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Identification of a Third Mn(II) Oxidase Enzyme in *Pseudomonas putida* GB-1

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ABSTRACT

The oxidation of soluble Mn(II) to insoluble Mn(IV) is a widespread bacterial activity found in a diverse array of microbes. In the Mn(II)-oxidizing bacterium *Pseudomonas putida* GB-1, two Mn(II) oxidase genes, named *mnxG* and *mcoA*, were previously identified; each encodes a multicopper oxidase (MCO)-type enzyme. Expression of these two genes is positively regulated by the response regulator MnxR. Preliminary investigation into putative additional regulatory pathways suggested that the flagellar regulators FleN and FleQ also regulate Mn(II) oxidase activity; however, it also revealed the presence of a third, previously uncharacterized Mn(II) oxidase activity in *P. putida* GB-1. A strain from which both of the Mn(II) oxidase genes and *fleQ* were deleted exhibited low levels of Mn(II) oxidase activity. The enzyme responsible was genetically and biochemically identified as an animal heme peroxidase (AHP) with domain and sequence similarity to the previously identified Mn(II) oxidase MopA. In the Δ*fleQ* strain, *P. putida* GB-1 MopA is overexpressed and secreted from the cell, where it actively oxidizes Mn. Thus, deletion of *fleQ* unmasked a third Mn(II) oxidase activity in this strain. These results provide an example of an Mn(II)-oxidizing bacterium utilizing both MCO and AHP enzymes.

IMPORTANCE

The identity of the Mn(II) oxidase enzyme in *Pseudomonas putida* GB-1 has been a long-standing question in the field of bacterial Mn(II) oxidation. In the current work, we demonstrate that *P. putida* GB-1 employs both the multicopper oxidase- and animal heme peroxidase-mediated pathways for the oxidation of Mn(II), rendering this model organism relevant to the study of both types of Mn(II) oxidase enzymes. The presence of three oxidase enzymes in *P. putida* GB-1 deepens the mystery of why microorganisms oxidize Mn(II) while providing the field with the tools necessary to address this question. The initial identification of MopA as a Mn(II) oxidase in this strain required the deletion of FleQ, a regulator involved in both flagellum synthesis and biofilm synthesis in *Pseudomonas aeruginosa*. Therefore, these results are also an important step toward understanding the regulation of Mn(II) oxidation.

Biomineralization, the formation of minerals by living things, is best known as the process by which hardened body structures, like bones or shells, are formed. However, this process is also thought to contribute to the formation of geological features, like the ferromanganese crusts and nodules found at the sediment-water column interface (1–3). While the transition metal manganese (Mn) can cycle abiotically between a soluble, reduced Mn(II) form and an insoluble, oxidized Mn(IV) form in the environment (4, 5), both the oxidation and reduction of Mn can be driven by microbial activity. For example, the dissimilatory metal-reducing bacteria *Shewanella* sp. and *Geobacter* sp. utilize Mn(III, IV) oxide minerals as terminal electron acceptors during anaerobic respiration, resulting in their reduction to soluble Mn(II) compounds (6, 7).

The ability to form Mn(III, IV) minerals through the oxidation of Mn(II) is found in a diverse array of bacteria, including Gram-positive and Gram-negative species, and can be found in many environments, including deep-sea vents, freshwater lakes, rivers, and soil (8, 9). There are, however, common themes among Mn(II)-oxidizing bacteria. In most cases, Mn(II) oxidation begins in stationary phase. The Mn(II) oxidase activity is localized to the outer surface of the cell, for example, the exosporium of *Bacillus* sp. SG-1 or the sheath of *Leptothrix discophora* SS-1 (8). As a consequence, the cells become covered in Mn minerals. The oxidation reaction is thermodynamically favorable, raising the possibility that bacteria could derive energy from Mn(II) oxidation (10). Recent studies have also shown that Mn(II)-oxidizing bacteria exhibit increased resistance to the reactive oxygen species hydrogen peroxide (11). Nonetheless, the physiological function of bacterial Mn(II) oxidation remains unclear.

The Mn(II) oxidase complex from *Bacillus* sp. PL-12 is made up of multiple subunits, including a multicopper oxidase (MCO) named MnxG. Because the active Mnx complex can be heterologously expressed in *Escherichia coli* (12), this Mn(II) oxidase is the best studied to date. The putative Mn(II) oxidase enzymes of *Leptothrix discophora* SS-1 and *Pedomicrobium* sp. ACM 3067 have been identified as MCO enzymes called MofA and MoxA, respectively (13, 14). However, the Mn(II) oxidases in *Aurantimonas manganoxydans* SI85-9A1, *Erythrobacter* sp. SD21 (15, 16), and *Roseobacter* sp. AzwK-3b (17) are animal heme peroxidases

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, characteristic, or construction	Antibiotic resistance[a]	Reference or source
Strain			
Pseudomonas putida			
GB-1	Manganese oxidizer, wild type	Amp^r	45
Δ*fleN*	GB-1 with in-frame deletion of *fleN*		24
Δ*mnxR*	GB-1 with in-frame deletion of *mnxR*		22
Δ*fleQ*	In-frame deletion of *fleQ* generated by conjugation of pKG289 into GB-1		This work
MopA-OE	In-frame deletion of *mnxG*, *mcoA*, and *fleQ* generated by conjugation of pKG289 into Δ*mnxG* Δ*mcoA*		This work
MopA-KO	In-frame deletion of *mnxG*, *mcoA*, *fleQ*, and *mopA* by conjugation of pKG168 into MopA-OE		This work
Δ*fleN* Δ*mnxR*	In-frame deletion of *fleN* and *mnxR* by conjugation of pKG214 into Δ*mnxR*		This work
Δ*mnxR* Δ*fleQ*	In-frame deletion of *mnxR* and *fleQ* by conjugation of pKG289 into Δ*mnxR*		This work
Δ*mcoA* Δ*mnxG*	GB-1 with in-frame deletions of *mcoA* and *mnxG*		21
Escherichia coli			
TAM1	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*		Active Motif
Plasmid			
pEX18Gm	Gene replacement vector; *oriT sacB*	Gm^r	46
pJET1.2/blunt	Commercial cloning vector	Amp^r	Thermo Scientific
pKG168	~500 bp upstream of *mopA* fused in frame with ~500 bp downstream cloned into pEX18Gm for making in-frame deletion	Gm^r	This work
pKG178	*mopA* amplified with mop1-F and mop2-R, cloned into pJET1.2/blunt and subcloned into pUCP22 with XbaI and XmaI	Amp^r Gm^r	This work
pKG214	~500 bp upstream of *fleN* fused in frame with ~500 bp downstream cloned into pEX18Gm for making in-frame deletion	Gm^r	24
pKG224	pUCP22 carrying *fleN* under the control of P_{lacZ}	Amp^r Gm^r	This work
pKG289	~500 bp upstream of *fleQ* fused in frame with ~500 bp downstream cloned into pEX18Gm for making in-frame deletion	Gm^r	This work
pRK2013	Helper plasmid for conjugation	Km^r	47
pRL27	Tn*5*	Km^r	48
pUCP22	Broad-host-range plasmid, ColE1 replicon	Amp^r Gm^r	49

[a] Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

(AHPs) which, in *A. manganoxydans* SI85-9A1 and *Erythrobacter* sp. SD-21, have been named MopA [Mn(II)-oxidizing peroxidase]. Similarly, species of white rot fungus employ MCO proteins called laccases and a variety of peroxidase family enzymes (Mn peroxidases and lignin peroxidases) in order to degrade lignin via an Mn(III) intermediate (18, 19). Thus, while the organisms that oxidize Mn(II) and the environments they inhabit are quite diverse, the variety of enzymes responsible appears to be limited to MCOs and peroxidases.

The gammaproteobacterium *Pseudomonas putida* GB-1 is a prominent model system for the investigation of bacterial Mn(II) oxidation (20). Recent work revealed the presence of two Mn(II) oxidase genes in this strain: *mnxG*, encoding an MCO with a low level of homology to the *Bacillus* sp. PL-12 Mn(II) oxidase subunit MnxG, and *mcoA*, encoding a second MCO distinct from MnxG (21). A strain from which both genes had been deleted failed to oxidize Mn(II) under all conditions tested. Quantitative PCR (qPCR) revealed that both *mnxG* and *mcoA* are upregulated in the presence of the σ^{54}-dependent response regulator MnxR (21). MnxR is part of a two-component regulatory pathway (the Mnx TCR) that is required for Mn(II) oxidation (22). Thus, a simple pathway could be proposed whereby the Mnx TCR, in response to an as-yet-unknown environmental signal, phosphorylates MnxR, stimulating its function and resulting in upregulation of *mnxG* and *mcoA* and, ultimately, in Mn(II) oxidase activity.

The physiological role of Mn(II) oxidation in bacteria remains unclear; however, elucidating the pathways that regulate expression of this activity may provide clues about this role. Therefore, we chose to further investigate the Mnx regulatory pathway using a genetic approach. Specifically, we screened for second site mutations that could suppress the oxidation defect of the Δ*mnxR* strain. Our results support a connection between Mn(II) oxidation and biofilm formation while revealing the surprising presence of a third Mn(II) oxidase enzyme in *P. putida* GB-1.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are shown in Table 1. *P. putida* GB-1 was grown at room temperature (RT) in LB or Lept medium (23). *Escherichia coli* strains were grown in LB medium at 37°C. Solid media contained 1.5% (wt/vol) agar. Antibiotics were used at the following concentrations: kanamycin (Km) 30 μg/ml, ampicillin (Amp) 100 μg/ml, and gentamicin (Gm) 50 μg/ml.

Transposon mutagenesis. pRL27, the plasmid carrying transposon Tn*5*, was moved into the *P. putida* GB-1 Δ*mnxR* strain (Table 1) by triparental mating using helper plasmid pRK2013, as previously described (24). The oxidation phenotype of the transposon (Tn) mutants was assessed by replica plating transconjugants onto Lept plates with 100 μM $MnCl_2$. The Δ*mnxR* strain failed to oxidize Mn(II) under these conditions; suppressors of the Δ*mnxR* oxidation defect were identified as colonies exhibiting visible Mn(IV) oxides after 4 days of incubation at RT. At least 5,000 colonies were screened in each of two independent conjugation

TABLE 2 Primers used in this study

Primer	Sequence
Per-upstream-F	5′CCTCCCTTTATCGCTAAGCGGG3′
Per-junction-F	5′TCTCCCGAAAGATCGAGGGCAGACTTCATCCTGGCGGGTTGA3′
Per-junction-R	5′TCAACCCGCCAGGATGAAGTCTGCCCTCGATCTTTCGGGAGA3′
Per-downstream-R	5′AGAAGAACCGCCTGGTGGC3′
fleQ_1-F	5′CTCAGCCTGCCAGCTGAAGG3′
fleQ_2-R	5′CGCTGAGTTCGTCAATGCGCTG3′
fleQ_3-F	5′TGCTATTGCATGTGGCGTGAAACCCAGGCAGAAGATTGACCTTGTTGGG3′
fleQ_4-R	5′CCCAACAAGGTCAATCTTCTGCCTGGGTTTCACGCCACATGCAATAGCA3′
mop1-F	5′CCCGGGACCCATGACCAGATACTGCGC3′, adds an XmaI site
mop2-R	5′TCTAGACCCCCGGCCTTTGACACC3′, adds an XbaI site

experiments. The sites of transposon insertion were determined as previously described (24).

Generation of in-frame deletions. The in-frame deletions of *mopA*, *fleQ*, and *fleN* were generated as described previously (22). Briefly, DNA upstream and downstream of the gene of interest was amplified (primers, Table 2) (24) and fused by PCR splicing by overhang extension (SOE) (25). The fusion construct was cloned into the gene replacement vector pEX18Gm (Table 1), which could then be moved via conjugation into various *P. putida* GB-1 backgrounds. Genes were deleted through double recombination with the fusion construct and verified by PCR amplification across the site of deletion.

Complementation. The *mopA* gene was PCR amplified with primers designed to incorporate an XmaI site at the 5′ end and an XbaI site at the 3′ end of the gene (Table 2). The PCR product was ligated into pJET1.2/blunt (Fermentas, Glen Burnie, MD) and then subcloned into pUCP22. The *mopA* gene is expressed from P_{lacZ} in this construct. The plasmid was moved into *P. putida* GB-1 strains via heat shock transformation (22), and complementation was assessed by Mn(II) oxidation assays and SDS-PAGE, with pUCP22 as the empty vector (EV) control.

Complementation by *fleN* was performed using the plasmid pKG224 (Table 1). This plasmid expresses *fleN* from a P_{lacZ} promoter in the pUCP22 backbone. The plasmids were transformed into the suppressor strains as described above, and Mn(II) oxidation was assessed on Lept plates after growth at RT for 5 days.

Motility assay. Selected strains were grown overnight in LB media and then diluted 25-fold into Lept media containing 100 μM $MnCl_2$. After approximately 2.5 h growth with shaking at RT, 10-μl aliquots were placed on a microscope slide and examined using a Leica DM750 compound microscope at ×100 magnification.

Quantification of Mn(II) oxidation. Selected strains were grown overnight at RT in LB (MopA-KO + empty vector and MopA-KO + pmopA were grown in LB with 50 μg/ml amp). The following morning, triplicate cultures were set up as follows: 40 μl was subcultured into 2 ml Lept with 100 μM $MnCl_2$ in 14-ml polystyrene snap-cap tubes. Cultures were incubated with shaking at RT for 3 days. To measure oxide production, 500 μl 0.016% leucoberbelin blue (LBB) (23, 26) in 1% acetic acid was added to each culture, and tubes were shaken at RT for 2 h. Then, 300 μl of this mixture was transferred to a 96-well plate, and absorbance was scanned from 360 to 750 nm with 4-nm steps in a SpectraMax M2e plate reader (Molecular Devices). The LBB absorbance at 624 nm was normalized to a baseline determined from the slope of the absorbance at 480 nm and 700 nm.

Generation of concentrated culture supernatants. Single colonies were inoculated into 5 ml LB and grown at RT overnight. The following morning, cultures were diluted 50-fold into Lept medium. These cultures were grown at RT with continuous shaking. After 48 h, cells were pelleted by centrifugation at 6,000 rpm for 15 min at 4°C. After transfer of the supernatants to clean 50-ml tubes, any remaining cells were removed by filtering through 0.2-μm-pore-size Acrodisc syringe filters (Pall Corporation). The ~34 ml of supernatant was concentrated using Amicon Ultra-15 50-kDa MWCO filters (Amicon) to a volume of 1 to 2 ml. Then, 500 μl of this was further concentrated using Amicon Ultra-0.5 30-kDa centrifugal filter devices to a final volume of ~60 μl. Concentrated culture supernatants were stored at 4°C.

Analysis of protein. Concentrated culture supernatants were loaded in duplicate and separated on a Mini-Protean TGX precast 4% to 15% PAGE gel (Bio-Rad) using 25 mM Tris, 192 mM glycine buffer (pH 8.3). After electrophoresis, the gel was rinsed three times in double-distilled water (dH_2O) and cut in half. One half of the gel was incubated in 10 mM HEPES (pH 7.5) and 100 μM $MnCl_2$ overnight at RT (23). Brown bands formed at the location of Mn(II)-oxidizing protein complexes and were recorded photographically. The gel was then rinsed in dH_2O, briefly submerged in 0.0032% LBB to stain oxides blue, and rephotographed. The other half of the gel was stained using imperial protein stain (Thermo Scientific), destained with H_2O, and photographed. Separation of proteins in the concentrated culture supernatants by denaturing gel electrophoresis was performed as described above, except the running buffer contained 0.1% SDS. Proteins were visualized by staining with imperial protein stain.

Liquid chromatography-tandem mass spectrometry. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed by the Oregon Health & Science University proteomics shared resource (http://www.ohsu.edu/xd/research/research-cores/proteomics/). The sample was reduced with dithiothreitol, alkylated with iodoacetamide, and digested overnight with trypsin (27). The digested sample was then separated using liquid chromatography with a NanoAcquity ultraperformance liquid chromatography system (Waters) and delivered to an LTQ Velos dual pressure linear ion trap mass spectrometer (Thermo Fisher). Samples were applied at 15 μl/min to a Symmetry C18 trap cartridge (Waters) for 10 min and then switched onto a 75-μm to 250-mm NanoAcquity BEH 130 C18 column with 1.7-μm particles (Waters) using mobile phases water and acetonitrile containing 0.1% formic acid, 7% to 30% acetonitrile gradient over 60 min, and a 300-nl/min flow rate. Comet (version 2015.01, revision 1) was used to search MS2 Spectra against a July 2015 version of the Uniprot *Pseudomonas putida* (strain GB-1) FASTA protein database, with concatenated sequence-reversed entries to estimate error thresholds and with 179 common contaminant sequences and their reversed forms. The database processing was performed with python scripts available at http://www.proteomicanalysisworkbench.com. Comet searches for all samples were performed with trypsin enzyme specificity (28, 29). Peptide-to-protein mapping and protein filtering were performed using PAW_results_7.py (version 7.0). The in-house Python scripts have been described previously (29).

RESULTS

Suppression of the oxidation defect of the Δ*mnxR* deletion strain. To further investigate the pathways that regulate Mn(II) oxidation, we screened for mutations that could suppress the oxidation defect of the *mnxR* deletion (Δ*mnxR*) strain. This was

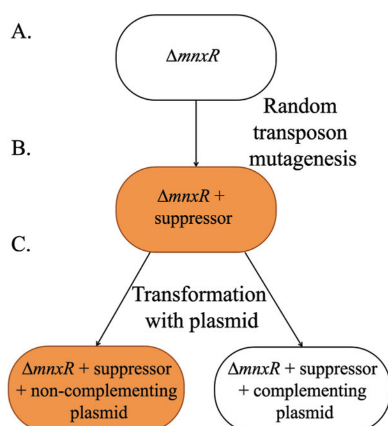

FIG 1 Suppression of Δ*mnxR*. (A) The nonoxidizing Δ*mnxR* strain is a white oval. (B) Random mutagenesis with the Tn*5* transposon resulted in Δ*mnxR* strains with Mn oxidase activity, i.e., suppressor strains. Mn(II)-oxidizing strains are represented by an orange oval. (C) Complementation, restoring to the strain a wild-type copy of the gene disrupted by the suppressor mutation, should result in the suppressor strain reverting to the nonoxidizing phenotype.

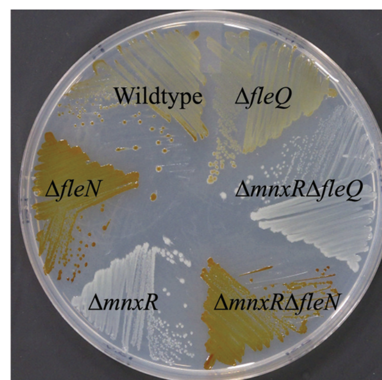

FIG 2 Suppression of Δ*mnxR*. The indicated strains were streaked onto Lept media and grown at RT for 3 days.

done by randomly mutagenizing the Δ*mnxR* strain with the transposon Tn*5* and screening for colonies with restored Mn(II) oxidation (Fig. 1A and B). In two separate screens, we identified colonies with Mn(II) oxidase activity, for a total of 29 Δ*mnxR*,Tn*5* suppressor strains (Table 3). We sequenced DNA flanking the transposon for a subset of the strains in order to determine the site of transposon insertion. Four transposons mapped to the gene *fleN*, and the remaining eight mapped to *flhF* (Table 3), a gene located immediately upstream of *fleN* on the *P. putida* GB-1 chromosome (30).

TABLE 3 Δ*mnxR* suppressors

Suppressor	Transposon insertion site	Mapping method
Sup3	*fleN*	Sequenced/complemented by pFleN
Sup4	*flhF*	Sequenced/complemented by pFleN
Sup5	*flhF*	Sequenced
Sup6	*fleN* or *flhF*	Complemented by pFleN
Sup7	*fleN* or *flhF*	Complemented by pFleN
Sup8	*flhF*	Sequenced
Sup9	*fleN* or *flhF*	Complemented by pFleN
Sup10	*fleN* or *flhF*	Complemented by pFleN
Sup11	*fleN* or *flhF*	Complemented by pFleN
Sup13	*fleN* or *flhF*	Complemented by pFleN
Sup14	*fleN* or *flhF*	Complemented by pFleN
Sup15	*fleN* or *flhF*	Complemented by pFleN
Sup17	*fleN* or *flhF*	Complemented by pFleN
Sup18	*fleN* or *flhF*	Complemented by pFleN
Sup19	*fleN* or *flhF*	Complemented by pFleN
Sup20	*fleN* or *flhF*	Complemented by pFleN
Sup21	*fleN* or *flhF*	Complemented by pFleN
Sup22	*fleN* or *flhF*	Complemented by pFleN
Sup23	*fleN* or *flhF*	Complemented by pFleN
Sup26	*fleN* or *flhF*	Complemented by pFleN
Sup27	*fleN* or *flhF*	Complemented by pFleN
KGsup1	*fleN*	Sequenced
KGsup2	*fleN*	Sequenced
KGsup3	*flhF*	Sequenced
KGsup4	*flhF*	Sequenced
KGsup5	*flhF*	Sequenced
KGsup6	*flhF*	Sequenced
KGsup7	*fleN*	Sequenced
KGsup8	*flhF*	Sequenced

Loss of the flagellar regulator FleN suppresses Δ*mnxR*. It is possible that the *flhF*::Tn*5* insertions affect expression of the downstream *fleN* gene through polar effects. Also, previous work showed that a strain with an in-frame deletion of *fleN* exhibited increased Mn(II) oxidation (24). Therefore, we hypothesized that disruption of FleN function was responsible for the restoration of Mn(II) oxidation observed in the Δ*mnxR*,Tn*5* suppressor strains. If so, it should be possible to complement the oxidation phenotype of the Δ*mnxR*,Tn*5* strains (Fig. 1C) with a plasmid expressing FleN (pKG224) (Table 1). In each case, pKG224 caused the Δ*mnxR*,Tn*5* strains to behave like the Δ*mnxR* strain, while the empty vector control did not; in other words, the Δ*mnxR*,Tn*5* strains carrying the FleN-expressing plasmid failed to oxidize Mn(II), while the Δ*mnxR*,Tn*5* strains carrying the empty vector continued to oxidize Mn(II). For controls, we also transformed pKG224 and the empty vector into suppressor strains for which the site of Tn*5* insertion had been mapped by sequencing (Sup3, *fleN*::Tn*5* and Sup4, *flhF*::Tn*5*) (Table 3). Both strains were complemented by the FleN-expressing plasmid. Because multicopy expression of FleN restored the Δ*mnxR* phenotype to each of the Δ*mnxR*::Tn5 strains, we concluded that the suppression of the Δ*mnxR* oxidation defect in each of the suppressor strains was due to disruption of FleN activity.

To verify that the loss of FleN suppressed the oxidation defect of the Δ*mnxR* strain, we generated a double-mutant strain in which both *fleN* and *mnxR* had been deleted. As previously published (22, 24), the Δ*mnxR* strain failed to oxidize Mn(II) and the Δ*fleN* strain exhibited increased Mn(II) oxidation on solid media relative to wild type (Fig. 2). The Δ*mnxR* Δ*fleN* mutant strain exhibited an oxidation phenotype on plates similar to that of the Δ*fleN* strain (Fig. 2). Therefore, consistent with the results of the transposon mutagenesis, deletion of *fleN* suppressed the oxidation defect of the Δ*mnxR* strain.

In *Pseudomonas aeruginosa* PAO1, FleN has been shown to regulate flagellar number through inhibition of the transcription factor FleQ (31, 32). The *P. putida* GB-1 genome encodes a gene with extensive similarity to *P. aeruginosa* PAO1 FleQ (PputGB1_3934, 84% identical). In-frame deletion of this gene resulted in cells that were nonmotile when viewed under a micro-

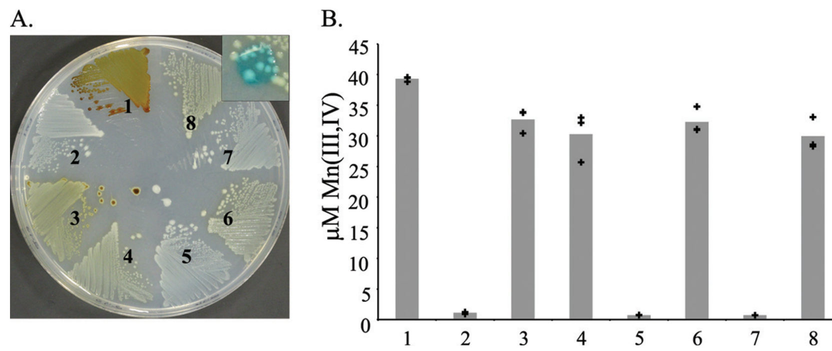

FIG 3 Deletion of *fleQ* reveals the presence of a third Mn(II) oxidase, MopA. (A) Strains were streaked onto Lept medium and grown at RT for 7 days. Inset shows colonies after 10 µl leucoberbelin blue was dropped on top. (B) Quantification of Mn(III, IV) oxides in liquid cultures after shaking at RT for 3 days. Bars represent the average of three replicates; crosses indicate individual values. Strains shown: 1, wild type; 2, Δ*mnxR*; 3, Δ*fleQ*; 4, Δ*mnxG* Δ*mcoA* Δ*fleQ*; 5, Δ*mnxG* Δ*mcoA* Δ*fleQ* Δ*mopA*; 6, Δ*mnxR* Δ*fleQ*; 7, Δ*mnxG* Δ*mcoA* Δ*fleQ* Δ*mopA* + pUCP22; 8, Δ*mnxG* Δ*mcoA* Δ*fleQ* Δ*mopA* + pKG178.

scope during the early exponential phase, supporting the identification of PputGB1_3934 as the master flagellar regulatory gene, *fleQ*. The Δ*fleQ* strain, when grown on solid media, appeared to oxidize Mn(II) to a slightly lesser extent than wild-type bacteria (Fig. 2). This slight decrease was also present in cultures grown in liquid media (Fig. 3). Thus, FleN may regulate Mn(II) oxidation at least in part through its inhibition of the positive regulator FleQ, in a manner similar to the regulation of flagella synthesis.

Δ*fleQ* reveals a third Mn(II) oxidase activity. If both FleQ and MnxR are positive regulators of Mn(II) oxidation, we predict that deletion of both genes—Δ*mnxR* Δ*fleQ*—would result in a nonoxidizing phenotype. This is initially what was observed (Fig. 2). However, after prolonged incubation on solid media (7 days), the Δ*mnxR* Δ*fleQ* mutant strain produced a diffuse brown color that resembled Mn oxides (Fig. 3A). We verified that the faint brown color was the result of Mn(III, IV) oxide formation by dropping a solution of leucoberbelin blue (LBB) (23, 26) onto the colonies; the reaction of LBB with Mn(III, IV) oxides produces a blue color (Fig. 3A, inset). Quantification of Mn(III, IV) oxide formation in liquid culture gave a similar result: the Δ*mnxR* Δ*fleQ* mutant strain produced nearly 30-fold higher concentration of Mn(III, IV) oxides than the Δ*mnxR* strain (Fig. 3B).

We identified two possible explanations for the Mn(II) oxidation seen in the Δ*fleQ* Δ*mnxR* strain: either one or both of the two oxidase genes are expressed in the Δ*fleQ* Δ*mnxR* mutant through an MnxR-independent regulatory pathway, or the Δ*fleQ* Δ*mnxR* mutant had unmasked a third Mn(II) oxidase activity. To test these possibilities, we generated a triple mutant in which the two known Mn(II) oxidase genes and *fleQ* had each been deleted (Δ*mnxG* Δ*mcoA* Δ*fleQ*) (Table 1). In previous work, the Δ*mnxG* Δ*mcoA* strain failed to oxidize Mn(II) (21). However, the Δ*mnxG* Δ*mcoA* Δ*fleQ* strain retained a level of Mn(II) oxidase activity similar to that of the Δ*fleQ* Δ*mnxR* double mutant (Fig. 3A and B) despite each of the known Mn(II) oxidase genes having been deleted from the chromosome. Therefore, the Δ*fleQ* deletion unmasked a third Mn(II) oxidase activity. While the role of FleN, FleQ, and MnxR in regulating Mn(II) oxidation is an important subject of ongoing research in our group, we here address our efforts to identify and characterize this third Mn(II) oxidase enzyme.

The third Mn(II) oxidase in *P. putida* GB-1 is MopA. Previous work (21) had shown that the *P. putida* GB-1 genome encodes an AHP similar to the AHP proteins implicated in Mn(II) oxidation in *A. manganoxydans* SI85-9A1, *Erythrobacter* sp. SD-21, and *Roseobacter* sp. AzwK-3b (15–17). However, deletion of this gene (*mopA*) from the *P. putida* GB-1 genome had little reproducible effect on Mn(II) oxidation. Furthermore, genes homologous to *mopA* appeared to be present in the genomes of both oxidizing and nonoxidizing pseudomonads (21). For these reasons, we had previously discounted a role for *mopA* in Mn(II) oxidation by *P. putida* GB-1 (21). To determine if MopA is responsible for the cryptic Mn(II) oxidation seen in the Δ*fleQ* Δ*mnxR* and Δ*mnxG* Δ*mcoA* Δ*fleQ* strains, we deleted *mopA* from the Δ*mnxG* Δ*mcoA* Δ*fleQ* triple mutant. This resulted in complete loss of Mn(II) oxidation (Fig. 3A and B). Oxidation activity could be restored to the quadruple mutant by a plasmid carrying *mopA* (pKG178) (Table 1). Therefore, MopA is a third Mn(II) oxidase enzyme in *P. putida* GB-1.

MopA is present in the culture supernatant. When wild-type cells oxidize Mn(II) on plates, the oxides remain associated with the cells, turning the colonies dark brown. In the *fleQ* mutants (especially Δ*mnxG* Δ*mcoA* Δ*fleQ* and Δ*mnxR* Δ*fleQ*), the oxides are light brown and diffuse into the agar of the plate (Fig. 3A, inset). This diffusion suggests that the oxidase is secreted away from the cells, into the culture medium. To test this, we isolated cell-free culture supernatants from wild type, Δ*mnxG* Δ*mcoA* Δ*fleQ*, and Δ*mnxG* Δ*mcoA* Δ*fleQ* Δ*mopA* strains and separated the protein by SDS-PAGE (Fig. 4). A large band that migrated above the 200-kDa marker was clearly visible in the supernatant prepared from the Δ*mnxG* Δ*mcoA* Δ*fleQ* strain but not from the wild-type or Δ*mnxG* Δ*mcoA* Δ*fleQ* Δ*mopA* + empty vector strains. This band was restored to the Δ*mnxG* Δ*mcoA* Δ*fleQ* Δ*mopA* strain by the pMopA plasmid (Fig. 4). MopA is 3,608 amino acids long, or roughly 373 kDa. Thus, this >200-kDa protein was likely MopA. The MopA band was also visible in whole-cell lysates prepared from the Δ*mnxG* Δ*mcoA* Δ*fleQ* and the Δ*fleQ* strains but not in wild-type whole-cell lysates (data not shown). Thus, the *fleQ* deletion resulted in substantially increased levels of MopA protein. In the remainder of the text, we refer to the Δ*mnxG* Δ*mcoA* Δ*fleQ* strain as the MopA overexpression (MopA-OE) strain and the Δ*mnxG* Δ*mcoA* Δ*fleQ* Δ*mopA* strain as the MopA knockout (MopA-KO). To confirm that the protein in the >200-kDa band was MopA, we excised it from the MopA-OE lane (Fig. 4, box) and submitted it for mass spectrometry. The major com-

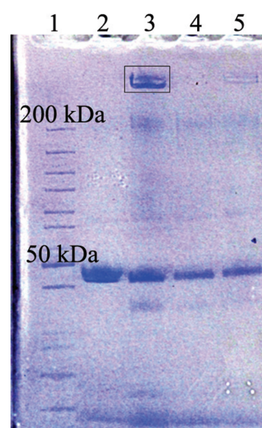

FIG 4 Deletion of *fleQ* results in overexpression and secretion of MopA. Lane 1, PAGERuler unstained protein ladder (Thermo Scientific); lane 2, wild type; lane 3, MopA-OE; lane 4, MopA-KO + EV; lane 5, MopA-KO + pmopA. The top protein in the marker lane is 200 kDa. The box indicates the portion of the gel submitted for LC-MS/MS.

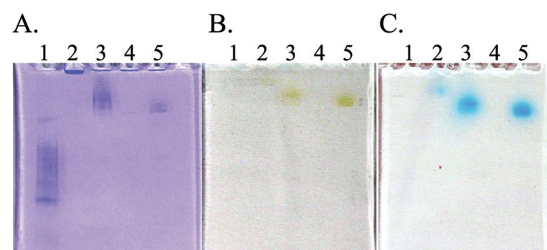

FIG 5 Native PAGE analysis results for protein stain (A); activity assay using gel incubated in 10 mM HEPES (pH 7.5) + 100 μM $MnCl_2$ overnight at RT (B); activity assay using gel described for panel B after soaking in 0.0032% LBB (C). Lane 1, PAGERuler unstained protein ladder; lane 2, wild type; lane 3, MopA-OE; lane 4, MopA-KO + EV; lane 5, MopA-KO + pmopA.

ponent of the mass spectra was MopA, with 1,345 peptides mapping to this protein. Protein sequence coverage was 40.7% (Table 4).

MopA actively oxidizes Mn(II). It is possible to detect Mn(II) oxidase activity in proteins separated by nondenaturing gel electrophoresis (17, 23, 33). Therefore, if the secreted MopA is an Mn(II) oxidase, this activity should be apparent after separation of culture supernatant on a native gel. After separating concentrated cell-free culture supernatant by native PAGE, we soaked the gel overnight in a buffer solution containing $MnCl_2$ (see Materials and Methods). A smear of brown Mn(IV) oxides was visible in the MopA-OE lane, and a sharper brown band was present in the MopA-KO + pmopA lane (Fig. 5B). After soaking the gel briefly in LBB, these brown bands turned blue, supporting their identification as Mn(III, IV) oxides (Fig. 5C). This result supported the conclusion that MopA is capable of oxidizing Mn(II). In addition, a faint blue spot was visible in the wild-type lane, migrating higher in the gel than those in the MopA-OE and MopA-KO + pmopA lanes (Fig. 5C). MnxG is predicted to weigh 209 kDa; McoA, 123 kDa. Therefore, this faint blue band at the top of the gel may represent a complex of two or three of the Mn(II) oxidase enzymes.

TABLE 4 LC-MS/MS peptide identification of proteins present in SDS-PAGE band

Locus tag	Protein description	Estimated molecular mass (kDa)	Total spectral counts	% coverage
PputGB1_3353	MopA	373	1,345	40.7
PputGB1_3075	Outer membrane adhesion-like protein	359	19	4.2
PputGB1_1837	Leucine-rich repeat protein	168	2	1.8
PputGB1_3392	Uncharacterized	248	2	1.9
PputGB1_0792	Probable malate:quinone oxidoreductase	54	2	6
PputGB1_0945	Uncharacterized	138	2	2.8
PputGB1_3939	Flagellin	49	4	10.4
PputGB1_1040	Uncharacterized	74	2	4.5

DISCUSSION

This work began as an effort to understand the regulation of Mn(II) oxidation in *Pseudomonas putida* GB-1 but ultimately revealed the presence of a third Mn(II) oxidase enzyme in this strain. Deletion of *fleQ* resulted in the overexpression of a large, secreted protein capable of oxidizing Mn(II), as shown by in-gel oxidase assays. Mass spectrometry and directed mutagenesis identified this protein as MopA, an animal heme peroxidase with sequence similarity to the MopA Mn(II) oxidase proteins of *A. manganoxydans* SI85-9A1 and other Mn(II)-oxidizing bacteria. Therefore, *P. putida* GB-1 employs three separate enzymes for the oxidation of Mn: the two MCOs, MnxG and McoA, and the AHP, MopA.

FleQ is a σ^{54}-dependent transcription factor that initiates the tightly controlled regulatory cascade that results in flagellum synthesis (34). In *P. aeruginosa* PAO1, FleQ also regulates extracellular polysaccharide synthesis and biofilm formation (35), and, in *P. putida* KT2440, FleQ has been shown to play a role in expression of the adhesion protein LapA (36). Thus, the *fleQ* deletion may have pleiotropic effects on the physiology of *P. putida* GB-1. In addition, the overexpression of MopA seen in the *fleQ* mutants could cause the enzyme to exhibit nonnative activity. This raises the question of whether Mn(II) oxidation is a normal function of MopA in *P. putida* GB-1. Deletion of *mopA* from a *fleQ*-positive strain does not reproducibly result in decreased Mn(II) oxidation. However, under normal laboratory growth conditions, the bulk of Mn(II) oxidation may be performed by either MnxG or McoA, making the contribution of MopA difficult to detect. In agreement with this, the oxidation defect of a *mnxG*-mutant strain is exacerbated by the further deletion of *mopA* (data not shown). Thus, removing one of the MCO Mn(II) oxidases renders it possible to detect a role for MopA in Mn(II) oxidation in a *fleQ*-positive strain background.

A connection between Mn(II) oxidation and flagellum synthesis has been previously identified (24). In a genetic screen for transposon insertions that resulted in increased Mn(II) oxidation, several insertions were identified within flagellar regulatory and structural genes. Curiously, these flagellar mutants exhibited very different oxidation phenotypes depending on the growth conditions, with some of the mutants failing to oxidize Mn(II) in liquid media while exhibiting increased Mn(II) oxidation on solid media. The results reported here suggest that deletion of *fleQ* results in increased expression of one Mn(II) oxidase gene (*mopA*)

(Fig. 4), while the activity of the other two oxidases may be decreased (suggested by the decreased oxidation of the Δ*fleQ* strain) (Fig. 3A and B). In addition, the precise amount of Mn(II) oxidation exhibited by strains with single oxidase gene deletions varies with growth conditions (21) and from experiment to experiment (data not shown). Therefore, the three oxidase genes are likely not expressed at the same times and under the same conditions. Future studies will need to focus on the regulation of individual genes, rather than the regulation of Mn(II) oxidation. Nonetheless, the fact that FleQ regulates not just flagellum synthesis but biofilm formation and surface adhesion suggests that the different Mn(II) oxidases play different roles in planktonic versus sessile lifestyles.

The AHP Mn(II) oxidase enzymes are characterized by the presence of one or two conserved heme-binding domains and multiple repeat-in-toxin family calcium-binding domains located carboxy-terminal to the heme domains (17). In *Roseobacter* sp. AzwK-3b, it has been proposed that one or more of its four secreted AHP enzymes indirectly oxidize Mn(II) through the production of superoxide and the removal of hydrogen peroxide (17). However, work done with the heterologously expressed heme-binding domain from *Erythrobacter* sp. SD-21 MopA showed that Mn(II) oxidation by this enzyme was insensitive to superoxide dismutase (16), suggesting that superoxide was not involved in oxidation by *Erythrobacter* sp.SD-21 MopA. Mn(II) oxidation by *Roseobacter* sp. AzwK-3b cultures and culture supernatants is stimulated by light (37) and by the reductant NADH (17, 38). Preliminary experiments examining Mn(II) oxidation by concentrated culture supernatants prepared from the MopA-OE strain indicated that the oxidase activity of GB-1 MopA is also stimulated by light (data not shown) but is completely inhibited by NADH (data not shown). Therefore, the mechanism(s) by which AHP enzymes contribute to Mn(II) oxidation remains unclear. However, the identification of a Mn(II) oxidation-associated AHP in the genetically tractable *P. putida* GB-1 strain may simplify investigation of these mechanisms.

The results reported here describe a Mn(II)-oxidizing bacterium utilizing both MCO and AHP Mn(II) oxidase enzymes. As previously reported (21), *Leptothrix cholodnii* SP-6 encodes in its genome two different putative MCO-type Mn(II) oxidases. In addition, *L. cholodnii* SP-6 has a potential *mopA* gene, while the *A. manganoxydans* SI85-9A1 genome has not just *mopA* but also a gene with similarity to an *mcoA* homolog (30) and two genes with sequence similarity to the *Pedomicrobium* sp. ACM 3067 Mn(II) oxidase MoxA (39). Therefore, *P. putida* GB-1 may not be unique in harboring Mn(II) oxidase enzymes of both the MCO and AHP families. In the basidiomycete fungus *Stropharia rugosoannulata*, degradation of lignin is accomplished through the cooperation of a laccase (an MCO-type enzyme) and a Mn peroxidase, with the peroxidase utilizing H_2O_2 generated during the oxidation of Mn(II) to Mn(III) by the laccase (19). Therefore, it is possible that MopA works together with one or both of the MCO-type Mn(II) oxidases during oxidation.

Mn(II) oxidation occurs on the outside surface of the cell, coating the cell with Mn oxides (8). The Mn(II) oxidase enzymes that have been identified so far are found loosely bound to the exosporium (40) or the outer membrane (15) or are secreted from the cell into the culture milieu (17). *P. putida* GB-1 MopA is secreted into the culture supernatant (Fig. 4); the cellular localization of MnxG and McoA is unknown, although the proteins are likely exported (41). Furthermore, the results shown here suggest that Mn(II) oxidation is regulated as part of the planktonic to biofilm lifestyle switch. Thus, Mn(II) oxidase enzymes can be considered a public good or a resource available to the bacterial biofilm community at large, not just the cell actively secreting the protein (42). What function does Mn(II) oxidation play in the bacterial community such that the cost of secreting large oxidase enzymes is evolutionarily affordable? Mn(II) oxidation has been shown to protect *P. putida* GB-1 from hydrogen peroxide (11). In the closely related plant-beneficial strain *P. putida* KT2440, deletion of the *mopA* homolog PP2561 resulted in increased sensitivity to compounds like tert butyl hydroperoxide (43), supporting a role for Mn(II) oxidation in the oxidative stress response. This mutant strain also had a competitive defect in colonization of plant hosts that may be linked to its increased sensitivity to reactive oxygen species (43, 44). Understanding how and why bacteria oxidize Mn(II) has been a long-standing goal in this field; the identification of the oxidase enzymes in *P. putida* GB-1 makes it possible to further address the question of the physiological role of Mn(II) oxidation.

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