed that D is a dimer that has the unusual property of preferentially binding LREEs over HREEs, though more weakly than LanM [8]. This may suggest that directional lanthanide affinity gradients exist in periplasmic $\mathrm{Ln}^{\mathrm{III}}$ trafficking, as has been shown for cytosolic copper trafficking [85]. B is probably an enzyme, with sequence homology to methylamine dehydrogenase [8], a protein that contains an unusual tryptophan tryptophyl quinone cofactor synthesized from two adjacent Trp residues [86]; indeed, a homology model of B indicates the presence of two Trp residues close in space and adjacent to a cluster of carboxylate residues, suggesting to us that a similar cofactor might form in this protein and bind a $\mathrm{Ln}^{\mathrm{III}}$ ion. If true, it would suggest the more general importance of Lewis acidic $\mathrm{Ln}^{\mathrm{III}}$ ions in facilitating biological quinone chemistry. Precisely where the other proteins fit into this picture is not yet known.

While cytosolic lanthanide uptake has been established, an important unanswered question is whether the $\mathrm{Ln}^{\mathrm{III}}$ necessary for metalation of periplasmic lanthanoenzymes (XoxF, ExaF, and possibly others) must be imported to the cytosol and then pumped back out. (The analogous question is also open in copper import in methanotrophs [79].) The ABC transporter genes are essential for lanthanide utilization [22,23], but this does not necessarily require cytosolic import followed by export if cytosolic $\mathrm{Ln}^{\mathrm{III}}$ ions serve an unknown regulatory function or essential cytosolic lanthanoproteins exist. The answer depends on the localization of the enzyme(s) presumably needed to liberate $\mathrm{Ln}^{\mathrm{III}}$ ions from the lanthanophore.

How these proteins fit into the activation pathway for ADHs is also not completely clear. In addition to the proposed involvement of XoxJ in XoxF activation, the mechanism of insertion of PQQ and metal ions into PQQ-dependent ADHs has long been an open question. This is especially true because PQQ is synthesized in the cytosol but the enzymes that use it as a cofactor reside in the periplasm. While theoretically XoxF could be loaded with PQQ and metalated in the cytosol, the protein possesses a predicted Sec secretion signal so it probably folds and cofactor is inserted in the periplasm. In this regard, two genes at the end of the *pqq* operon, *orf6* and *orf7*, encode putative periplasmic proteins that are essential for Ln-mediated growth [23]. We suggest that, because Orf6 possesses a carboxylate-rich N-terminus that would be exposed following cleavage of the signal peptide, it may bind a metal ion, which in turn may be bound to PQQ (perhaps in complex with Orf7) as a chaperone for periplasmic cofactor insertion. An ABC transporter (META1p2359) that is a putative exporting ATPase [87] was also identified in the transposon screen, a candidate for mediating PQQ export to the periplasm [23]. There is more to be learned about cellular PQQ trafficking as well, as one of the few genes upregulated by lanthanides [22,58] has been characterized by our group as a PQQ-binding SBP named PqqT, putatively involved in PQQ uptake from the periplasm [88].

Finally, given the above results and established storage pathways for many biologically used metals (for example, acidocalcisomes and cuprosomes) [89,90], it seemed likely that analogous lanthanosomes might exist. Recently, Skovran and Martinez-Gomez [23] demonstrated that one destination for the cytosolically internalized lanthanides in *M. extorquens* is storage, through the use of transmission electron microscopy (TEM) coupled with energy dispersive X-ray spectroscopy (EDS). Their results revealed that $\mathrm{La}^{\mathrm{III}}$ can be stored as crystalline metal deposits, with EDS analysis suggesting $\mathrm{La}^{\mathrm{III}}$ complexed with phosphate. Further experiments are necessary to fully elucidate the lanthanide storage pathway, although this storage mechanism has potential to be exploited as a useful biotechnology for lanthanide concentration.

6. Regulation

Many open questions also exist regarding regulation of lanthanide-dependent systems (see ref. [91] for a detailed discussion). It seems clear that substantial diversity in regulatory mechanisms exists across organisms, although the evidence to date supports a unified theme of lanthanide sensing localized in the periplasm. The signal is transduced to the cytosol for gene regulation *via* two-component systems, which consist of a membrane-bound sensor kinase and a cytosolic response regulator [92].

In organisms that are able to utilize both lanthanide- and calcium-

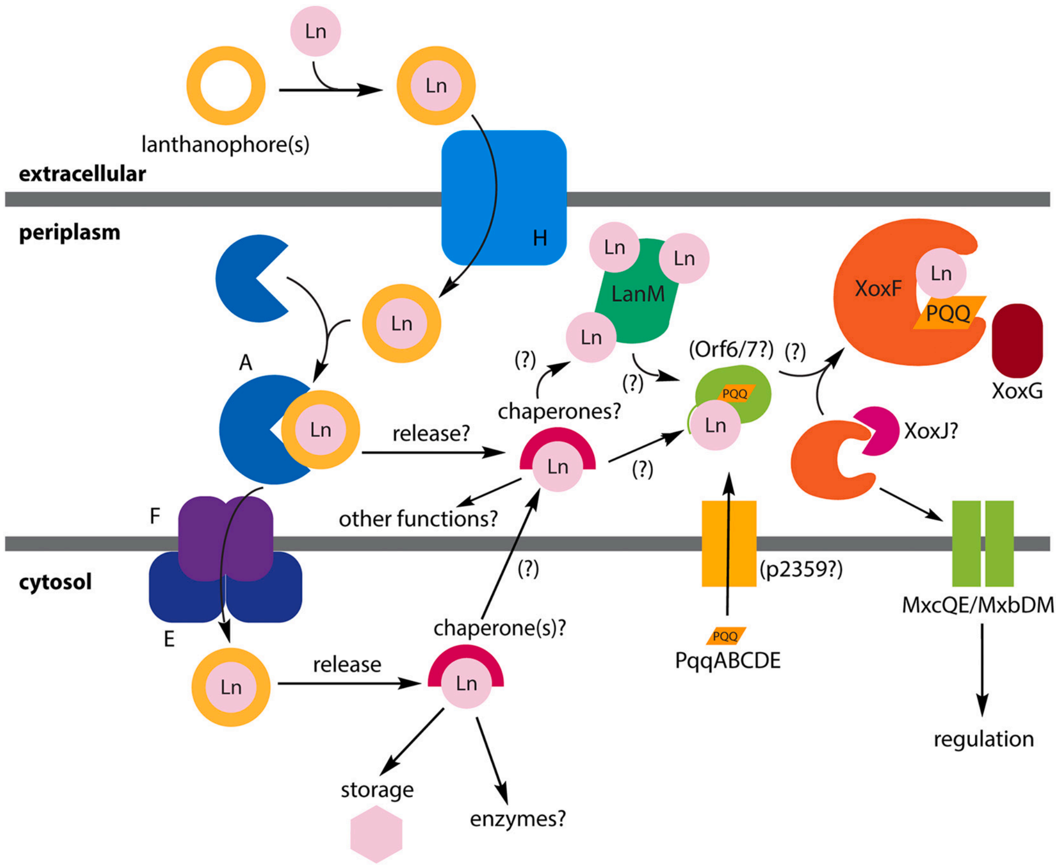

Fig. 6. Model for lanthanide uptake, trafficking, and utilization in *M. extorquens*, involving the genes shown in Fig. 5, and based on the work described in the text. Postulated functions are indicated with question marks. Adapted and updated from ref. [7].

dependent carbon metabolism, these systems are regulated by the so-called "lanthanide switch" [93–95]. In the general mechanism, the presence of lanthanides represses transcription of the Ca-MDH and activates transcription of the Ln-MDH. The mechanism by which this transcriptional regulation occurs differs according to the organism. In *M. extorquens*, the regulatory mechanism is complex and involves two two-component systems, MxbDM and MxcQE, as well as the response regulator MxaB [91]. The response regulator MxbM is necessary for the repression of the *xox1* operon in the absence of lanthanides [96]. Furthermore, *xoxF1* is required for expression of MxaFI and the MxbDM two-component system, in addition to repression of itself [59,96]. As little as 25 nM La^{III} was required to observe significant upregulation of the *xox1* operon, and repression of the *mxa* operon occurred in a dose-dependent manner up to 250 nM La^{III}, even in the presence of calcium [59]. Therefore, *M. extorquens* actively senses and responds to presence of lanthanides, favoring them over calcium when available. Because of the requirement for *xoxF1* for MxaFI expression, it was suggested that apo-XoxF may be involved in periplasmic lanthanide sensing through interactions with either MxcQ or MxbD, or both, whereas metalation of the enzyme with Ln^{III} relieves the repression of *xox1* and leads to *mxa* repression [59]. In *M. buryatense*, the homologs of MxbDM and MxcQE are absent, and a different sensor kinase is present, MxaY [97], which might sense Ln^{III} that are either "free" or bound to an unknown protein or small molecule. In the non-methylotroph *P. putida*, regulation of the PedH Ln-ADH and PedE Ca-ADH is accomplished using the PedR2/S2 two-component system, but it is also not known in what form lanthanides are sensed by this system [98]. Further studies will be necessary in all of these systems and others [99] to elucidate the cellular signals for lanthanide-replete and lanthanide-deficient states, the full regulatory pathways, and the complex interactions of these metal sensing mechanisms with those for other important metals such as copper [94] and iron [82].

7. Conclusions and outstanding biochemical issues

The field of lanthanide biochemistry is not quite a decade old, but the last few years have seen a rapid intensification in efforts to understand the biology, biochemistry, and chemistry of lanthanide-utilizing organisms. Much progress has been made, as described in the present review, but important questions remain to be addressed at many levels: coordination chemistry, elucidation of biochemical pathways, the scope of lanthanide-utilizing organisms, and applications.

The principles of biological coordination chemistry of lanthanides are still in the early days of interrogation. The discussion here has highlighted analogies between lanthanide biochemistry and the bioinorganic chemistry of other metals: Ca^{II} for catalysis and trafficking, Fe^{III} and Cu^{II} for acquisition mechanisms. Perhaps the most intriguing question is how biology achieves selectivity for the LREEs, as man-made ligands with this preference are rare [40]. Not only do ADHs exhibit highest activity *in vitro* with LREEs, but it appears that the entire Ln^{III} uptake and trafficking machinery is biased toward LREEs in conformational response and/or intrinsic binding, as highlighted by characterization of LanM [21,76], a second periplasmic Ln-binding protein [8], and what can be inferred to date about lanthanophores [7,8]. The greater complexity of many biomolecules likely enforces specific metal coordination-induced interactions in the second coordination sphere and other distal parts of the molecule, in order to finely tune the ligand to ionic radius and override the typical thermodynamic preference for HREEs. Detailed structural and biophysical characterization of these and other, yet to be characterized, molecules will be fascinating as well as potentially useful (see below). It will also be intriguing to unravel the extent to which individual ligands are responsible for an organism's selectivity within the lanthanide series; or whether entire uptake and trafficking pathways (lanthanophore, receptor, transporter, chaperones) are involved in a multi-stage separation scheme, filtering out less efficiently transferred Ln^{III} ions at each step, and accumulating the large preference for La^{III} over Nd^{III} in metalation of XoxF that has been observed in *M. extorquens*, for example [60].

Genetic and biochemical studies have rapidly uncovered the existence of various lanthanide-linked pathways and the molecular players in them, and work will be necessary to fill in the details: uptake, chaperones, cofactor assembly, sensing, regulation, other lanthanoenzymes, storage, whether there are cytosolic functions of lanthanides apart from storage, and more. It remains to be seen how similar these pathways are (or whether all of them are present at all) in a broader range of lanthanide-utilizing organisms. Although unified in the preference for the more abundant LREEs (at least to date – organisms preferring other REEs may exist), differences in the specific sets of lanthanides utilized by various bacteria [6] imply diversity in the details of uptake, trafficking, and utilization, which in turn are likely linked to the distinct environmental niches of these organisms. The need to characterize structures and binding properties of lanthanophores from different organisms is obvious, but this imperative extends to other ligands involved in lanthanide recognition as well. The level of sophistication of these ligands may depend on the difficulty of Ln^{III} extraction from the organism's environment. For example, *M. fumariolicum* was isolated from an acidic volcanic mudpot with micromolar levels of REEs [18], conditions in which a lanthanophore should not be necessary to enhance bioavailability, unless small-molecule-mediated selectivity against the rarer HREEs is still required; however, LREE-selective proteins would be able to achieve the same goal, in principle [8,21,76]. It will be intriguing to see, as more lanthanide uptake and trafficking systems are characterized, what the most important driving forces are for evolution of these systems. It will also be important to view these findings in the context of lanthanide geochemistry, in order to better understand how lanthanide availability through time may have contributed to the evolution of biological lanthanide utilization.

More broadly, whereas most mechanistic work to date has focused on the role of lanthanides in bacteria, the lessons learned can be applied to other biological systems and even in the fields of biometallurgy and medicine. Tantalizing clues point toward functions of lanthanides in plants and even in higher organisms (briefly reviewed [7,36]). Furthermore, since the discovery of the use of REEs in biology, it has been clear that there might be timely and significant translational impact from using lanthanide-utilizing bacteria [35] or the ligands that they synthesize (small molecules, proteins) [7,76] to address challenges in REE extraction and separations [40] and in REE-based medical imaging and therapeutic applications [100,101]. These applications likely extend to the actinides as well [76]. Having begun to uncover the fundamentals of biology's highly evolved chemistry of lanthanides, scientists can now start to use and evolve this chemistry ourselves.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CRediT authorship contribution statement

Both authors contributed to the writing of this manuscript at all stages: original draft, review, and editing.

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