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Title
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Rare earth element alcohol dehydrogenases widely occur among globally distributed, numerically abundant and environmentally important microbes

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Abstract
Lanthanides (Ln^{3+}), known as rare earth elements, have recently emerged as enzyme cofactors, contrary to prior assumption of their biological inertia. Several bacterial alcohol dehydrogenases have been characterized so far that depend on Ln^{3+} for activity and expression, belonging to the methanol dehydrogenase clade XoxF and the ethanol dehydrogenase clade ExaF/PedH. Here we compile an inventory of genes potentially encoding Ln^{3+}-dependent enzymes, closely related to the previously characterized XoxF and ExaF/PedH enzymes. We demonstrate their wide distribution among some of the most numerically abundant and environmentally important taxa, such as the phylogenetically disparate rhizobial species and metabolically versatile bacteria inhabiting world's oceans, suggesting that reliance on Ln^{3+}-mediated biochemistry is much more widespread in the microbial world than previously assumed. Through protein expression and analysis, we here more than double the extant collection of the biochemically characterized Ln^{3+}-dependent enzymes, demonstrating a range of catalytic properties and substrate and cofactor specificities. Many of these enzymes reveal propensity for oxidation of methanol. This observation, in combination with genome-based reconstruction of methylotrophy pathways for select species suggests a much wider occurrence of this metabolic capability among bacterial species, and thus further suggests the importance of methylated compounds as parts of the global carbon cycling.

Introduction

Metals are important elements in organic life, beside carbon, oxygen and nitrogen, as they serve as enzyme cofactors. Over years of research, a group of metals has been established of universal significance for life, and these became known as life metals. These include Na, K, Mg, Ca, Fe, Zn, Mn, Cu, Co and Mo [1]. At the same time, some

metals, such as rare earth elements (REEs), while revealing attractive catalytic properties in in vitro manipulations, leading to their wide use in industrial and medical applications [2–4], have been assumed biologically inert [5]. This assumption has been dramatically reversed recently with the demonstration of the activity of REEs lanthanides (Ln^{3+}) as cofactors of methanol dehydrogenases (MDH), enzymes essential in metabolism of single-carbon compounds such as methane and methanol [6–14]. An example of a Ln^{3+}-dependent alcohol dehydrogenase (ADH) in a non-methylotroph has also been reported [15, 16]. In addition to their catalytic function, Ln^{3+} also act in regulation of gene expression, through a mechanism known as the 'Ln^{3+} switch' [10–17]. The understanding of the evolution of REE-linked enzymes is lacking, but it has been noted that they are more diverse than their Ca^{2+}-dependent counterparts [18, 19]. It has been proposed that REE-linked enzymes may possess higher catalytic efficiencies compared to Ca^{2+}-dependent counterparts [5], and some experimental data have been collected in support of this proposal [9, 20–22]. However, only a handful of REE-dependent enzymes

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| | Ca²⁺-dependent enzymes | | Ln³⁺-dependent enzymes | | |
Order	MxaF	ExaA/PedE	ExaF/PedH	XoxF5	XoxF4
● Methylococcales (green)	blue	gray	gray	blue	gray
● Methylophilales (green)	blue	gray	gray	gray	blue
○ Rhizobiales (yellow)	blue	blue	blue	blue	gray
○ Rhodocyclales (yellow)	blue	blue	blue	blue	gray
○ Thiotrichales (yellow)	blue	blue	blue	blue	gray
○ Burkholderiales (yellow)	blue	blue	blue	blue	gray
○ Rhodobacterales (yellow)	blue	blue	gray	blue	gray
● Rhodospirillales (gray)	blue	blue	blue	blue	gray
● Alteromonadales (purple)	blue	blue	blue	gray	gray
● Xanthomonadales (purple)	blue	blue	blue	gray	gray
● Chromatiales (purple)	gray	blue	blue	blue	gray
● Oceanospirillales (purple)	gray	blue	blue	blue	gray
● Vibrionales (purple)	gray	blue	blue	blue	gray
● Nevskiales (purple)	gray	blue	blue	blue	gray
● Salinisphaerales (purple)	blue	gray	blue	blue	gray
● Neisseriales (purple)	gray	blue	blue	gray	gray
● Pseudomonadales (purple)	gray	blue	blue	gray	gray
● Sphingomonadales (purple)	gray	blue	blue	gray	gray
● Campylobacterales (purple)	gray	blue	gray	gray	gray
● Caulobacterales (purple)	gray	gray	gray	blue	gray

Fig. 1 Occurrence and distribution of REE-dependent alcohol dehydrogenases among Proteobacteria, compared to the occurrence and distribution of their calcium-dependent counterparts. Taxa are shown at the order level. In green, taxa that only contain methylotroph species [18]. In yellow, taxa that contain both methylotroph and non-mehylotroph species [18]. In gray, taxa that contain species capable of methylotrophic co-metabolism [55]. In purple, taxa that are not known to contain any methylotroph species. Blue, gene present, gray, gene absent

have been characterized so far [7, 9, 15, 20, 23, 24], and no consensus exists at this point.

As the essential role of REE-linked ADH enzymes has been firmly established for the methylotrophy metabolism [25, 26], the important questions remain as to whether REEs are important in the context of other metabolisms and whether they are widespread beyond the known methylotrophs. In this study, we employed a recombinant system based on a *Methylorubrum extorquens* AM1 (formerly *Methylobacterium extorquens* AM1) mutant, to express and purify several REE-dependent enzymes from organisms representing some of the major taxa within Proteobacteria.

These represent both, functional groups including methylotrophs and functional groups not known for methylotrophic life style. Our data demonstrate that, in general, these enzymes display similar properties across a range of organisms, while variations occur at the level of the downstream electron-accepting redox proteins. Straightforward bioinformatic inferences suggest that these enzymes, while appearing auxiliary in many taxa, are widely occurring in environmentally important and globally distributed microbes. The data we present here further suggest the importance of Ln^{3+} in microbial life, and potentially beyond, and firmly position these metals as true life metals.

Results and discussion

Lanthanide-dependent alcohol dehydrogenases of the XoxF5 and ExaF/PedH clades are widely distributed among Proteobacteria

While the role of Ln^{3+} has been already well established among the bona fide methylotrophs, it remains unclear how widespread are Ln^{3+}-dependent biochemistries among bacteria in general, and, potentially, even beyond. Further investigations are required to understand the role of Ln^{3+} in life, and the significance of Ln^{3+} as substitutes for Ca^{2+} ions (or vice versa), including the evolutionarily aspects of the alternative biochemistries. To approach these questions through bioinformatics, we used sequences of the so far characterized XoxF5 enzymes [7, 23, 24], to query the genomes available in the IMG/M database, which includes finished and draft genomes of cultivated organisms, as well as genomic bins assembled from metagenomes (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). We determined that representatives of 13 orders within Proteobacteria, a total of 698 genomes, encoded XoxF5-type enzymes that displayed amino acid identities over 65% with the characterized enzymes, placing them within the XoxF5 clade on XoxF phylogenetic trees [18, 19, 27], a total of 738 genes (some genomes encoding multiple homologs; Supplementary Tables 1 and 2). Of the 13 orders, only 1, the Methylococcales, was represented exclusively by the methylotrophs, organisms reliant on methanol oxidation for their major ecophysiological function [28] (Fig. 1). Five orders were represented by both species characterized as methylotrophs and species not known to be capable of methylotrophy, one by species capable of co-metabolism of methanol, while the remaining six orders were represented only by species not known for methylotrophic life styles (Fig. 1). This simple analysis demonstrates the wide distribution of XoxF5-type enzymes among alpha-, beta- and gammaproteobacteria, including some universally widespread, numerically abundant and ecologically significant functional guilds. These

include the phylogenetically disparate rhizobia, the members of Vibrionales, known for parasitic relationships with multiple species, the metabolically versatile Rhodobacterales, abundant in world oceans, the ammonia-oxidizing *Nitrosococcus*, as well as other species within Chromatiales. In comparison, genes for the classic, Ca^{2+}-dependent MDH large subunit, MxaF, were much less represented in the same database, occurring in species belonging to 11 orders, mostly known methylotrophs, these always present in a single copy per genome, a total of 254 genes (Fig. 1, Supplementary Tables 3 and 4). XoxF5 and MxaF co-occurred in 8 orders of Proteobacteria (Fig. 1). Note that MxaF proteins belonging to the methanotrophs of the NC10 phylum phylogenetically cluster together with proteobacterial MxaF proteins, and thus these were also counted (Supplementary Tables 3 and 4).

Importantly, a different form of XoxF, XoxF4, is encoded and expressed by Proteobacteria of the order Methylophilales, and XoxF4 and XoxF5 appear to be mutually exclusive [23]. Properties of the two of several subclasses of XoxF4 enzymes have recently been described, confirming them as Ln^{3+}-dependent MDHs [23]. A total of 64 Methylophilales genomes/genomic bins have been identified in the IMG database, encoding 127 XoxF4 enzymes (multiple copies of XoxF4 are encoded in most Methylophilales genomes; Supplementary Tables 5 and 6).

Additional clades of XoxF enzymes (i.e., XoxF1, XoxF2 and XoxF3) are also known, but their distribution is limited to specific taxa [18, 19]. A single XoxF2 representative has been characterized as a bona fide Ln^{3+}-dependent MDH, from a verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* SolV [9]. Likely, all the verrucomicrobial methanotrophs encode and express this enzyme. However, only a handful of these organisms are available in cultures, and, thus, no statistical meaning can be gained from analysis of the current databases. The only study on the properties of a representative of XoxF1, from the NC10 phylum methanotroph *Candidatus* 'Methylomirabilis oxyfera', reported Ca^{2+} dependence for this enzyme, despite the presence of the motif for Ln^{3+} binding [29]. Thus, further data are needed to determine metal specificities for the enzymes of this clade. No biochemical data are available for XoxF3 clade enzymes.

We further queried the occurrence of the ExaF/PedH enzymes that have been characterized as Ln^{3+}-ethanol dehydrogenases (EDH) [15, 20]. These were found in 14 orders of Proteobacteria (a total of 898 genes belonging to 888 genomes; Supplementary Tables 7 and 8), in 9 of these co-occurring with XoxF5 (Fig. 1). In comparison, the Ca^{2+}-dependent counterparts ExaA/PedE/Mdh2 forms were detected in 16 orders of Proteobacteria (a total of 954 genes/951 genomes; Supplementary Tables 9 and 10), in the 14 orders ExaA/PedE co-occurring with ExaF/PedH/Mdh2

Table 1 Enzymes expressed in this study and their sources

Protein	Organism	NCBI Taxon ID	Locus Tag (IMG/M)
XoxF5_M.e.1	*Methylorubrum extorquens* AM1	272630	MexAM1_META1p1740
XoxF5_M.e.2	*Methylorubrum extorquens* AM1	272630	MexAM1_META1p2757
XoxF5_M.d.	*Methyloversatilis discipulorum* FAM1	1119528	MetFAM1DRAFT_3630
XoxF5_S.m.	*Sinorhizobium meliloti* 5A14	1194707	SM5A14DRAFT_08183
XoxF5_B.d.	*Bradyrhizobium diazoefficiens* USDA 110	224911	GA0133328_113011
XoxF5_R.k.	*Rhodovulum kholense* DSM 19783	453584	Ga0183464_12212
XoxF5_T.c.	*Tistlia consotensis* DSM 21585	1321365	Ga0170457_10916
XoxF5_G.m.	*Grimontia marina* CECT 8713	646534	Ga0125493_102428
ExaF/PedH_M.s.	*Methylopila* sp. 107	1101190	A3OUDRAFT_2942
ExaF/PedH_M.d.	*Methyloversatilis discipulorum* FAM1	1119528	MetFAM1DRAFT_1172

(Fig. 1). From these analyses, we conclude on the wide occurrence of REE-dependent ADH enzymes within Proteobacteria. Relative to Ca^{2+}-dependent ADHs, they occur at similar frequencies. Importantly, in numerous species, enzymes with alternative inferred metal specificities are encoded within the same genome. Also of note, many genomes encode multiple predicted REE-dependent enzymes. All these observations suggest a much wider occurrence and distribution of REE-dependent enzymes than previously appreciated.

Methylorubrum extorquens mutant defective in methanol/ethanol utilization serves as a platform for expressing REE-dependent enzymes

The wide distribution of XoxF5 enzymes, especially among bacteria not known for methylotrophic life styles, made us question whether these XoxF5 enzymes would reveal properties similar to the properties of enzymes from bona fide methylotrophs. While it has been claimed that Ln^{3+} enzymes might be catalytically superior to their Ca^{2+} counterparts [9, 20, 22], the data on the properties of these enzymes are still very scarce. Moreover, a novel function has been recently proposed for XoxF5 from an *Advenella* species (Burkholderiales), in sulfur metabolism [30]. Remarkably, Ln^{3+} were not required for its activity in converting reduced sulfur compounds, as part of a newly proposed hypothetical metabolic cycle [30].

To resolve some of these controversies, we set up to purify and study properties of several XoxF5 enzymes, representing divergent alpha-, beta- and gammaproteobacteria. *Methylorubrum extorquens* AM1 was chosen as a major model methylotroph, in which the roles of different MDH enzymes have been previously investigated [17, 31]. *Methyloversatilis discipulorum* FAM1 was chosen as another bona fide methylotroph, representative of Rhodocyclales, known for the diversity of ADH enzymes encoded in their genomes, some with proven functions in methylotrophy [32, 33]. As the presumed non-methylotroph models, we employed *Sinorhizobium* and *Bradyrhizobium* species, representing phylogenetically disparate groups of rhizobia, known for their ecological function in nitrogen-fixing legume symbioses [34] (Table 1), a *Rhodovulum* species, a photosynthetic marine bacterium representing a broad diversity of Rhodobacterales [35] and a *Tistlia* species, another marine bacterium representing the metabolically versatile Rhodospirillales [36]. We also selected a representative of a broadly distributed order Vibrionales, a *Grimontia* (formerly *Vibrio*) species representing an important family within gammaproteobacteria containing both parasitic and free-living species [37]. To express representatives of the ExaF/PedH type enzymes, we elected to use the genes from *M. discipulorum* FAM1 and *Methylopila* sp. 107, the latter being a third methylotroph model [38].

As we have previously encountered problems with utilizing *Escherichia coli* as an expression host [23], we here utilized *M. extorquens* AM1 as an expression host, specifically the quadruple mutant variant with deletions in the *mxaF*, the two *xoxF5* and the *exaF* genes, unable to grow on either methanol or ethanol [17]. The genes of interest were synthesized using the current JGI pipeline and cloned into a modified expression vector pCM80 (see Materials and methods), previously successfully used as an expression system in *M. extorquens* AM1 [39]. The respective plasmids were cloned into *E. coli* and subsequently conjugated into the quadruple mutant. The resulting progenies were selected for tetracycline resistance on succinate as a carbon source, and these were then tested for the ability to grow on methanol and ethanol, in the absence or in the presence of a range of Ln^{3+} (see Materials and methods).

The constructs expressing the two native *M. extorquens* AM1 XoxF5 enzymes [17, 40], XoxF_M.e.1 and XoxF5_M.e.2, served as positive controls, as these were expressed in their native host. Both complemented growth of the mutant host on both methanol and ethanol, when REEs La^{3+} through Nd^{3+} were supplied, but not when the
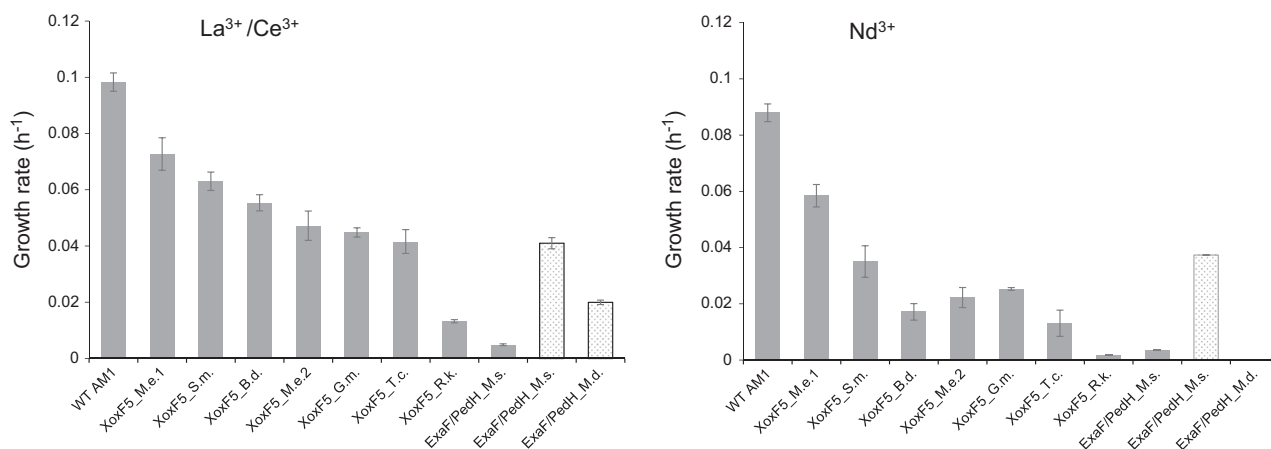
Fig. 2 Growth rates of constructs expressing XoxF or ExaF/PedH enzymes in the presence of La^{3+}, Ce^{3+} and Nd^{3+}. Metals were tested individually at concentration of 30 µM. As data for La^{3+}, Ce^{3+} were very similar, they were joined together. Gray columns, growth on methanol (0.5% V/V); hatched columns, growth on ethanol (0.15% V/V). Cultures were incubated with shaking at 30 °C, with the exception of the construct expressing XoxF5_R.k., which was incubated at 20 °C. Data represent the means of two replicate tests, with error bars. Additional data are presented in Supplementary Table 11 and Supplementary Fig. 1

heavier REEs were supplied (Fig. 2, Supplementary Figure 1 and Supplementary Table 11). These results confirmed that both XoxF5 proteins encoded by the *M. extorquens* AM1 genome possessed the MDH/EDH enzymatic activities, even though these proteins have been reported to have differential roles in methylotrophy, XoxF5_M.e.1 appearing to be a more important enzyme compared to XoxF5_M.e.2 [17]. Seven of the heterologously expressed genes were also able to complement growth of the host on methanol and/or ethanol (Fig. 2 and Supplementary Fig. 1 and Supplementary Table 11). Plate and liquid culture growth experiments in the presence or in the absence of a range of Ln^{3+} revealed a total of four complementation patterns, as follows: (1) XoxF5 enzymes from *Bradyrhizobium diazoefficiens*, *Grimontia marina*, *Rhodovulum kholense*, *Sinorhizobium meliloti* and *Tistlia consotensis* showed the same pattern as the enzymes from *M. extorquens* AM1, supporting growth on both methanol and ethanol, in the presence of REEs ranging from La^{3+} to Nd^{3+}. (2) XoxF5 from *Methyloversatilis discipulorum* did not complement growth of the host. (3) ExaF/PedH enzyme from *M. discipulorum* only complemented growth on ethanol, only when La^{3+} or Ce^{3+} were present. (4) Remarkably, ExaF/PedH from *Methylopila* sp. restored growth on both methanol and ethanol in all conditions, even in the absence of Ln^{3+}. However, growth was significantly stimulated by REEs (La^{3+} through Sm^{3+}).

Effect of REEs on growth on methanol or ethanol has been reported before for several organisms [7, 9, 14, 15, 17, 20, 23, 41, 42], as well as for natural populations [43], and our data are in general in line with the published data. XoxF5, XoxF2 and XoxF4-2 enzymes display narrow specificity for Ln^{3+}, selecting for the early Ln^{3+} (La^{3+}–Nd^{3+}), while others, such as XoxF4-1 and PedH display specificity toward a broader range of lanthanides (La^{3+}–Gd^{3+}). The decrease in catalytic efficiency with later Ln^{3+} has recently been experimentally demonstrated [14, 42]. While Ln^{3+} specificity of ExaF/PedH_M.s. we report here is similar to the one reported for PedH from *Pseudomonas* [15], it is not strictly dependent on Ln^{3+}. This identifies it as a promiscuous REE enzyme that, in the absence of REEs, can apparently use Ca^{2+}. Note that we exclude the possibility of contamination with REEs, as all the constructs were tested on the same plate for each condition, and thus nine of the strains served as negative controls for growth (Supplementary Fig. 2).

REE alcohol dehydrogenases reveal a range of catalytic properties

In this study, we focused on the kinetic properties of the XoxF5 enzymes, to test whether the enzymes from the presumed non-methylotrophs revealed propensity for oxidizing methanol. The eight XoxF5 proteins were expressed in the presence of Ce^{3+} (see Materials and methods). In crude extracts, MDH activities of 0.07 to 1.31 U were measured using the artificial dye assay (Fig. 3, Supplementary Fig. 3). Notably, the enzyme from *M. discipulorum* FAM1 showed activity within this range. Thus, the reason for negative complementation phenotype for XoxF5_M.d. is obviously not the lack of catalytic activity. More likely, the lack of complementation is due to the absence of compatible electron acceptors or other components of the electron transfer chain in the host strain. This speculation of course requires experimental testing, in the future. The two *xoxF* genes, present in a tandem on the chromosome of *M. discipulorum* FAM1 form a cluster with a *xoxJ* homolog, which frequently form operons with *xoxF* genes [9, 17], while there are no genes encoding cytochromes in the vicinity [33]. In *M. extorquens* AM1 and in many other
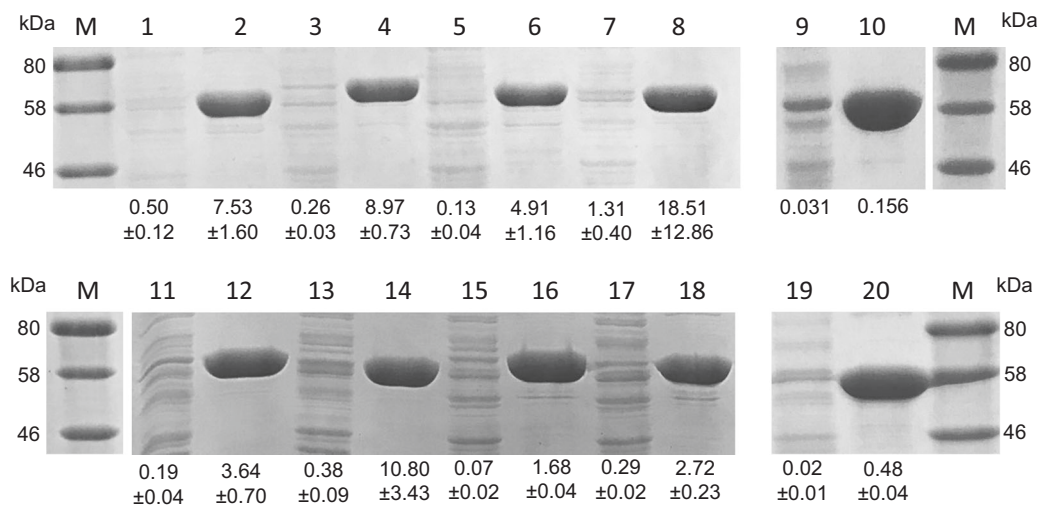
Fig. 3 Denaturing polyacrylamide gel electrophoresis of crude extracts (lanes with odd numbers) and respective purified enzymes (lanes with even numbers), and respective specific activities measured with methanol as a substrate for XoxF5 enzymes and with ethanol for ExaF/ PedH enzymes (Units/mg, defined as μM substrate/min/mg protein). 1, 2, XoxF5_T.c.; 3, 4, XoxF5_S.m.; 5,6, XoxF5_M.d.; 7, 8, XoxF5_G. m.; 9, 10, ExaF/PedH_M.d.; 11, 12, XoxF5_M.e.2; 13, 14, XoxF5_M. e.1; 15, 16, XoxF5_R.k.; 17, 18, XoxF5_B.d.; 19, 20, ExaF/PedH_M. s. M denotes marker protein lanes. Respective molecular masses are indicated. Full length gels are presented in Supplementary Fig. 3

Table 2 Summary of kinetic parameters determined for REE-enzymes in this study, compared to previously published data

Organism	Enzyme	V_{max} (Umg^{-1})	K_m (mM)	K_{eff} (s^{-1} mM^{-1})[a,b]	Reference
Bradyrhizobium diazoefficiens USDA 110	XoxF5_B.d.	2.7 ± 0.1	15 ± 2	371 ± 4	This study
Bradyrhizobium sp. MAFF 211645	XoxF5	15.0	29	1 090	[7]
Grimontia marina CECT 8713	XoxF5_G.m.	14.9 ± 0.2	24 ± 2	1 296 ± 175	This study
Methylomicrobium buryatense 5G	XoxF5	0.18	–	–	[24]
Methylomonas sp. LW13	XoxF5	5.97 ± 0.94	39 ± 11	342 ± 99	[23]
Methyloversatilis sp. FAM1	XoxF5_M.d.	3.1 ± 0.1	15 ± 2	434 ± 51	This study
Methylorubrum extorquens AM1	XoxF5_M.e.1	12.5 ± 0.2	21 ± 1	1 252 ± 135	This study
	XoxF5_M.e.1	5.7 ± 0.13	44 ± 5	272 ± 31	[46]
	XoxF5_M.e.2	3.2 ± 0.1	12 ± 2	564 ± 110	This study
Rhodovulum kholense DSM 19783	XoxF5_R.k.	1.6 ± 0.03	17 ± 1	194 ± 24	This study
Sinorhizobium meliloti 5A14	XoxF5_S.m.	4.4 ± 0.1	23 ± 2	408 ± 44	This study
Tistlia consotensis DSM 21585	XoxF5_T.c.	5.3 ± 0.3	30 ± 6	366 ± 46	This study
Methylotenera mobilis JLW8	XoxF4–1	0.65 ± 0.56	55 ± 32	27 ± 20	[23]
	XoxF4–2	0.78 ± 0.3	42 ± 18	42 ± 17	[23]
Methylacidiphilum fumariolicum SolV	XoxF2	4.4	0.8	11 600	[9]
Methylopila sp. M107	ExaF/PedH_M.s.	0.45 ± 0.01	16 ± 3	57 ± 2	This study
Methylorubrum extorquens AM1	ExaF	6.4	0.9	14 500	[20]
Pseudomonas putida KT2440	PedH	10.6	177	66	[15]

[a]K_{eff} is defined as K_{cat}/K_m

[b]Kinetic constants reported here were deduced from two to four independent biological replicate experiments

Fig. 4 Phylogenetic tree representing relationships among the REE-enzymes from this (in red bold) and prior (in black bold) studies, with additional enzymes used for refining the tree. Clade designations are indicated. Bar, percent of amino acid divergence
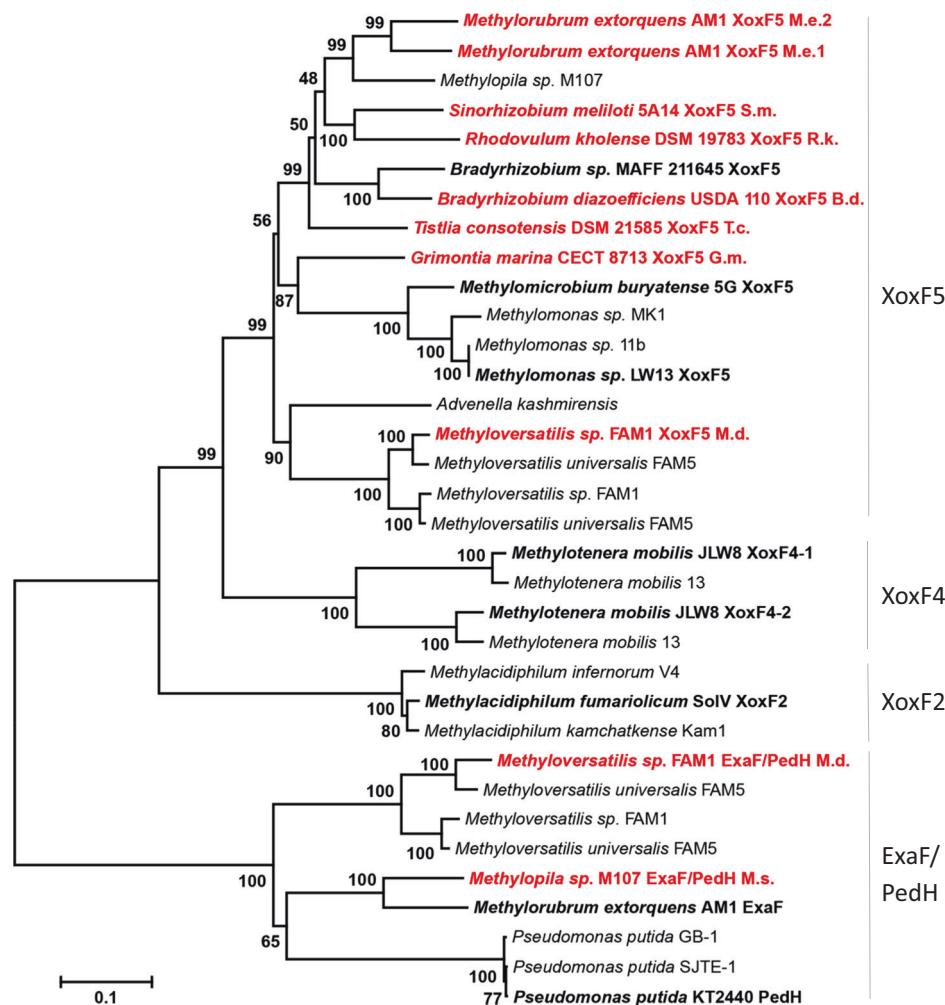
organisms, a gene encoding a cytochrome *c*, named *xoxG*, is located between *xoxF* and *xoxJ* (the second *xoxF* gene in *M. extorquens* AM1 is an orphan [40]). We hypothesize that, while the XoxF5 proteins from *M. extorquens* AM1 and *M. discipulorum* FAM1 are highly homologous (~70% amino acid identity), the latter may not be able to transfer electrons to XoxG of *M. extorquens* AM1. Interestingly, *G. marina* also lacks recognizable *xoxG* in its genome. Instead, the *xox* gene cluster in this organism contains a gene encoding a plastocyanin, another type of a small redox protein, which likely serves as a natural electron acceptor from XoxF5_G.m. Nevertheless, XoxF5_G.m. appears to be able to transfer electrons to XoxG of *M. extorquens* AM1. The diversity of the putative electron acceptors from XoxF enzymes has been noted before [13, 44], and this study provides further evidence toward the complexity of XoxF/electron acceptor pairings. Moreover, an interaction between XoxF5 and particulate methane monooxygenase has been recently reported for a methanotrophic bacterium [24].

Pure XoxF5 proteins revealed specific activities in the range of 1.68 to 18.51 U with methanol as a substrate (Fig. 3, Supplementary Fig. 3). The enzymes were relatively unstable even in the presence of glycerol and dithiothreitol, losing 12–80% of initial activity within 24 h when kept on ice (Supplementary Table 12). The enzymes showed typical UV-Vis absorption spectra, with a maximum of 355 nm and a slight shoulder at 400 nm, previously reported for redox cofactor pyrroloquinone quinone (PQQ) in XoxF type enymes [9, 14]. The PQQ peak decreased somewhat over time, potentially indicative of protein denaturation (Supplementary Fig. 4). We determined pH optima and ammonia requirements for the eight enzymes, and found that all required ammonia for the in vitro activity assay, and all had a pH optimum between 9.0 and 9.5 (Supplementary Fig. 5), similarly to the properties of the previously described XoxF5 from *Methylomonas* sp. LW13 [23]. The artificially high pH and the ammonia ion requirements have been known for the Ca^{2+}-dependent MDHs and for other PQQ-dependent

Table 3 Enzymes and pathways for C1 metabolism deduced from representative genomes

Organism	XoxF5 MDH	GSH-formaldehyde oxidation	H₄F-C1 transfer	Formate oxidation	CBB cycle	Choline oxidation	Sarcosine oxidation	TMA oxidation	TMAO demethylation	DMA oxidation	MA oxidation (MADH)	MA oxidation (NMG pathway)	Glycine betaine demethylation	Dimethylglycine oxidation
B. diazoefficiens	+	+	+	+	+	+								
S. meliloti	+	+	+	+	+	+	+	+	+	+		+	+	
T. consonensis	+	+	+	+	+	+	+	+		+	+	+	+	+
R. kholense	+	+	+	+	+	+	+						+	+
G. marina	+	+	+	+	+	+	+					+		
A. kashmirensis	+	+	+	+	+	+	+				+			

MDH methanol dehydrogenase; *GSH* glutathione, *H₄F* tetrahydrofolate, *TMA* trimethylamine, *TMAO* trimethylamine N-oxide, *DMA* dimethylamine, *MA* methylamine, *MADH* MA dehydrogenase, *CBB* Calvin–Benson–Bassham

dehydrogenases for decades, but the biochemical bases for these requirements remained a mystery [45].

We further determined substrate specificities for the eight enzymes, and they also fell into similar ranges, all enzymes revealing high activities with the saturating concentrations of methanol, ethanol, 1-propanol, 1-butanol, 1-hexanol and formaldehyde (Supplementary Fig. 6), again, similar to the properties determined for XoxF5 from *Methylomonas* sp. LW13 [23]. The kinetic constants were determined for a range of alcohols and for formaldehyde (Table 2, Fig. 4, Supplementary Fig. 6), and these, again, were in line with the constants previously determined for XoxF5 enzymes, demonstrating that these enzymes had affinities for a broad range of alcohols, and that the affinities for formaldehyde were significantly lower than for alcohols. K_m values for alcohols generally varied between 0.012 ± 0.002 and 0.130 ± 0.015 mM, while K_m values for formaldehyde were in general higher (0.072 ± 0.07 to 0.182 ± 0.041 mM), as previously observed for XoxF5 from *Methylomonas* sp. LW13 [23]. One exception was XoxF5_R.k., which revealed poor affinities for propanol, butanol and hexanol (Supplementary Fig. 6). Respectively, K_{eff} values (the proxy for enzyme catalytic efficiency, defined as K_{cat}/K_m), while varied enzyme to enzyme, were four- to eleven-fold higher for methanol compared to formaldehyde (Supplementary Fig. 6).

Interestingly, the two XoxF5 enzymes from the host organism, while 88% identical and 94% similar, demonstrated different kinetic properties. XoxF5_M.e.1 revealed a much higher maximal velocity (V_{max}) rates with all substrates employed in this study, as well as much higher K_{eff} values compared to XoxF5_M.e.2, and it showed highest affinity toward methanol and hexanol as substrates, while XoxF5_M.e.2 revealed highest affinity for methanol (Supplementary Fig. 6). While this manuscript was in revision, kinetic parameters have been presented for XoxF5_M.e.1 by another group [46]. While similar K_m values were reported for methanol and formaldehyde, K_m for ethanol appeared 16-fold higher compared to the value we obtained. In addition, much lower V_{max} values were reported, resulting in K_{eff} values 4.5 to 27 lower than the ones we are reporting here [46]. These discrepancies highlight the difficulty of comparing kinetic data from different studies.

As a XoxF5 type enzyme from *Advenella kashmirensis* has been implicated recently in dehydrogenation of thiosulfate [30], we measured this activity with the eight XoxF5 enzymes purified in this study, utilizing the published assay protocol [30]. No thiosulfate dehydrogenase activity was detected for any of the eight enzymes (Supplementary Fig. 7).

ExaF/PedH_M.s. revealed higher affinities for ethanol and propanol compared to other alcohols, differing this

enzyme from the XoxF5 enzymes, while the affinity for formaldehyde, again, was much lower than for alcohols (Supplementary Fig. 6). However, the difference in catalytic efficiency displayed with methanol versus ethanol was not nearly as dramatic as has been reported for a similar enzyme from *M. extorquens* AM1 (7250-fold) [20]. The K_{eff} value for methanol was only three-fold lower compared to ethanol, in agreement with the robust growth of the host strain on both substrates (Supplementary Table 11). Differently from the XoxF5 enzymes, ExaF/PedH_M.s. did not reveal strict requirement for ammonia, but its activity was significantly stimulated by it (Supplementary Figs. 1 and 2). We were not able to determine kinetic constants for the ExaF/PedH_M.d. due to low specific activity and rapid enzyme activity loss over the course of the purification protocol (Fig. 3, Supplementary Fig. 3; Incubation with either Ln^{3+} or both Ln^{3+} and PQQ could not improve the performance of any of the enzymes we purified in this or in our previous study [23], contrary to reports by others [15, 20]). However, from the complementation experiments and from activity measurements with crude cell extracts, this enzyme revealed properties very different from the ones of other characterized ExaF/PedH enzymes, most importantly, showing no affinity for methanol (Supplementary Table 13). Remarkably, of all the ExaF/PedH enzymes described so far, only ExaF/PedH_M.s. revealed a promiscuous metal dependence, showing (reduced) growth on methanol/ethanol (Supplementary Fig. 1) and (reduced) enzymatic activity (Supplementary Fig. 8) in the absence of Ln^{3+}. Low activity in the absence of Ln^{3+} has previously been reported for purified XoxF5_Me1 [47], and low activity of XoxF4 enzymes in *Methylotenera* species was implicated from growth experiments [48].

We measured metal contents in protein preparations from cultures grown in the presence of both Ln^{3+} and Ca^{2+}, and found that all proteins bound Ln^{3+} (up to 0.9 mol per mol of the enzyme protomer). Several enzymes were purified from cultures grown with cocktails of lighter Ln^{3+}, and in each of these cases, the lightest of the Ln^{3+} were preferentially bound (Supplementary Table 14).

Metabolic reconstruction implicates methylotrophy capabilities for the numerically abundant and environmentally important microbial guilds

As the properties of XoxF5 enzymes from non-methylotrophs were determined here to be rather similar to the properties of XoxF5 enzymes from true methylotrophs, with some variations on catalytic activities and substrate specificities, their function as bona fide MDH is likely, despite the fact that some of these enzymes do not necessarily reveal specificity toward methanol. We analyzed the genomes of *B. diazoefficiens*, *G. marina*, *R. kholense*, *S.*

meliloti, *T. consotensis*, as well as the genome of *A. kashmirensis*, in attempts to reconstruct the potential methylotrophy metabolic pathways, and these could be easily identified. All six genomes encoded, in addition to XoxF5 MDH enzymes, enzymes for formaldehyde oxidation to formate, via the glutathione-linked pathway and further to CO_2, via formate dehydrogenases, and a pathway for CO_2 assimilation via the Calvin–Benson–Bassham cycle, the metabolic scheme typical of some bona fide methylotrophs, such as certain *Paracoccus* species [18] (Table 3). Genes for degradation of an array of other methylated compounds (choline, glycine betaine, dimethylglycine, sarcosine, trimethylamine, trimethylamine N-oxide, dimethylamine, methylamine) were identified in some of the genomes (Table 3), these pathways producing either formaldehyde or methyl-groups as intermediates. This suggests a substantial potential for these organisms in gaining both energy and carbon from single-carbon compounds, thus significantly expanding our understanding of the occurrence of methylotrophy in abundant, globally distributed microbes and the significance of Ln^{3+} in their environmental fitness.

Conclusion

In this study, we more than doubled the number of extant biochemically characterized enzymes that require REEs for their activity and expression, demonstrating that enzymes representing diverse taxa of globally distributed, abundant and environmentally important bacteria likely rely on REEs for their central metabolisms that involve but may not be limited to conversions of alcohols. The wide distribution of REE-dependent enzymes, along with the recently discovered high-affinity mechanisms involved in their acquisition by bacterial cells [49–51] position REEs as true life metals in, and potentially beyond, the bacterial domain of life.

Materials and methods

Bioinformatics

To assess the presence of alternative alcohol dehydrogenases in bacteria, enzymes with previously characterized properties were employed [7, 9, 15, 20, 23, 24]. The IMG/MER database (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi) was searched using the Homolog Display tool, in an iterative fashion as follows. First an enzyme of interest was used to display top homologs in the IMG/M database, and sequences with more than 65% amino acid identity were selected. Based on prior phylogenetic analyses, this cutoff selects members of specific clades of REE

enzymes [18, 19, 27]. Then, the most dissimilar sequence was used to display its top homologs and additional sequences were selected, iterated until the database for each homology group was saturated and no new sequences were identified.

Phylogenetic analysis

Protein sequences were aligned using the CLUSTAL W algorithm, and phylogenetic trees were constructed using the Maximum-Likelihood method, as implemented in the MEGA7 software [52]. Statistical support was obtained from 1000 bootstrap replicates.

Gene synthesis and expression vector construction

The vector used for protein expression in this study was a modified pCM80 vector previously successfully used for expressing genes in *M. extorquens* AM1 [39]. The modified vector, pCM80-PxoxF, contains a 411 bp synthetic DNA fragment inserted between the *mxa* operon promoter (PmxaF) and lacZa. This DNA fragment contains a presumed promoter for the native *xoxF* gene in *M. extorquens* AM1, a ribosome-binding site, a unique *Nco*I restriction site for cloning genes of interest, a TEV cleavage site, and 8x His-tags (Supplementary File 1). The selected gene homologs were codon-optimized for expression in *M. extorquens* AM1 using an empirically derived codon usage table. Codon optimization and vendor defined synthesis constraints removal were performed using BOOST [53]. Synthetic DNA were obtained from Twist Biosciences and cloned into the *Nco*I site of the pCM80-PxoxF vector using the Gibson Assembly method (NEBuilder HiFi, NEB). The *Escherichia coli* Top10 transformants were plated on lysogeny broth (LB) agar plates supplemented with tetracycline ($10 \mu g ml^{-1}$). All gene constructs were sequence-verified using the PacBio RSII sequencing platform (Pacific Biosciences).

Plasmid transfer into host strain

As the expression host strain, we utilized the quadruple mutant of. *M. extorquens* AM1, unable to grow on either methanol or ethanol [17]. Plasmid constructs were transferred From *E. coli* Top10 to the host via three-way conjugation, employing a helper strain *E. coli* 2073. Matings were carried out on nutrient agar plates as described before [54], and progenies were selected on minimal medium plates supplemented with succinate (0.2% W/V) as a substrate and tetracycline ($10 \mu g ml^{-1}$) and rifampicin ($50 \mu g ml^{-1}$) for, respectively, selecting for the plasmid-borne function and counter-selecting for *E. coli* presence.

Growth complementation experiments

The ability of the host strain to grow on methanol (0.5% V/V) or ethanol (0.15% V/V) was tested on plates and in liquid medium, comparing growth on standard minimal media and media supplemented with 30 μM of either of the following Ln^{3+}: La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Dy^{3+}, Yb^{3+}. Plates were incubated at room temperature (~25 °C), and growth patterns were monitored daily for up to 6 weeks. Liquid cultures were incubated in tubes (20×150 mm) filled with 6 ml cultures, and these were shaken on a rotating shaker at 200 rpm at a 45° angle, at 30 °C, with the exception of the construct expressing XoxF5_R.k., which was incubated at 20 °C, as it demonstrated better growth and higher MDH activity at lower temperatures.

Protein expression and purification

For protein expression, 300-ml cultures were grown in shake flasks with succinate to late exponential phase and collected by centrifugation at $4700 \times g$ for 10 min. Pellets were transferred to 100 ml of fresh minimal medium supplemented with methanol (0.5% V/V) and Ce^{3+} (30 μM), and these were incubated overnight at 30 °C with shaking at 200 rpm. Cells were harvested by centrifugation, as above, and used immediately or kept at −80 °C. For XoxF5_R.k., best results were achieved when expression was carried out as above, but at 20 °C, for 2 days. Protein purification was carried out using the previously described protocol [23]. Briefly, cell pellets were resuspended in 0.1 M Tris-HCl buffer pH 9.0 and broken using the French Pressure Cell (Sim-Aminco), at 20,000 psi. His-tagged proteins were purified by Ni-NTA agarose by successive wash steps, finishing with elution step, using standard buffers (start buffer: 0.1 M Tris–HCl pH 9.0, 150 mM NaCl, 5 mM imidazole, 1 mM β-mercaptoethanol (β-ME), a PierceTM protease inhibitor tablet (Thermo Fisher Scientific); wash buffer: 0.1 M Tris–HCl pH 9.0, 150 mM NaCl, 30 mM imidazole, 1 mM β-ME; elution buffer: 0.1 M Tris–HCl pH 9.0, 150 mM NaCl, 250 mM imidazole, 1 mM β-ME.) with one exception. A higher concentration of sodium salts, up to 350 mM, was required for XoxF5_R.k., in all the purification buffers. Proteins were desalted and concentrated via a series of dilution/concentration steps until the concentration of imidazole reached below 1 μM. Purified proteins denatured by sodium dodecyl sulfate (SDS) were analyzed by electrophoresis in 12.5% polyacrylamide gel run with a standard buffer (1× Tris/Glycine/SDS; Bio-Rad Laboratories, Hercules, CA, USA). Protein concentrations were determined using the bicinchonic acid Protein Assay kit (Pierce, Thermo Fisher Scientific), with serum albumin as a standard. Proteins were kept in a storage buffer (0.1 M Tris–

HCl pH 9.0, 1 mM dithiothreitol, 10% glycerol) on ice. Kinetic analyses were carried out within hours of purification.

Methanol dehydrogenase assay

Methanol dehydrogenase activity was measured by monitoring the phenazine methosulfate (PMS; 1 mM)-mediated reduction of 2,6-dichlorophenol-indophenol (DCPIP; 150 μM; $\varepsilon_{600} = 21.0$ mM^{-1}cm^{-1}) as described as previously [23]. Assays were performed at room temperature (~25 °C in a total volume of 0.8 ml in plastic cuvettes (1 cm path length). One unit (U) of specific enzyme activity was defined as 1 μM DCPIP reduced per minute (determined at 600 nm) and was expressed as U per milligram of protein. To determine optimum pH values, standard buffers were used as follows: 100 mM sodium/potassium phosphate (pH range 6.0–8.0), 100 mM Tris-HCl (pH range 7.5–9.0), 100 mM sodium carbonate/ sodium bicarbonate (pH range 9.0–10.0). The effect of ammonia was examined in the standard assay including or omitting 45 mM NH$_4$Cl. Substrate specificities were determined for several alcohols and aldehydes using optimal assay conditions for each enzyme, as follows: 0.1 M Tris–HCl buffer pH 9.0 was used for XoxF5_T.c., XoxF5_M.e.1, XoxF5_R.k., XoxF5_B.d., XoxF5_G.m. and ExaF/PedH_M.s.; 0.1 M Na$_2$CO$_3$/ NaHCO$_3$ buffer pH 9.0 was used for XoxF5_M.e.2 and XoxF5_M.d.; and 0.1 M Tris–HCl buffer pH 9.5 was used for XoxF5_S.m. All substrates were purchased from Sigma Aldrich. Formaldehyde was prepared by incubating paraformaldehyde in water at 95 ℃ for approximately 1 h, until all the powder has been completely dissolved. Enzyme activity was determined at three time points, at 0, 4 and 24 h, using freshly prepared PMS and DCPIP.

Enzyme kinetics

Kinetic parameters were determined using the respective optimal assay conditions, as above, using varying concentrations of select substrates (0.005 to 1 mM). The values of K_m and V_{max} were obtained using the Michaelis–Menten equation, with a GraphPad Software (GraphPad Inc., USA). Averages of kinetic constants were determined for each enzyme based on two to four biologically independent experiments.

Metal analysis

Metals were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) on an ELAN DRC-e (PerkinElmer, Shelton, CT). Standard curves were generated with a dilution series of elements (Ce^{3+}, La^{3+}, Nd^{3+}, Ca^{2+}), with concentrations of 0, 500, 1000, 1500, and 3000 nM in 1%

nitric acid (Thermo Fisher Scientific, Waltham, MA, USA). Ammonia was used in the DRC mode to measure Ca^{2+}. For metal content in proteins, 50–300 μl of purified enzyme (0.5 to 4 mgml^{-1}) was destructed with nitric acid (5 to 10%) at 90 °C for 1 h and diluted to 5 ml with water.

Author contributions LC and JH conceived the study. LC compiled inventories of the ADH genes and carried out plasmid transfer, LC and JH carried out complementation tests. JH and ZY carried out enzyme expression, purification and analysis tasks. ZY carried out phylogenetic analyses. JG carried out metal content quantification. YY supervised the synthetic component of the study. JFC carried out vector and synthetic DNA design. AT generated the expression constructs and carried out sequence verification. LC and JH wrote the manuscript, and all authors approved the final version.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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