Pyrroloquinoline Quinone Ethanol Dehydrogenase in *Methylobacterium extorquens* AM1 Extends Lanthanide-Dependent Metabolism to Multicarbon Substrates

Nathan M. Good,[a] Huong N. Vu,[b] Carly J. Suriano,[a] Gabriel A. Subuyuj,[b] Elizabeth Skovran,[b] N. Cecilia Martinez-Gomez[a]

Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan, USA[a]; Department of Biological Sciences, San José State University, San José, California, USA[b]

ABSTRACT

Lanthanides are utilized by microbial methanol dehydrogenases, and it has been proposed that lanthanides may be important for other type I alcohol dehydrogenases. A triple mutant strain (*mxaF xoxF1 xoxF2*; named MDH-3), deficient in the three known methanol dehydrogenases of the model methylotroph *Methylobacterium extorquens* AM1, is able to grow poorly with methanol if exogenous lanthanides are added to the growth medium. When the gene encoding a putative quinoprotein ethanol dehydrogenase, *exaF*, was mutated in the MDH-3 background, the quadruple mutant strain could no longer grow on methanol in minimal medium with added lanthanum (La^{3+}). ExaF was purified from cells grown with both calcium (Ca^{2+}) and La^{3+} and with Ca^{2+} only, and the protein species were studied biochemically. Purified ExaF is a 126-kDa homodimer that preferentially binds La^{3+} over Ca^{2+} in the active site. UV-visible spectroscopy indicates the presence of pyrroloquinoline quinone (PQQ) as a cofactor. ExaF purified from the Ca^{2+}-plus-La^{3+} condition readily oxidizes ethanol and has secondary activities with formaldehyde, acetaldehyde, and methanol, whereas ExaF purified from the Ca^{2+}-only condition has minimal activity with ethanol as the substrate and activity with methanol is not detectable. The *exaF* mutant is not affected for growth with ethanol; however, kinetic and *in vivo* data show that ExaF contributes to ethanol metabolism when La^{3+} is present, expanding the role of lanthanides to multicarbon metabolism.

IMPORTANCE

ExaF is the most efficient PQQ-dependent ethanol dehydrogenase reported to date and, to our knowledge, the first non-XoxF-type alcohol oxidation system reported to use lanthanides as a cofactor, expanding the importance of lanthanides in biochemistry and bacterial metabolism beyond methanol dehydrogenases to multicarbon metabolism. These results support an earlier proposal that an aspartate residue near the catalytic aspartate residue may be an indicator of rare-earth element utilization by type I alcohol dehydrogenases.

Methylotrophy is the capability of organisms to metabolize reduced carbon compounds lacking carbon-carbon bonds as the sole source of carbon and energy (1). The genus *Methylobacterium* is comprised of aerobic facultative methylotrophs that can metabolize single-carbon compounds, such as methanol and methylamine, as well as multicarbon substrates like ethanol, acetate, ethylamine, pyruvate, and succinate (2, 3). Members of the genus *Methylobacterium* are wide-spread plant epiphytes (4, 5) that utilize their metabolic flexibility to gain an advantage in the phyllosphere, an oligotrophic environment with transient substrate availability (6, 7).

Methanol dehydrogenase (MDH) is an essential enzyme for the methylotrophic metabolism of methanol and methane (8). In Gram-negative methylotrophic bacteria, MDHs are soluble, periplasmic proteins with pyrroloquinoline quinone (PQQ) as the prosthetic group (9, 10). The best studied PQQ-containing MDHs are $\alpha_2\beta_2$ tetramers consisting of the MxaF and MxaI proteins (11–14) that contain calcium (Ca^{2+}) in the active site (15, 16). Studies have provided evidence for the physiological role of a second type of PQQ-dependent MDH, XoxF, which has ~50% amino acid identity to MxaF from MxaFI-type MDHs (17). Metagenomic and environmental proteomics studies have demonstrated that *xoxF* is more widespread than *mxaF* in environmental samples (18–21). Phylogenetic analysis of putative PQQ-containing MDHs has shown that XoxF-type MDHs are genetically diverse with at least

five distinct clades, and it has been suggested that MxaFI-type MDHs represent a minor fraction of these MDHs (8, 22). It has been further proposed that MxaFI-type MDHs may be the result of a second evolutionary event, with an ancestral XoxF-type MDH prototype (22). Together, these suppositions suggest that XoxF-type MDHs may be the primary MDHs for methylotrophy. For the few that have been studied to date, production of functional XoxF-type MDHs requires the presence of lanthanides in the growth medium (23–27) and all reported XoxF-type MDHs that have been biochemically characterized thus far contain a lanthanide such as cerium (Ce^{3+}) or lanthanum (La^{3+}), rather than Ca^{2+} in the active site (25, 27). Limited kinetic studies of XoxF-type MDHs have shown an increased efficiency when oxidizing

methanol compared to that of MxaFI-type MDHs (summarized in reference 22). Functional MxaFI-type MDHs may be produced in the absence of lanthanides in organisms that have both XoxF- and MxaFI-type MDHs, although the number of studies reporting this is limited (28–30).

The genome of the model methylotroph *Methylobacterium extorquens* AM1 contains genes encoding three known PQQ-containing MDH homologs: one MxaFI-type MDH and two XoxF-type MDHs (12). The MxaFI-type MDH is required for growth with methanol in the absence of exogenous lanthanides (25, 29). Of the two XoxF-type MDHs, a catalytic role has been demonstrated only for XoxF1, not for XoxF2 (25), although genetic studies demonstrate the importance of *xoxF2* for growth with methanol in the absence of *xoxF1* (29, 31). XoxF1 and XoxF2 share ~90% amino acid identity and are required for functional expression of the *mxaFI* genes (31). Vu et al. (29) demonstrated that when lanthanides were present in the growth medium, a triple mutant strain (*mxaF xoxF1 xoxF2*; referred to subsequently as MDH-3) of *M. extorquens* AM1 that lacks all known MDHs was still able to grow in methanol medium but with a severe reduction in growth rate (29). This finding suggested that an additional lanthanide-dependent enzyme or pathway that can oxidize methanol exists in *M. extorquens* AM1. This supposition was confirmed by detection of methanol oxidation activity in cell extracts from the MDH-3 mutant strain grown in methanol medium with exogenous La^{3+} (29).

Aside from the aforementioned MDHs, the genome of *M. extorquens* AM1 contains genes encoding two additional predicted PQQ-dependent ADHs. One is annotated as a putative general type I ADH encoded by the open reading frame *META1_4973* (12). This general type I ADH does not contain the second aspartate residue proposed for lanthanide coordination (22). The second enzyme is a possible quinoprotein ethanol dehydrogenase (QEDH) annotated as ExaA, encoded by *META1_1139*. Biochemical studies with ExaA homologs purified from *Rhodopseudomonas* and *Pseudomonas* strains demonstrated that ExaA enzymes are calcium-dependent dehydrogenases capable of oxidizing a broad range of alcohols, including methanol (32–35). Like the MxaFI-type MDH, *in vitro* activity assays require a high pH (~pH 9) and ammonia or a primary amine as an activator (32, 35–37). Despite the methanol-oxidizing capability of these enzymes, only low growth yields with methanol have been reported under aerobic conditions, calling into question whether or not ExaA is capable of supporting growth with methanol for the MDH-3 mutant of *M. extorquens* AM1. Intriguingly, however, ExaA from *M. extorquens* AM1 contains an aspartate residue proposed to be properly positioned for lanthanide coordination.

We examined the predicted ExaA and type I ADH from *M. extorquens* AM1 as potential lanthanide-utilizing alcohol dehydrogenases. In this work, we report that the ExaA quinoprotein ethanol dehydrogenase, and not the type I ADH, is responsible for methanol oxidation in the MDH-3 mutant strain. ExaA was purified and biochemically characterized. Unlike other ExaA homologs reported, ExaA from *M. extorquens* AM1 utilizes La^{3+} rather than Ca^{2+} as a cofactor. Due to its La^{3+}-dependent nature, we propose to rename ExaA in *M. extorquens* AM1 as ExaF to distinguish it from other ExaA homologs. We show that ExaF is functionally an ethanol dehydrogenase, with secondary activities with formaldehyde, methanol, and acetaldehyde. Our *in vitro* data suggest that ExaF may catalyze the sequential oxidations of etha-

nol to acetaldehyde and then to acetate. We identify ExaF as the newest member of the lanthanide-containing enzymes, demonstrating for the first time that lanthanides are utilized by enzymes other than XoxF-type MDHs and showing that lanthanides are relevant beyond single-carbon microbial metabolism.

MATERIALS AND METHODS

Chemicals. Potassium phosphate trihydrate was purchased from Acros Organics (New Jersey). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

Bacterial strains and plasmids. *M. extorquens* AM1 strains and *Escherichia coli* strains and plasmids used in this work are described in Table 1. Null mutations in *pqqF*, *exaF*, and *META1_4973* were generated using the allelic-exchange suicide plasmid pCM184 (38) with the wild-type strain as a recipient. The kanamycin (Km) resistance cassette in the *mxaF xoxF1 xoxF2*::Km mutant strain described in a previous study (29) was deleted using the *cre* expression vector pCM157 as described previously (38). *META1_4973* and *exaF* were individually mutated in the MDH-3 mutant background to generate the MDH-3 *META1_4973*::Km and MDH-3 *exaF*::Km strains (Table 2). Diagnostic colony PCR was used to confirm the Km insertion and deletion. The Km resistance cassette was removed from the MDH-3 *exaF*::Km strain as described above, and the quadruple mutant was complemented using pNG265 (see Tables 3 and 4).

Protein production constructs were generated using the *mxa* and *xox1* promoter regions, *exaF*, and sequence encoding 6 histidines. The PCR products were assembled using Gibson Assembly master mix (39, 40) (New England BioLabs, Ipswich, MA). Primers used for plasmid construction are listed in Table 2 and were designed to generate insert fragments with 20-bp overlapping regions to the respective backbone fragments. Briefly, the promoterless, kanamycin-selectable IncP cloning vector pAWP78 (Addgene, Cambridge, MA) was amplified as a linear PCR fragment using the pAWP_backbone primers. The lanthanide-inducible promoter region of *xoxF1* (P*xox1*) (29) was amplified from *M. extorquens* AM1 wild-type genomic DNA using the pLB01 primers. The pLB01 reverse primer contains a hexahistidine sequence and Factor Xa protease cleavage site for tag removal. The two fragments were joined together, generating plasmid pLB01. pLB01 was used as the template DNA to PCR amplify the pAWP78 backbone DNA with P*xox1* using the pAWP78_backboneFor and the pLB01_backboneRev primers. The gene encoding the putative quinoprotein ethanol dehydrogenase (*exaF*) was amplified from *M. extorquens* AM1 genomic DNA using the *exaF* forward and reverse primers. The reverse primer contains a hexahistidine sequence and Factor Xa protease cleavage site. The backbone and insert fragments were assembled to generate pNG265. Using pNG265 as the template DNA, a linear DNA fragment of pAWP78 with P*xox1* was generated using the pNG267 forward and reverse primers. The fragment was recircularized using assembly methods to generate the "empty" plasmid pNG267. Using pNG265 as the template DNA, a backbone DNA fragment was generated containing the promoterless, linearized plasmid. The *mxa* promoter region (41) was PCR amplified using the P*mxa* forward and reverse primers. The backbone and insert fragments were assembled, generating pNG271.

E. coli TOP10 cells (Sigma-Aldrich, St. Louis, MO) were used for cloning and plasmid propagation as reported previously (42). All plasmids were verified by colony PCR and sequencing. Mating of plasmids into *M. extorquens* AM1 was performed using *E. coli* S17-1 (Sigma-Aldrich, St. Louis, MO) cells for conjugal transfer as reported previously (41).

Growth conditions. *E. coli* cultures were grown in culture tubes with Luria-Bertani broth purchased from BD (Franklin Lakes, NJ). For *M. extorquens* AM1 cultures, glassware was prepared as reported in reference 29. *M. extorquens* AM1 cultures were grown in minimal medium (43) in borosilicate culture tubes, polystyrene round-bottom tubes, or shake flasks at 30°C with 15 mM succinic acid, 34 mM ethanol, or 125 mM methanol added as the growth substrate. A 2 μM concentration of

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
TOP10	Competent cells for cloning	Invitrogen
S17-1	Helper strain for conjugation	57
M. extorquens		
AM1	Rifamycin-resistant derivative	58
AM1 *exaF*::Km	Deletion mutant	This study
AM1 *META1_4973*::Km	Deletion mutant	This study
AM1 *pqqF*::Km	Deletion mutant	This study
AM1 MDH-3	Δ*mxaF* Δ*xoxF1* Δ*xoxF2* deletion mutant	This study
AM1 MDH-3 *exaF*::Km	Deletion mutant	This study
AM1 MDH-3 *exaF*	Deletion mutant	This study
AM1 MDH-3 *META1_4973*::Km	Deletion mutant	This study
Plasmids		
pNG265	P*xox1-exaF*-hexahistidine tag	This study
pNG267	P*xox1*-hexahistidine tag	This study
pNG271	P*mxa-exaF*-hexahistidine tag	This study
pLB01	P*xox1-xoxF1*-hexahistidine tag	This study
pAWP78	IncP-based broad-host-range replicating vector	Addgene
pCM184	Km^r Ap^r Tc^r allelic-exchange vector	38
pHV23	pCM184::*xoxF*; donor for *xoxF1*::Km	This study
pHV24	pCM184::*exaF*; donor for *exaF*::Km	This study
pHV25	pCM184::*META1_4973*; donor for *META1_4973*::Km	This study

LaCl$_3$ was added to the growth medium when stated. When antibiotics were necessary, kanamycin (Km) was added to a final concentration of 50 μg/ml.

For purification of enzyme, 200-ml cultures were grown in shake flasks with succinate to late exponential phase and then transferred to 10 liters of fresh minimal medium with methanol and kanamycin in a 10-liter New Brunswick Celligen/BioFlo 115 bioreactor (Edison, NJ). For purification in the presence of La^{3+}, 20 μM LaCl$_3$ was added to the medium. Cultures were grown at 30°C with 2 standard liters-per-minute (SLPM) airflow and the agitation rate set at 1,000 rpm.

Phenotypic analysis. Growth curve measurements were performed as follows: glassware was cleaned of residual lanthanides as described previously (29). Briefly, 3 ml of minimal medium plus 125 mM methanol was inoculated with the wild-type strain grown to mid-exponential phase. Cultures were grown to the maximal optical density at 600 nm (OD$_{600}$) and discarded, and the glassware was washed and sterilized. Next, 2 ml of minimal medium was inoculated from isolated colonies into the lanthanide-free borosilicate culture tubes with 15 mM succinate as the growth substrate. Cultures were incubated at 30°C with shaking at 200 rpm until they reached mid-exponential phase (~OD$_{600}$ 1.0), and then 0.12 to 0.2 ml of preculture was used to inoculate 3 to 6 ml of minimal medium in a new culture tube with 125 mM methanol or 34 mM ethanol as the growth substrate. A 2 μM concentration of La^{3+} was added for Ca^{2+}-plus-La^{3+} conditions. Tube cultures were shaken at 200 rpm at 30°C, and the OD$_{600}$ was monitored over time using an Ultraspec 10 cell density meter (Amersham Biosciences, Little Chalfont, UK) or a Spectronic 20D spectrophotometer (Milton Roy Company, Warminster, PA) (29). Three or four biological replicates were measured for each condition.

Purification of ExaF. Cultures were grown to a final OD$_{600}$ of 3.2 to 4.0 in 2.8-liter baffled flasks with 1.5 liter of methanol minimal medium, both with and without 20 μM LaCl$_3$, and cells were harvested by centrifugation using a Sorvall RC6+ centrifuge (Thermo Fisher Scientific, Waltham, MA) at 21,000 × g at 4°C for 10 min. The supernatant was discarded, and cell pellets were flash frozen with liquid nitrogen and stored at −80°C until needed. Cell pellets were resuspended in a double volume of 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM imidazole and disrupted

using a French pressure cell (Aminco, Haverhill, MA) at 20,000 lb/in^2. Cell debris was removed by ultracentrifugation using a Sorvall WX Ultra 80 centrifuge (Thermo Fisher Scientific, Waltham, MA) for 1 h at 4°C at 100,000 × g. Protein was purified by metal ion affinity chromatography (IMAC) as follows. The supernatant was loaded onto an equilibrated 2 ml Ni-nitrilotriacetic acid (NTA) Superflow resin (Qiagen, Hilden, Germany). A wash step of 5 column volumes was performed with buffer containing 50 mM imidazole, and elution was performed using buffer containing 400 mM imidazole. One-milliliter fractions were collected and analyzed using a 4 to 12% SDS-PAGE gel (Life Technologies, Carlsbad, CA). Pure fractions were pooled, concentrated with an Amicon 10-kDa ultracentrifugal unit (Millipore, Billerica, MA), and desalted on a PD-10 column (GE Healthcare, Pittsburgh, PA) that was equilibrated with 25 mM Tris-HCl (pH 8.0), 150 mM NaCl.

The hexahistidine tag was removed using Factor Xa protease (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Enzyme used for kinetic measurements was reconstituted by combining the purified enzyme with an equimolar concentration of LaCl$_3$ and 20 μM PQQ and incubating the mixture overnight at 4°C. The reconstituted enzyme was desalted and concentrated as mentioned above.

Methanol dehydrogenase activity measurements. Methanol dehydrogenase activity was measured by monitoring the phenazine methosulfate (PMS)-mediated reduction of 2,6-dichlorophenol-indophenol (DCPIP) ($\varepsilon_{600} = 21$ mM^{-1} cm^{-1}) (44). Initial assays were performed as described by Anthony and Zatman (45) with the modifications reported by Vu et al. (29). The assay protocol was then modified as follows: 100 mM Tris-HCl (pH 9.0) was combined with either 100 μM CaCl$_2$ or 100 μM LaCl$_3$, 5 mM methylamine or 15 mM NH$_4$Cl, 10 mM PQQ, 100 μM DCPIP, 100 μM PMS, and 6.25 to 12.5 μl of cell extract (concentrations ranging from 4 to 30 mg/ml) or 0.4 to 1.0 μg of pure enzyme. When cell extracts were used, the reaction mixture was incubated for 5 to 10 min at 30°C to mitigate any methanol-independent reduction of DCPIP. Methanol-independent reduction of DCPIP was not observed with pure enzyme. The reaction was initiated by adding the assay mixture to 10 μl of 1 M methanol in a microplate well, resulting in a final reaction volume of 185 μl. The decrease in absorbance of DCPIP was monitored in a BioTek

TABLE 2 Primers used in this study

Construct or mutation	Primer	Sequence (5′–3′)
Constructs		
pLB01	pAWP_backboneFor	TTGTCGGGAAGATGCGTGATCTG
	pAWP_backboneRev	CAGCTCACTCAAAGGCGGTAATAC
	Pxox1_xoxF1For	CGTATTACCGCCTTTGAGTGAGCTGCTGAATTTAGCAGGCAAGTTTCCTG
	Pxox1_xoxF1_6xhisRev	ATCAGATCACGCATCTTCCCGACAATTAGTGGTGATGGTGATGATGACG ACCTTCGATGTTGTTCGGCAGCGAGAAGACCG
pNG265	pLB01_backboneRev	GGATTCCTCCGACAAGTCTTATCCG
	exaFFor	TAAGACTTGTCGGAGGAATCCATGAGAATGCGGAACCATTTCCTG
	exaFRev	ATCAGATCACGCATCTTCCCGACAATTAGTGGTGATGGTGATGATGACG ACCTTCGATTCGGGAGGCGAGCGCCTTC
pNG267	Forward	CTTGTCGGAGGAATCCATCGAAGGTCGTCATCATCACCA
	Reverse	GGATTCCTCCGACAAGTCTTATCCG
pNG271	backboneFor	TTGTCGGGAAGATGCGTGATCTG
	backboneRev	ATCAGATCACGCATCTTCCCGACAATTAGTGGTGATGGTGATGATGACG ACCTTCGATTCGGGAGGCGAGCGCCTTC
	PmxaFor	TACCGCCTTTGAGTGAGCTGCCCGCTTGGTCGG
	PmxaRev	CAGCTCACTCAAAGGCGGTAATAC
Null mutations		
exaF	exaF_1139ULEcoRI	GTGAATTCCCTCCGACAGCCTACTGATGAAC
	exaF_1139URKpnI	CAGGTACCGTCTTGGCGTCGTTGAGGATGTC
	exaF_1139DLHpaI	CAGTTAACGCCGTTCGTGTCCAACATCAACTG
	exaF_1139DRSacI	TAGAGCTCGTTGATGCCAGCCGTCGTCTTGG
META1_4973	adh_4973ULEcoRI	TGAATTCAGCAGAGTGACACGCCGCACGAAG
	adh_4973URKpnI	CAGGTACCACGAACCTTGGACCGGCTTGTC
	adh_4973DLHpaI	TGTTAACCGAGATCCTCTGGCGCTTCCAGT
	adh_4973DRSacI	TGAGCTCTCCACTACATCCATTACGGCTGG
pqqF	pqqF_2330ULEcoRI	TAGAATTCACCGTCCCGCCATCACCTATCGC
	pqqF_2330URKpnI	ATGGTACCGTGATTCAGGCCTCGCTGCGGTC
	pqqF_2330DLHpaI	TAGTTAACGGTGACCGGCTACCTGACCAAGG
	pqqF_2330DRSacI	TAGAGCTCGTAGCGCAGGCTGGCGATCATCT
xoxF1	xoxF_1740ULEcoRI	TAGAATTCGCCCCGATGGCAGGAATTAAAGT
	xoxF_1740URKpnI	ATGGTACCCTGCTTCGGCTCGTACTTCCACA
	xoxF_1740DLHpaI	TAGTTAACGCCTGTTCTACGTCCCCACCAAC
	xoxF_1740DRSacI	TAGAGCTCCTGCACCGACGAGACCAAGAAGA

EpochII microplate reader (BioTek, VT). Methanol dehydrogenase activity was also monitored using cytochrome c isoform 1 from *Saccharomyces cerevisiae* as the final electron acceptor and monitoring its reduction at 550 nm under oxic conditions. For this assay, 3.5 to 30 μg of pure enzyme was added to a final assay volume of 1 ml. Protein concentrations were measured using the bicinchoninic acid assay (Sigma-Aldrich, St. Louis, MO) (46) according to the manufacturer's instructions.

Enzyme kinetics. Kinetic parameters for ExaF were determined using the second assay detailed above with various substrate concentrations. This assay was used due to the minimal or complete absence of substrate-independent reduction of DCPIP. If background activity was detected, it was subtracted from enzyme activity measurements. Data were fitted by nonlinear regression, and kinetic parameters were calculated according to the Michaelis-Menten equation using Prism GraphPad 6 (GraphPad Software, La Jolla, CA).

M_r determination. The molecular weight of the purified protein sample was determined by size exclusion chromatography-multiangle light scattering (SEC-MALS) using a Superdex 200 Increase 10/300 GL column (Bio-Rad, Hercules, CA) with 25 mM Tris-HCl buffer (pH 8) containing 150 mM sodium chloride and a miniDAWN TREOS multiangle light scattering detector with an Optilab T-rEX refractometer (Wyatt, Santa Barbara, CA). Data analysis was performed using Astra software according to the manufacturer's specifications (Wyatt, Santa Barbara, CA).

Identification of cofactor and metal content. The absorbance spectrum of the enzyme as purified was recorded in a 1-cm-path-length cuvette at room temperature using a UV-2600 UV-Vis spectrophotometer (Shimadzu, Columbia, MD). The PQQ concentration and content were calculated using the molar absorption coefficient of 9,620 $M^{-1} \cdot cm^{-1}$ (47). The metal content of the enzyme as purified was determined by inductively coupled plasma mass spectrometry (ICP-MS) at the Michigan State University Laser Ablation ICP-MS Facility using a Thermo Fisher Scientific ICAP Q ICP-MS (Waltham, MA) in collision cell mode.

ExaF homology model. The amino acid sequence for ExaF was submitted to the Phyre2 Web portal (48) and analyzed in intensive mode for structural homology. The template with the highest homology, PDB 1FLG, was chosen for further analysis. Overlay of our ExaF homology model with PDB 1FLG was performed using PyMOL (49).

RESULTS

ExaF supports growth with methanol in the presence of lanthanum. Growth studies demonstrated that the MDH-3 mutant strain, deficient in the three known MDHs, is still capable of growth in methanol minimal medium when lanthanides are exogenously supplied (29) (Fig. 1; Table 3). Besides the genes known to encode MDH homologs (*mxaF*, *xoxF1*, and *xoxF2*), the *M. extorquens* AM1 genome contains genes encoding a putative quinoprotein alcohol dehydrogenase (META1_4973) and a possible quinoprotein ethanol dehydrogenase (ExaF; META1_1139). Null mutations were constructed for each putative quinoprotein in the

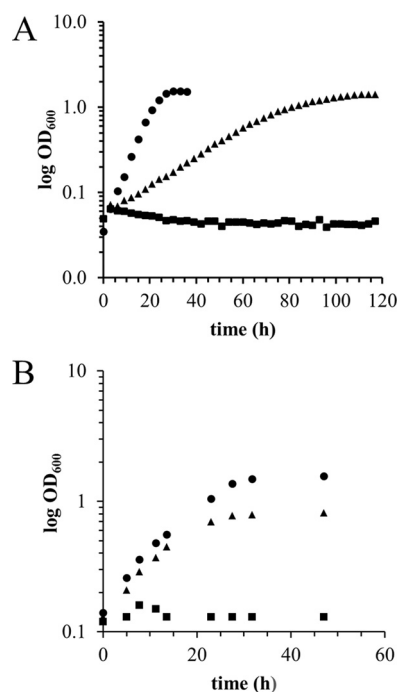FIG 1 La^{3+}-dependent methylotrophic growth with methanol and ethanol. Optical density was monitored over time for cultures grown in minimal medium with 125 mM methanol (A) or 34 mM ethanol (B) as the growth substrate. The growth medium was supplemented with 2 μM LaCl$_3$. Represented strains are wild-type *M. extorquens* AM1 (circles), MDH-3 (triangles), and MDH-3 *exaF*::Km (squares). The results are representative of three biological replicate cultures for each strain.

MDH-3 mutant strain background, and growth in methanol medium with La^{3+} was measured (Fig. 1; Table 3). The additional loss of *META1_4973* encoding the putative type I ADH showed no additional decrease in growth rate from the MDH-3 mutant strain (Table 3). In contrast, a quadruple mutant lacking the predicted quinoprotein ethanol dehydrogenase (*exaF*; *META1_1139*) eliminated growth, indicating that ExaF is responsible for the unknown methanol oxidation activity reported by Vu et al. (29) (Fig. 1; Table 3). The MDH-3 *exaF* mutant was complemented for growth with both methanol and ethanol using pNG265. The mutant was not complemented for growth with either substrate using

TABLE 3 Growth rate constants of *M. extorquens* strains grown with methanol as the carbon source

Strain	Growth rate constant (h^{-1}) for strain grown in[a]:	
	CH$_3$OH (no La)	CH$_3$OH (+La)
Wild-type strain AM1	0.147 (1.6)	0.152 (1.5)
AM1 MDH-3	No growth	0.042 (1.5)
AM1 *pqqF*::Km	No growth	No growth
AM1 *META1_4973*::Km	0.151 (1.6)	0.158 (1.5)
AM1 *exaF*::Km	0.161 (1.6)	0.152 (1.5)
AM1 MDH-3 *META1_4973*::Km	No growth	0.042 (1.5)
AM1 MDH-3 *exaF*::Km	No growth	No growth
AM1 MDH-3 *exaF*/pNG265	No growth	0.039 (0.3)

[a] Results are the mean from three or four biological replicates. Final growth yields determined by light scattering at OD$_{600}$ are shown in parentheses. Variances in growth rates and yields among replicates for each strain are <10%.

TABLE 4 Growth rate constants of *M. extorquens* strains grown with ethanol as the carbon source

Strain	Growth rate constant (h^{-1}) for strain grown in[a]:	
	C$_2$H$_6$O (no La)	C$_2$H$_6$O (+La)
Wild-type strain AM1	0.085 (1.3)	0.095 (0.9)
AM1 MDH-3	No growth	0.097 (1.6)
AM1 *exaF*::Km	0.080 (1.4)	0.080 (0.8)
AM1 MDH-3 *exaF*::Km	No growth	No growth
AM1 MDH-3 *exaF*/pNG265	No growth	0.061 (1.4)

[a] Results are the mean from three or four biological replicates. Final growth yields determined by light scattering at OD$_{600}$ are given in parentheses. Variances in growth rates and yields among replicates for each strain are <10%.

pNG271 (Tables 3 and 4). The strain lacking only *exaF* did not exhibit a growth defect compared to the wild-type strain in the presence of La^{3+}. These results suggest that while ExaF can contribute to methanol growth with La^{3+}, it is not required in the wild-type strain, and the methanol oxidation capacity of the MDH-3 mutant may be due to a promiscuous activity of ExaF using methanol as an alternative substrate. To demonstrate that ExaF requires PQQ as a cofactor, a gene encoding an essential PQQ synthesis protein, *pqqF* (*META1_2330*) (50, 51), was mutated. The *pqqF* mutant was unable to grow in methanol medium with La^{3+}, suggesting that PQQ is essential for all La^{3+}-dependent methanol oxidation enzymes in *M. extorquens* AM1, including ExaF (Table 3).

MDH activity of ExaF in cell extracts. MDH activity was measured in cell extracts from the MDH-3 mutant strain grown with 125 mM methanol and La^{3+} (29) but found to be very low (9 nmol · mg^{-1} · min^{-1} compared to 81 nmol · mg^{-1} · min^{-1} for the wild-type strain) when the traditional DCPIP-linked assay was used as described by Anthony and Zatman (45). By modifying the DCPIP-linked MDH assay (see Materials and Methods), including the use of methylamine rather than ammonium as the activator, we detected 5-fold-higher activity of ExaF in cell extracts of the MDH-3 mutant strain (45 ± 2 nmol · mg^{-1} · min^{-1}) than what was previously reported (29). This finding indicates that methylamine is a better activator for ExaF *in vitro* than ammonium. With our modifications to the assay, we detected minimal to no methanol-independent reduction of DCPIP in the "no-substrate" control assay preparations with PMS as an artificial electron acceptor and methylamine as an activator, making it an improved protocol for measuring the enzyme kinetics for ExaF.

Purification of ExaF. As growth of the MDH-3 mutant with methanol as a substrate is dependent on the addition of exogenous La^{3+} to the medium, biochemical investigation of ExaF was performed to directly test the metal specificity.

His-tagged (C-terminal) ExaF was expressed in wild-type *M. extorquens* AM1 from the La^{3+}-inducible promoter P*xox1* in the presence of La^{3+} (29, 30) or from P*mxa* in the absence of La^{3+} in minimal methanol medium. MDH activity was not detectable in extracts from cultures lacking La^{3+} when methylamine was added as the activator for the assay. ExaF-His$_6$ from both cultures, with (ExaF-La) and without (ExaF-Ca) La^{3+}, was purified (Fig. 2A), and the histidine tag was cleaved. Purified ExaF-La had a specific activity for methanol of 6.5 ± 0.2 μmol · mg^{-1} · min^{-1} as measured following the reduction of DCPIP. A 40% decrease in activity was observed for ExaF-La when ammonium replaced methyl-
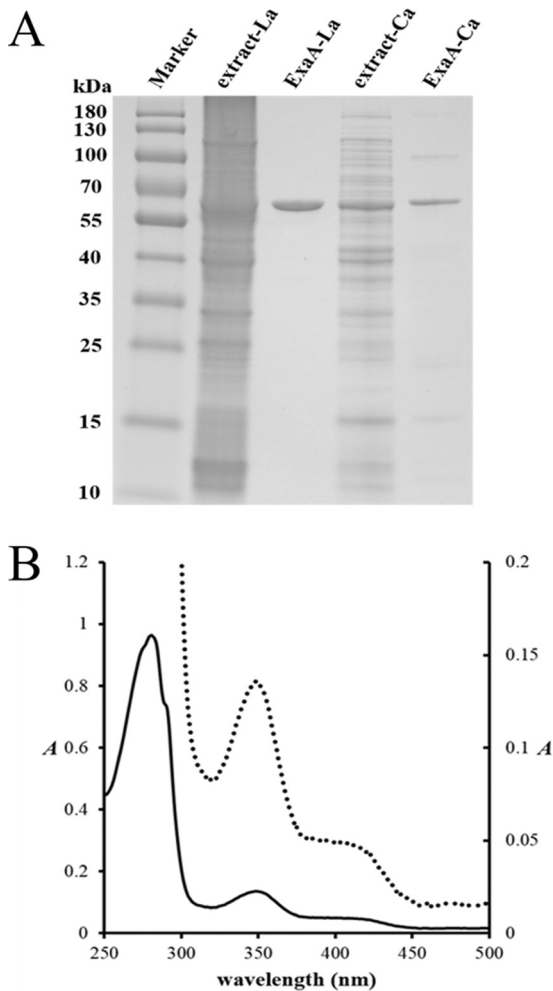
FIG 2 Production, purification, and UV-Vis analysis of ExaF. (A) SDS-PAGE analysis of ExaF produced with or without La³⁺. ExaF was produced in wild-type *M. extorquens* AM1 using either pNG265 with the La-inducible *xox1* promoter or pNG271 with the *mxa* promoter, which is constitutive in the absence of exogenous lanthanides. Cells were grown in methanol minimal medium with (pNG265) or without (pNG271) La³⁺ and purified by IMAC. Lanes (left to right): protein marker, cell extract with La³⁺, purified ExaF from culture grown with La³⁺, cell extract without La³⁺, and purified ExaF from culture grown without La³⁺. (B) UV-visible spectrum of purified ExaF. Enzyme (0.9 mg/ml) was prepared in 25 mM Tris-HCl, pH 8.0. The solid line corresponds to the left axis. The dotted line corresponds to the right axis.

amine as the activator. Specific activities of 6 ± 2 (pH = 7) and 37 ± 4 (pH = 9) nmol $\cdot$ mg^{-1} $\cdot$ min^{-1} for ExaF-La were measured following the reduction of cytochrome *c*. Purified ExaF-Ca did not exhibit any oxidation activity toward methanol. ExaF-La was stable when kept on ice at 4°C for at least 72 h, retaining >90% activity. After 7 days under the same conditions, the enzyme retained >50% of the initial activity. ExaF-La that had been frozen with 10% glycerol and stored at −80°C was no longer active after 12 h, but activity could be recovered by incubating the thawed enzyme with an equimolar concentration of LaCl₃ and 20 μM PQQ.

Analytical characterization of ExaF. ExaF exhibited an apparent molecular mass of ~61 kDa for the denatured protein (Fig. 2A), consistent with the expected value based on the gene sequence. The native molecular mass of ExaF was determined by SEC-MALS to be 126.4 ± 2.3 kDa, corresponding to a dimer. The UV-visible absorption spectrum of the purified enzyme (14.2 μM) is consistent with spectra of other PQQ-containing enzymes (32–34), with a broad shoulder and maximum absorbance from 345 to 350 nm in addition to transitions near 280 nm from the aromatic residues (Fig. 2B). Using the molar extinction coefficient of 9,620 M^{-1} $\cdot$ cm^{-1} (47) for PQQ, the concentration of bound quinone was 13.2 μM, indicating a 1:1 molar ratio to protomer of enzyme. The pronounced absorbance at 345 nm was suggestive of a metal ion in the enzyme active site (52, 53). ICP-MS measurements of the purified enzyme from culture grown with La³⁺ determined 1.3 mol of La³⁺ per mol of ExaF protomer, indicating a 1:1 ratio of La³⁺ to protomer of enzyme.

Enzyme kinetics of ExaF with one-carbon substrates. The capacity to generate formate as the end product of methanol oxidation has been suggested for XoxF-MDH (22, 27). Our initial biochemical and phenotypic studies suggested that ExaF may exhibit a similar catalytic property, thus warranting further biochemical investigation of the enzyme. To gain further insight into the catalytic properties of ExaF with one-carbon substrates, we measured kinetic parameters of ExaF with methanol and formaldehyde. V_{max} and K_m values are reported in Fig. 3C. The maximal reaction velocity of ExaF-La for formaldehyde was 23% greater than for methanol as the substrate. The K_m for formaldehyde was in the micromolar range and ~100 times lower than the K_m for methanol. Thus, k_{cat}/K_m determinations show that ExaF-La is 100 times more efficient at oxidizing formaldehyde than methanol. Activity

C

substrate	K_m (μM)	V_{max} (U·mg^{-1})	k_{cat}/K_m (mM^{-1}s^{-1})
methanol	5,980	6.6	2
formaldehyde	66	8.1	259

D

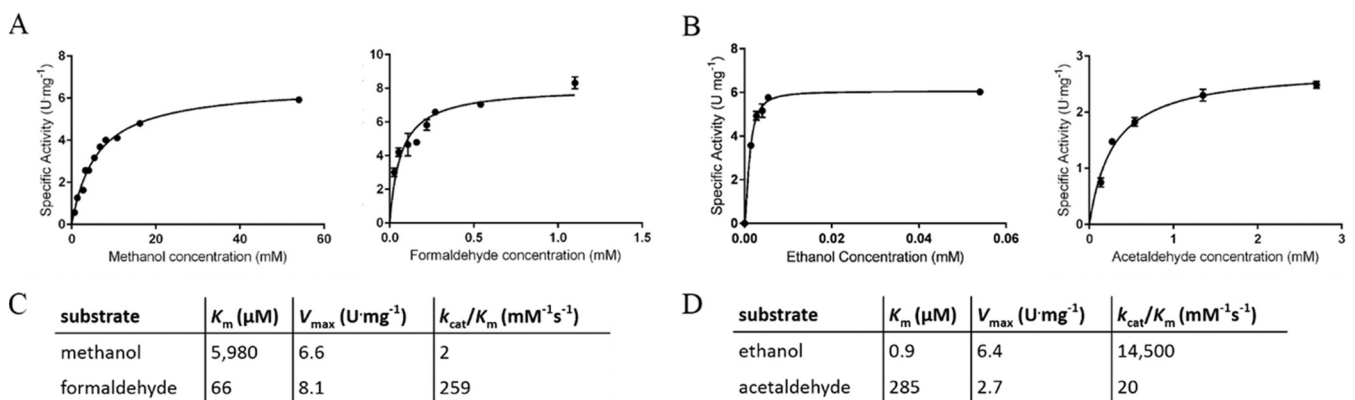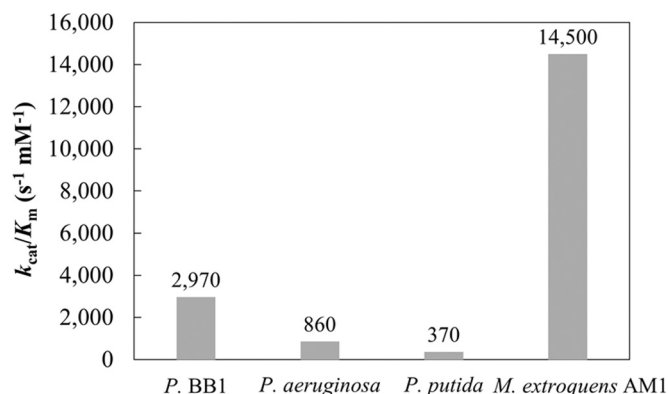| substrate	K_m (μM)	V_{max} (U·mg^{-1})	k_{cat}/K_m (mM^{-1}s^{-1})
ethanol	0.9	6.4	14,500
acetaldehyde	285	2.7	20

FIG 3 ExaF kinetic parameters with one- and two-carbon substrates. Michaelis-Menten plots are shown for methanol and formaldehyde (A) and ethanol and acetaldehyde (B). Kinetic parameters are given for one-carbon substrates (C) and two-carbon substrates (D).

FIG 4 Catalytic efficiencies of quinoprotein ethanol dehydrogenases reported as k_{cat}/K_m. Enzymes are represented as follows: *P.* BB1, QEDH (54); *P. aeruginosa*, QEDH (32); *P. putida*, QEDH (37); *M. extroquens* AM1, ExaF (QEDH) (this study). Values are for ethanol as the substrate.

FIG 5 ExaF homology model. (A) Predicted structure of ExaF (shown in cartoon view; gray) using the crystal structure of the quinoprotein ethanol dehydrogenase QEDH from *P. aeruginosa* (green; PDB 1FLG) (59) as the template. (B) Expanded view of the catalytic site of ExaF. PQQ is shown in yellow. La³⁺ metal ligand is shown in magenta. The La³⁺ position was modeled after the Ca²⁺ in the QEDH structure. Amino acids predicted to coordinate with La³⁺ are numbered according to their positions in the structure. The aspartate residue (D319) that has been suggested to be necessary for lanthanide coordination is highlighted in green, next to the predicted catalytic aspartate (D317).

was not detected for ExaF-Ca with methanol. The kinetic measurements indicate that ExaF-La can catalyze the oxidation of formaldehyde and support the possibility that ExaF-La is capable of oxidizing methanol to formate *in vivo*. Interestingly, ExaF-La is also an efficient formaldehyde dehydrogenase (Fig. 3C), suggesting a possible role in formaldehyde metabolism.

Enzyme kinetics with two-carbon substrates. The low efficiency of ExaF methanol oxidation suggests that methanol is not the primary substrate of this enzyme. Because ExaF is a homolog of the quinoprotein ethanol dehydrogenases, we investigated the kinetic properties of ExaF with the two-carbon substrates, ethanol and acetaldehyde. V_{max} and K_m values for ExaF-La are reported in Fig. 3D. The V_{max} with ethanol was 6.3 U · mg⁻¹, similar to the maximal velocity with methanol; however, the K_m for ethanol was determined to be 0.9 μM, compared to 14, 11, and 163 μM for homologous enzymes from *Pseudomonas* strains (32, 37, 54), making it the lowest value ever reported for a quinoprotein ethanol dehydrogenase. When limiting concentrations of ethanol were added to the assay, approximately two times the molar equivalent of DCPIP was reduced (~230%), indicating that ethanol was being oxidized to the level of acetic acid. The V_{max} with acetaldehyde was 2.7 U · mg⁻¹, 3-fold lower than the maximal velocity observed with formaldehyde, while the K_m for acetaldehyde was over 4-fold higher than that of formaldehyde. In comparison, ExaF-Ca oxidation activities with ethanol (7 nmol · mg⁻¹ · min⁻¹) or acetaldehyde (24 nmol · mg⁻¹ · min⁻¹) as the substrate for this form of the enzyme were considerably lower. Taken together, the kinetic analyses demonstrate that ExaF is a highly efficient, lanthanide-dependent ethanol dehydrogenase.

Phenotypic analysis of ExaF-dependent growth with ethanol. Because our kinetic studies strongly indicated that ethanol is its primary substrate, we assessed the role of ExaF in ethanol metabolism. Growth rate constants were determined for the wild-type, MDH-3, and the MDH-3 *exaF*::Km strains using ethanol as their sole carbon source (Table 4; Fig. 1B). The wild-type strain grew similarly in the presence and absence of La³⁺; however, the MDH-3 mutant strain grew only when exogenous La³⁺ was added to the growth medium. Additional loss of *exaF*::Km in the MDH-3 mutant background eliminated this growth, suggesting a contribution by ExaF during ethanol growth when La³⁺ is available. The single *exaF* mutant did not exhibit a growth defect compared to
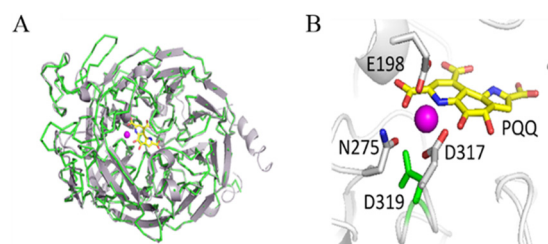
the wild-type strain with or without exogenous La³⁺, indicating that either XoxF-type MDH (in the presence of La³⁺) or MxaFI-type MDH (in the absence of La³⁺) can support growth with ethanol as well.

DISCUSSION

A role for lanthanides in microbial metabolism has only recently been discovered, and its study continues to uncover surprises. Prior to this work, XoxF-type MDHs were the only enzymes known to contain lanthanides in their active sites (27). ExaF (sometimes referred to as QEDH) homologs from *P. aeruginosa*, *Rhodopseudomonas*, and *Acinetobacter* have all been reported to contain Ca²⁺ (32, 33, 35, 37, 55, 56). We were unable to detect oxidation activity for ExaF from *M. extorquens* AM1 in cell extracts from culture grown without La³⁺. Pure ExaF-Ca did not exhibit activity with methanol and exhibited minimal activity with ethanol. ICP-MS measurements confirmed that ExaF preferentially binds La³⁺, and kinetic analyses showed that ExaF oxidizes ethanol and methanol at similar rates but that it has a clear substrate preference for ethanol (Fig. 3A and C). Interestingly, activity following the reduction of cytochrome *c* at neutral pH was detected under oxic conditions, providing insights to design an activity assay independent of redox dyes. Compared to the other quinoprotein ethanol dehydrogenases, ExaF is a remarkably efficient enzyme (Fig. 4). ExaF is most similar to the quinoprotein ethanol dehydrogenase from *P. aeruginosa*. Their amino acid sequences are 57% identical, and structural homology is over 90% with a root mean square deviation of 0.246 (Fig. 5). However, of the quinoprotein ethanol dehydrogenases, only QEDH from *Pseudomonas putida* has an aspartate residue positioned for lanthanide coordination.

It has been proposed that La³⁺ and lanthanides in general may function as more potent Lewis acids than Ca²⁺, generating a stronger polarization of PQQ for catalysis (22). It has also been proposed that incorporation of a lanthanide into the active site may modify the kinetic properties of the enzyme, as suggested by two XoxF-type MDHs that demonstrate relatively higher formaldehyde oxidation efficiencies than those of MxaF-type MDHs (22, 27). Formaldehyde is a highly toxic intermediate that is integral to methylotrophy. ExaF can also oxidize formaldehyde with relatively high efficiency, suggesting a possible contribution by ExaF

to methylotrophy. This result contrasts ExaF with other reported QEDH enzymes that are generally inefficient catalysts for formaldehyde oxidation (32, 33, 35, 56).

Our phenotypic analyses show that in the MDH-3 mutant strain, ExaF supports growth with methanol when exogenous La^{3+} is added to the medium. The kinetic analysis with methanol indicates that ExaF is not an efficient methanol dehydrogenase, suggesting that the methanol oxidation detected *in vivo* in the MDH-3 mutant strain is due to a promiscuous activity of ExaF. This low activity is a likely explanation for the lower growth rate with methanol observed for the MDH-3 mutant. It is unclear at this time why growth yields are 2-fold higher in the MDH-3 strain than in the wild type when grown in medium with ethanol and La^{3+}. One possibility is that loss of the MDHs affects expression of *exaF*, as XoxF has previously been shown to affect expression of *xoxF* and *mxaF* (31). If *exaF* expression is upregulated in the MDH-3 mutant, this may allow for increased growth yields, as ethanol may be converted to acetate, preventing the loss of valuable carbon as volatile acetylaldehyde. Alternatively, the metabolic network may be altered in the MDH-3 mutant, redirecting carbon and nutrient flow. A systems biology approach would be helpful in identifying the cause of yield difference.

The lack of a growth rate defect in the MDH-3 strain grown with ethanol as a sole carbon source demonstrates the contribution of ExaF to ethanol oxidation *in vivo*. Therefore, we add ExaF to the lanthanide-utilizing enzymes and demonstrate that the role of lanthanides in microbial metabolism extends beyond XoxF-type MDHs and one-carbon metabolism. It is unclear, however, whether or not ExaF is the primary dehydrogenase utilized to oxidize ethanol when La^{3+} is available, since the *exaF* single mutant does not exhibit a growth defect with ethanol compared to the wild-type strain. It is likely that XoxF also contributes to ethanol growth with La^{3+}, although kinetic studies of XoxF1 from *M. extorquens* AM1 are needed to determine its capacity for oxidizing substrates other than methanol. Further studies involving genetic and phenotypic growth analyses might determine which enzyme, if either, is the predominant catalyst for ethanol oxidation *in vivo* in the presence of La^{3+}. Alternatively, both ExaF and XoxF may be produced to provide more robust alcohol oxidation capacity than a single dehydrogenase. *Methylobacterium* species are often associated with the aerial parts of the plant, or the phyllosphere, which is considered a dynamic and oligotrophic environment. Some phyllosphere metabolites can be utilized for growth by these facultative methylotrophic plant epiphytes and include carbohydrates, amino acids, and organic volatile compounds, such as methanol, ethanol, and acetate. The metabolic capability for methanol metabolism in *Methylobacterium* confers a fitness advantage *in situ* (6, 7). In the presence of La^{3+}, the XoxF-type MDH is the primary methanol dehydrogenase; however, its capacity for ethanol oxidation is unknown. Producing an arsenal of enzymes to utilize multiple substrates in a nutrient-poor environment such as the phyllosphere may confer a metabolic advantage as well.

ACKNOWLEDGMENTS

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