Elimination of Manganese(II,III) Oxidation in *Pseudomonas putida* GB-1 by a Double Knockout of Two Putative Multicopper Oxidase Genes

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Bacterial manganese(II) oxidation impacts the redox cycling of Mn, other elements, and compounds in the environment; therefore, it is important to understand the mechanisms of and enzymes responsible for Mn(II) oxidation. In several Mn(II)-oxidizing organisms, the identified Mn(II) oxidase belongs to either the multicopper oxidase (MCO) or the heme peroxidase family of proteins. However, the identity of the oxidase in *Pseudomonas putida* GB-1 has long remained unknown. To identify the *P. putida* GB-1 oxidase, we searched its genome and found several homologues of known or suspected Mn(II) oxidase-encoding genes (*mnxG*, *mofA*, *moxA*, and *mopA*). To narrow this list, we assumed that the Mn(II) oxidase gene would be conserved among Mn(II)-oxidizing pseudomonads but not in non-oxidizers and performed a genome comparison to 11 *Pseudomonas* species. We further assumed that the oxidase gene would be regulated by MnxR, a transcription factor required for Mn(II) oxidation. Two loci met all these criteria: PputGB1_2447, which encodes an MCO homologous to MnxG, and PputGB1_2665, which encodes an MCO with very low homology to MofA. In-frame deletions of each locus resulted in strains that retained some ability to oxidize Mn(II) or Mn(III); loss of oxidation was attained only upon deletion of both genes. These results suggest that PputGB1_2447 and PputGB1_2665 encode two MCOs that are independently capable of oxidizing both Mn(II) and Mn(III). The purpose of this redundancy is unclear; however, differences in oxidation phenotype for the single mutants suggest specialization in function for the two enzymes.

In the environment, the redox activities of microorganisms profoundly affect the geochemical cycling of the transition metal manganese which, in turn, affects the availability and turnover of other metals and organic compounds due to the absorptive and reactive nature of the Mn(III,IV) oxides (1). The oxidation of Mn(II) by fungi and bacteria has been identified in both aquatic and terrestrial niches (2). In some soil fungi, the product of oxidation is Mn(III) complexed with organic ligands, which in turn oxidizes lignin, breaking down this otherwise recalcitrant organic molecule (3). Other fungi may produce a Mn(IV) oxide (4–6). In bacteria, Mn(II) oxidation leads primarily to the formation of Mn(IV) oxides, the function and benefit of which remain speculative (7).

Putative Mn(II) oxidases have been identified in numerous bacterial species: MoxA in *Pedomicrobium* sp. strain ACM 3067 (8), MofA in *Leptothrix discophora* SS-1 (9), MnxG in *Bacillus* sp. strain SG-1 and related strains (10), and MopA in *Aurantimonas manganooxydans* SI85-9A1 and *Erythrobacter* sp. strain SD-21 (11). Except for the alphaproteobacterial enzymes produced by SI85-9A1 and SD-21, the Mn(II) oxidases reported to date belong to the multicopper oxidase (MCO) family of enzymes. MCOs are all characterized by four to six conserved copper binding sites and the capacity for at least a one-electron oxidation of substrates (12). Included in the MCO family are laccases, identified as the Mn(II)-to-Mn(III) oxidases of fungi. In bacteria, however, Mn(II) oxidation is a two-electron oxidation of the Mn(II) substrate to Mn(IV), and there is evidence that a bacterial Mn(II)-oxidizing MCO is capable of performing both of the electron transfers (13). In *Bacillus* sp. strain SG-1, for example, a transposon insertion in the *mnxG* gene (encoding the MCO MnxG) disrupted both oxidation steps (14).

The MopA enzymes of *A. manganooxydans* SI85-9A1 and *Eryth-*

robacter sp. SD-21 belong to the family of Ca²⁺-binding heme peroxidases (11). A different family of peroxidases, the Mn peroxidases, has also been implicated in Mn(II) oxidation by fungi. The white rot basidiomycete *Phanerochaete chrysosporium*, for example, employs a Mn peroxidase and hydrogen peroxide to generate Mn(III) (3). In some cases, MCOs and Mn peroxidases have been shown to work together, with the MCO producing the hydrogen peroxide substrate for the Mn peroxidase (15). Other reactive oxygen species also play a role in Mn(II) oxidation (16). For example, in the marine alphaproteobacterium *Roseobacter* sp. strain AzwK-3b, Mn(II) is indirectly oxidized by enzymatically produced superoxide (17, 18). Superoxide has also been shown to oxidize Mn(II) in seawater (16).

The gammaproteobacterium *Pseudomonas putida* GB-1 and the closely related strain MnB1 have long been used as model organisms for the study of Mn(II) oxidation, with work in MnB1 dating as far back as the 1970s (19). Despite this extensive history, the Mn(II) oxidase in *P. putida* has not been conclusively identified. Random-mutagenesis investigations have been repeatedly

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performed to identify the enzyme (20–25), an approach which was successful in *Bacillus* sp. strain SG-1 (26). Indeed, an MCO identified as CumA was initially thought to be the oxidase when a strain carrying a Tn5 transposon insertion in the *cumA* gene was shown to be defective in Mn(II) oxidation (20). Subsequently, a second, spontaneous point mutation in a two-component regulatory pathway was discovered. It was this mutation, not the *cumA*::Tn5 disruption, that caused the loss-of-function phenotype (27). A number of other genes, including components of the tricarboxylic acid (TCA) cycle, the cytochrome *c* synthesis operon, genes involved in tryptophan synthesis, and genes encoding a modified type II secretion system pathway (21–23), have been shown to affect Mn(II) oxidation in *P. putida*, but direct Mn(II) oxidase activity has not been demonstrated.

Some clues to the nature of the Mn(II) oxidase in *P. putida* GB-1 can be derived from previous work. The addition of copper to *P. putida* GB-1 cultures has been shown to stimulate Mn(II) oxidation (20), suggesting that the oxidase in this organism is a copper-binding enzyme. Biochemical isolation of the Mn(II) oxidase activity followed by separation on a native gel identified two bands that were each capable of oxidizing Mn(II) (28). These observations, coupled with the inability to identify the sole oxidase gene by random transposon mutagenesis despite much effort from numerous researchers, suggest that *P. putida* may employ multiple Mn(II) oxidases, at least one of which is stimulated by copper, as would be predicted for an MCO.

With the failure of traditional genetics to identify the Mn(II) oxidase, biochemical isolation and identification would be the next logical approach. However, this also has proven difficult, possibly due to the enzyme's low abundance (29). Starting with what is known about Mn(II) oxidation in other organisms, we undertook a reverse genetics approach to identify the Mn(II) oxidase in *P. putida* GB-1. Here we describe the resulting *P. putida* GB-1 mutant, showing a complete loss of Mn(II) oxidation phenotype, and demonstrate for the first time a direct link between two partially redundant MCO genes encoding the two Mn(II) oxidases necessary for Mn(II) oxidation in *P. putida* GB-1 and other putative Mn(II)-oxidizing pseudomonads.

MATERIALS AND METHODS

Strains and culture conditions. The strains and plasmids used in this study are shown in Table 1. *P. putida* GB-1 strains were grown at room temperature (RT) or 30°C in LB or Lept medium (34). *Escherichia coli* strains were grown in LB medium at 37°C. The following concentrations of antibiotics were used: ampicillin (Amp) (100 µg/ml), kanamycin (Kn) (30 µg/ml), and gentamicin (Gm) (50 µg/ml in LB, 2.5 µg/ml in Lept). For oxidation assays, MnCl₂ [Mn(II)] or Mn(OAc)₃ [Mn(III)] were added to Lept medium at a final concentration of 100 µM. Mn(OAc)₃ solutions were prepared in the presence of 5 molar excess sodium pyrophosphate (13).

Genomic comparisons. Orthologous gene products shared between *P. putida* GB-1 and other members of the genus *Pseudomonas* (the genomes compared are listed in Results) were identified using the reciprocal smallest-distance (RSD) algorithm (threshold E value = 1e-05; sequence divergence = 0.4) (35, 36). Genome sequence information in GenBank and protein amino acid file formats were downloaded from the Joint Genome Institute's Integrated Microbial Genome system. Results from the pairwise RSD analyses were merged into a single tab-delimited file showing shared orthologs from each pseudomonad genome in relation to the GB-1 genome. The comprehensive ortholog comparison file is provided as Table S1 in the supplemental material.

Quantification of Mn(II) oxidation in liquid culture. (i) Mn(II) ox-

idation in cultures used to generate RNA. Wild-type and KG127 (Table 1) cells containing pUCP22 were grown overnight at RT in LB with Gm in triplicate. The optical density at 600 nm (OD₆₀₀) was measured for each culture, and 1 ml was pelleted and resuspended in Lept liquid medium. The cells were diluted to a final volume of 5 ml and an OD₆₀₀ of ~0.1 in Lept with Gm and 100 µM MnCl₂. Cultures were grown with shaking at RT for 24 h, at which point a 1.5-ml sample was removed and pelleted, and the pellet was stored at -80°C for subsequent RNA purification. To the remaining culture, 1 ml leucobercin blue (LBB) reagent dissolved in acetic acid (34) was added, and then the mixture was vortexed vigorously. A 1-ml aliquot was removed from the culture-LBB mixture and pelleted by centrifugation. Two hundred microliters of supernatant was removed and used to determine the OD₆₁₈, i.e., the relative amount of Mn(III,IV) oxides. The pellet was then washed once with 1 ml Lept and resuspended in 1 ml H₂O. To determine relative protein concentration, 100 µl of washed, resuspended cell pellet was mixed with 100 µl Coomassie Plus protein assay reagent (Thermo Scientific, Waltham, MA), and the OD₅₉₅ was measured. To normalize the amount of oxides to the amount of protein, the OD₆₁₈ from the LBB assay was divided by the OD₅₉₅ from the Coomassie assay.

(ii) **Mn(II) oxidation by strains bearing oxidase deletions.** Quantification was performed as described above except that cells were diluted to an OD₆₀₀ of 0.05 in Lept with 100 µM MnCl₂ and split into two 3-ml cultures per strain. At the 1- and 2-day time points, 1 ml LBB was added to one set of the 3-ml cultures. Mn(III,IV) oxides and protein were measured as described above.

Quantification of Mn(II) oxidation on plates. To generate a lawn of cells, 100 µl of an overnight LB culture was spread onto a Lept with 100 µM MnCl₂ plate and incubated at RT. At the 2- and 3-day time points, 2.5 ml of 10 mM HEPES (pH 7.5) buffer was pipetted onto one set of lawns. Cells were resuspended from the plate by scraping with a pipet tip and pipetting up and down. Five hundred microliters of the resuspended cells was transferred to a 1.5-ml tube and mixed with 500 µl LBB with vortexing. Quantification proceeded as described above for the liquid assays.

RNA extraction. RNA was extracted from the pellets generated as described above using 1 ml Triagent (Sigma-Aldrich, St. Louis, MO) followed by chloroform extraction. The resulting RNA was then treated with 4 units RQ1 DNase (Promega, Madison, WI) and 40 units RNase Out recombinant RNase inhibitor (Invitrogen, Carlsbad, CA) in a final volume of 200 µl for 90 min at 37°C, followed by phenol-chloroform-isomyl alcohol extraction and ethanol precipitation.

Generation of cDNA. RNA concentration was estimated using a Nanodrop spectrophotometer (ND-1000 v3.7; Thermo Fisher Scientific). Five hundred nanograms of RNA was mixed with 0.5 mM final deoxynucleoside triphosphates (dNTPs) and 3.3 ng random primers (Invitrogen, Carlsbad, CA), heated to 65°C for 5 min, and then placed on ice. Next, 5 mM dithiothreitol, and First Strand Buffer (Invitrogen, Carlsbad, CA) were added to a final volume of 60 µl, and the mixture was split in half. To one half, 50 units RNase Out recombinant RNase inhibitor and 300 units SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) were added (+RevT reaction mixtures); to the other half, an equal volume of H₂O was added (-RevT reaction mixtures). Both mixtures were then incubated at 25°C for 10 min, 50°C for 3 h, and 70°C for 10 min and finally stored at 4°C. Semiquantitative PCRs were performed with the +RevT and -RevT reaction mixtures and primers specific for the S15 housekeeping gene (Table 2), and the lack of visible product in the -RevT controls after 30 cycles (data not shown) was taken to indicate minimal contamination of the RNA with genomic DNA.

Quantitative PCR. One microliter of the +RevT cDNA product (see above) was mixed with Sybr green master mix (Clontech, Mountain View, CA), ROX reference dye, and a 0.5 µM concentration of each forward and reverse primer (Table 2). Triplicate reactions were performed for each of the three cDNAs, for a total of nine reactions per strain. A standard curve was generated for each target using a linearized plasmid carrying the gene of interest (Table 1) as the template at known copy numbers ranging from

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, characteristics, or construction	Antibiotic resistance ^a	Reference or source
Strains			
<i>Pseudomonas putida</i>			
GB-1	Manganese oxidizer, wild type	Amp ^r	28
KG51	GB-1; Δ 2447, generated by conjugation of pFM47 into wild-type cells		This work
KG127	GB-1; Δ <i>mnxR</i>		27
KG152	GB-1; Δ 2665, generated by conjugation of pKG170 into wild-type cells		This work
KG180	GB-1; Δ 2665 Δ 2447, generated by conjugation of pKG174 into KG152		This work
KG182	GB-1; Δ 2447 Δ 2665, generated by conjugation of pKG170 into KG51		This work
KG202	GB-1; Δ 2665, Δ 2447::2665, generated by conjugation of pKG263 into KG180		This work
KG208	GB-1; Δ 2665 Δ 2447::2447, generated by conjugation of pKG264 into KG180		This work
<i>Escherichia coli</i>			
UQ950	DH5 α λ pir host for cloning; F [−] Δ (<i>argF-lac</i>)69 ϕ 80 <i>dlacZ</i> 58(Δ M15) <i>glnV44</i> (AS) <i>rfbD1</i> <i>gyrA96</i> (NaI ^r) <i>recA1</i> <i>endA1</i> <i>spoT1</i> <i>thi-1</i> <i>hsdR17</i> <i>deoR</i> λ pir ⁺		L. Dietrich, Caltech
TAM1	<i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i>		ActiveMotif
WM3064	Donor strain for conjugation: <i>thrB1004</i> <i>pro</i> <i>thi</i> <i>rpsL</i> <i>hsdS</i> <i>lacZ</i> Δ M15RP4-1360 Δ (<i>ara-bad</i>)567 Δ <i>dapA1341</i> ::[<i>erm</i> <i>pir</i> (wt)]		L. Dietrich, Caltech
Plasmids			
pBBR1MCS-5	Broad-host-range cloning vector	Gm ^r	30
pEX18Gm	Gene replacement vector; <i>oriT</i> <i>sacB</i>	Gm ^r	31
pFM47	1.8-kb fusion PCR fragment containing Δ <i>mnxG</i> flanking regions cloned into the <i>SpeI</i> site of pSMV10	Gm ^r	This work
pJET1.2/blunt	Commercial cloning vector	Amp ^r	Fermentas
pKG170	PputGB1_2665 deletion construct generated using 2665_upstream, downstream and junction primers (Table 2), cloned into pEX18Gm	Gm ^r	This work
pKG174	PputGB1_2447 deletion construct generated using 2447_3–2447_6 (Table 2), cloned into pEX18Gm	Gm ^r	This work
pKG193	PCR amplified PputGB1_2447 with 2447_11-F and 2447_12-R, cloned product into pJET1.2/blunt, subclone <i>Bgl</i> II fragment into <i>Bam</i> HI cut pBBR1MCS-5	Gm ^r	This work
pKG196	PCR amplified PputGB1_2665 with 2665_1-F and 2665_2-R, cloned into pJET1.2/blunt, subcloned <i>Xba</i> I/ <i>Xma</i> I fragment into <i>Xba</i> I/ <i>Xma</i> I cut pBBR1MCS-5	Gm ^r	This work
pKG223	S15 amplified with S15_3-F and S15_4-R and cloned into pJET1.2/blunt	Amp ^r	This work
pKG263	PputGB1_2665 complementation construct generated by PCR amplification with 2665_upstream-F and 2665_downstream-R primers (Table 2), cloned into pEX18Gm	Gm ^r	This work
pKG264	PputGB1_2447 complementation construct generated by PCR amplification with 2447_3-F and 2447_6-R (Table 2), cloned into pEX18Gm	Gm ^r	This work
pRK2013	<i>ColE1</i> replicon, <i>mobRK2</i> , <i>traRK2</i>	Kn ^r	32
pSMV10	Mobilizable suicide vector; <i>oriR6K</i> <i>mobRP4</i> <i>sacB</i>	Kn ^r , Gm ^r	D. Lies, Caltech
pUCP22	Broad-host-range vector, <i>ColE1</i> replicon	Gm ^r	33

^a Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Kn^r, kanamycin resistance.

8.7 to 2.8×10^6 , with six 10-fold serial dilutions. Transcript levels for the ribosomal protein S15 were assumed to be unaffected by the presence of the regulator MnxR and thus were used to normalize transcript levels between the two conditions. Reaction conditions were 7 min initial denaturation followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

Generation of in-frame deletions. In-frame deletions of selected genes were generated as described previously (27). Briefly, ~500 bp of DNA flanking the gene of interest was PCR amplified (primers are listed in Table 2) and fused using PCR with splicing by overlap extension (SOEing) (37). The fusion construct was cloned into the gene replacement vector pEX18Gm (Table 1), which was moved by conjugation (27) into wild-type or mutant *P. putida* GB-1. Double recombinants were isolated by counterselection against the *sacB* locus on the gene replacement vector, and the deletion of the target gene was verified by PCR amplification across the site of the deletion.

Complementation. The genes PputGB1_2447 and PputGB1_2665 were amplified along with ~500 bp of flanking sequencing by PCR (primers are listed in Table 2), and the resulting products were ligated into pJET1.2/blunt (Fermentas, Glen Burnie, MD). The genes plus the flanking regions were then excised with restriction enzyme *Bgl*II and subcloned into *Bam*HI-cut pEX18Gm to make pKG264 and pKG263 (Table 1). These plasmids were then moved by conjugation into the Δ *mcoA* Δ *mnxG* strain KG180, double recombinants were isolated, and the restoration of either *mcoA* or *mnxG* was verified by PCR.

RESULTS

Genomic comparisons to identify putative Mn(II) oxidase genes. To identify candidates for the Mn(II) oxidase gene in *P. putida* GB-1, we searched its genome for homologues to each of

TABLE 2 Primers used in this study

Purpose and primer	Sequence
In-frame deletions	
2665_upstream-F	CCAGGTCGGCTCGTTCTGGCG
2665_downstream-Rs	AGGCCATCGATCCACAGCCCCAG
2665_junction-F	CTGCCGTGATTCACCCGAACCGGGGGC
	AAAGTGTGACGGTGCCG
2665_junction-R	CGGCACCGTCACACTTTGCCCCGGTT
	CGGGTGAATCACGGCAG
2447_3-F	TATCGTCGGGCCCCGTGAC
2447_4-F	GACTACGCCACGCAAAGGCCAGTGAC
	GGAAAAGGAGCGC
2447_5-R	GCGCTCCTTTCCGTCCTGCTGCTTT
	GCGTGGCGTAGTC
2447_6-R	CCGACACAGTCGGGTGCGATC
2447_US-F.2	ATTACTAGTTGCGGTGACCTATATCGTCG
2447_US-R.1	CCCCTCCACTAAATTTAAATACAGGTTG
	GTCAGGTTGACCG
2447_DS-F.1	TATTTAAATTTAGTGATGGGCCACTG
	CACAACACCAAGC
2447_DS-R.1	TATACTAGTAGGTTTCGGTGTGATCACC
qPCR	
S15_1-F	TCAAATCGTTACCGACTTCCA
S15_2-R	TCTTTGCCCTTCAGGTAGTC
S15_3-F	GATCCGTTATCAGGAGAAGC
S15_4-R	ATGACATGCTCATAGACAACG
2447_11-F	TCCCGGAGGTGCAGCATGAC
2447_12-R	CATGCTCGCGTCCTTTTCCG
2447_20-F	GATGATGACGACGATGAGGA
2447_21-R	CGATCCAGAATGGGTAACCT
2665_1-F	CCCGGGACAGCCGTGAGCGAGGC
2665_2-R	TCTAGACCGCACAGCGCCCGTTTC
2665c-F	CCGTGATTCACCCGAACCGG
2665c-R	AACAACGGGCTGGGCTTGC

the putative Mn(II) oxidase genes previously identified in other bacterial species (GI sequence identification numbers and species are listed in Table 3) using the BLAST function on the IMG website (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>). For most cases, we categorized a gene as a potential homolog if the E value was less than 10^{-5} . There was no homolog of *mofA* in the *P. putida* GB-1 genome that fit this criterion; however, the MCO-encoding locus PputGB1_2665 has very weak homology ($E = 2$). In total, seven genes encoding potential homologs to the four putative Mn(II) oxidases were identified (Table 3). One of these, the *moxA* homolog *cumA* (PputGB1_1031), was previously implicated as encoding the Mn(II) oxidase (20) but was shown to have no effect on Mn(II) oxidation (27). Our group has also generated in-frame deletions of the *lapA* gene (PputGB1_0186) and the *mopA* homolog

(PputGB1_3353) and shown that the resulting strains are capable of Mn(II) oxidation (C. Butterfield, personal communication; also data not shown). Therefore, sequence homology revealed four potential Mn(II) oxidase genes in *P. putida* GB-1: PputGB1_0017, PputGB1_1828, PputGB1_2447, and PputGB1_2665.

To further narrow our focus, we reasoned that the gene(s) encoding the Mn(II) oxidase(s) would be conserved in pseudomonad species that oxidize Mn(II) but be absent in nonoxidizers. Using the reciprocal smallest-distance algorithm, orthologs to *P. putida* GB-1 genes were identified in 11 other Mn(II)-oxidizing, nonoxidizing, and unreported *Pseudomonas* species (reference 38 and our unpublished results). The species for which the Mn(II) oxidase activity status was unknown were tentatively designated oxidizers or nonoxidizers based on the presence or absence of *mnxR*, the gene encoding Mn_xR, a transcription factor essential for Mn(II) oxidation (27). Three species were strains of *P. putida* (F1, KT2440, and W619); the other pseudomonads compared were *P. entomophila* L48, *P. fluorescens* PfO-1 and PF5, *P. mendocina* ymp, *P. stutzeri* A1501, *P. syringae* pv. tomato DC3000 and T1, and *P. aeruginosa* PAO1. Using the merged RSD ortholog comparison file (see Table S1 in the supplemental material), we observed that only the putative *mnxG* homolog PputGB1_2447 displayed the predicted pattern of conservation in oxidizing organisms and absence in nonoxidizers (Table 4). The putative *mofA* homolog PputGB1_2665 was present in all but one of the oxidizers and absent in all of the nonoxidizers. Not surprisingly, *cumA*, which has been shown not to play a role in Mn(II) oxidation, had orthologs in all species examined. A similar comparative genomics study of *P. putida* strains KT2440, F1, W619, and GB-1 detected the same patterns of conservation for PputGB1_2447 and PputGB1_2665 (39).

Regulation of putative oxidase genes by the Mn(II) oxidation transcription factor Mn_xR. Because the response regulator Mn_xR, a predicted σ^{54} -dependent transcription factor (27), is required for Mn(II) oxidation, we investigated whether the expression of genes encoding potential Mn(II) oxidases were under Mn_xR control. As genes regulated by Mn_xR are necessarily transcribed from σ^{54} -dependent promoters, we visually examined the regions upstream of the genes listed in Table 3 and queried the sequences against the Prodigal Database (<http://www.prodigal.de/vfp/index2.php>) for the conserved σ^{54} binding site [CTGGNA–6 bp–TTGCA (40)]. For PputGB1_1828, we also scanned the sequence upstream of PputGB1_1829, as the genes' orientation suggests that the two may be cotranscribed from a promoter upstream of PputGB1_1829. Of the seven candidates, only PputGB1_2447 and PputGB1_2665 had predicted σ^{54} promoters within 250 bp of their start codons (Table 4).

To confirm Mn_xR regulation of the putative Mn(II) oxidase

TABLE 3 List of *P. putida* GB-1 genes with homology to putative Mn(II) oxidases from other organisms

Gene	Organism	Locus tag	Predicted function in <i>P. putida</i> GB-1	E value
<i>moxA</i> (GI:82940269)	<i>Pedomicrobium</i> sp. strain ACM 3067	PputGB1_1828	Copper resistance protein CopA	3×10^{-22}
		PputGB1_1031	Multicopper oxidase CumA	1×10^{-21}
		PputGB1_0017	Copper resistance protein CopA	9×10^{-21}
<i>mofA</i> (GI:294345251)	<i>Leptothrix discophora</i> SS-1	PputGB1_2665	Multicopper oxidase (<i>mcoA</i>)	2
<i>mnxG</i> (GI:149182216)	<i>Bacillus</i> sp. strain SG-1	PputGB1_2447	Hypothetical protein (<i>mnxG</i>)	1×10^{-22}
<i>mopA</i> (GI:90417986)	<i>Aurantimonas manganoxydans</i> SI85-9A1	PputGB1_3353	Animal heme peroxidase (<i>mopA</i>)	0
		PputGB1_0186	Adhesion protein LapA	4×10^{-6}

TABLE 4 Conservation of putative oxidase genes among oxidizing and nonoxidizing pseudomonads^a

Locus tag	Conservation in:											σ^{54} promoter ^b
	Oxidizing strains					Nonoxidizing strains						
	F1	KT2440	W619	PfO-1	L48	ymp	PF5	A1501	DC3000	T1	PAO1	
<i>mnxR</i>	+	+	+	+	+	—	—	—	—	—	—	ND
PputGB1_0017	+	+	+	+	—	+	+	+	+	+	+	No
PputGB1_0186 (<i>lapA</i>)	+	+	+	+	+	—	+	—	+	+	—	No
PputGB1_1031 (<i>cumA</i>)	+	+	+	+	+	+	+	+	+	+	+	No
PputGB1_1828	—	+	—	—	+	—	—	—	—	—	—	No
PputGB1_2447 (<i>mnxG</i>)	+	+	+	+	+	—	—	—	—	—	—	Yes
PputGB1_2665 (<i>mcoA</i>)	+	+	—	+	+	—	—	—	—	—	—	Yes
PputGB1_3353 (<i>mopA</i>)	+	+	—	—	—	+	—	—	+	+	—	Possible ^c

^a Oxidizing organisms are *P. putida* F1, *P. putida* KT2440, *P. putida* W619, *P. fluorescens* PfO-1, and *P. entomophila* L48. Nonoxidizing organisms are *P. mendocina* ymp, *P. fluorescens* PF5, *P. stutzeri* A1501, *P. syringae* pv. tomato strain DC3000, *P. syringae* pv. tomato strain T1, and *P. aeruginosa* PAO1.

^b The last column indicates whether a predicted σ^{54} -dependent promoter was detected within the 250 bp upstream of the start codon. ND, not determined.

^c Upstream of this gene is a possible σ^{54} binding site with 4 of 5 matches to the consensus sequence at the -12 element and 3 of 5 at the -24 element.

genes PputGB1_2447 and PputGB1_2665, wild-type and $\Delta mnxR$ cultures were grown to their respective robustly oxidizing and nonoxidizing phenotypes (Fig. 1A). Total RNA was extracted from these cultures, converted to stable cDNA, and used to perform quantitative PCR (Q-PCR) to determine the differential expression levels in the wild-type and $\Delta mnxR$ backgrounds relative to the housekeeping gene *S15*. For both PputGB1_2447 and PputGB1_2665, expression was substantially lower in the $\Delta mnxR$ culture than in the robustly oxidizing culture (Fig. 1B): PputGB1_2447 is downregulated 7-fold, while PputGB1_2665 expression is cut in half. This expression profile, considered in light of the conserved σ^{54} promoter motif found upstream of both genes, indicates that PputGB1_2447 and PputGB1_2665 are regulated by the Mn(II) oxidation regulator MnxR.

In-frame deletion of putative oxidase genes. PputGB1_2447 and PputGB1_2665 both fulfill the expectations for the Mn(II) oxidase gene: homology to known Mn(II) oxidases, conservation among oxidizers but not nonoxidizers, and regulation by MnxR. To determine which one of these genes encodes the Mn(II) oxidase, we generated in-frame deletions of each gene and screened for the resulting strains' capacity to oxidize Mn(II). However, both deletion strains retained oxidase activity on solid medium and in liquid culture, as indicated by the presence of brown oxides

(Fig. 2A to C) and reaction with leucoberbelin blue (data not shown). Curiously, the PputGB1_2665 deletion strain has a severe defect in Mn(II) oxidation on solid medium but behaves similarly to the wild type in liquid (see below). The fact that neither deletion strain resulted in a complete failure to oxidize Mn(II) could indicate that neither gene encodes the oxidase but that each instead encodes accessory factors that enhance, but are not required for, Mn(II) oxidation. Alternatively, it is possible that these genes encode partially redundant Mn(II) oxidases. The existence of two oxidases in *P. putida* GB-1 has been hinted at by the failure of random genetic screens to identify the oxidase and by the presence of two active protein bands on a nondenaturing gel. If these two genes each encode Mn(II) oxidases, deletion of both genes would be predicted to result in a complete loss of oxidase activity. This is in fact the phenotype observed for the $\Delta 2665 \Delta 2447$ double mutant: an inability to oxidize Mn(II) both on plates and in shaking liquid culture (Fig. 2A to C). We also screened for oxidation in static liquid culture, on plates at 30°C and 10°C, and on plates grown in a chamber containing a CampyPak to decrease O₂ levels, and in all cases the $\Delta 2665 \Delta 2447$ mutant failed to oxidize Mn(II) to Mn(IV) (data not shown). To be certain that the loss of oxidation observed in the $\Delta 2665 \Delta 2447$ double mutant was due to the gene deletions and not fortuitous second-site mutations, we re-

FIG 1 (A) Mn(II) oxidation by cultures from which RNA was purified. The dashed line indicates the limit of detection, 0.01. Cultures were grown in triplicate, error bars represent the standard deviation. (B) Quantitative PCR measurement of transcript levels relative to levels of the housekeeping gene, *S15*. Values represent triplicate reactions for each of the triplicate cultures used for panel A; error bars represent standard deviations.

FIG 2 Mn(II) oxidation by *P. putida* GB-1 strains. (A) Strains were grown in Lept + 100 μM MnCl_2 at RT with shaking for 24 h. (B) The same cultures after 48 h. (C) Bacteria were streaked onto a Lept plate containing 100 μM MnCl_2 and incubated at RT for 4 days. The $\Delta 2665 \Delta 2447::2447$ and $\Delta 2665 \Delta 2447::2665$ strains have had the PputGB1_2447 and PputGB1_2665 loci, respectively, restored to the chromosome via recombination. (D) Bacteria were streaked onto a Lept plate containing 100 μM Mn(III) acetate and 500 μM sodium pyrophosphate and incubated at RT for 5 days.

made the double mutant, this time by deleting PputGB1_2665 from the $\Delta 2447$ strain. This new double mutant, the $\Delta 2447 \Delta 2665$ strain, also was defective for Mn(II) oxidation (data not shown). Furthermore, using genetic recombination to restore full-length PputGB1_2447 or PputGB1_2665 to their native locations on the chromosome restored Mn(II) oxidation to the $\Delta 2665 \Delta 2447$ strain: the $\Delta 2665 \Delta 2447::2447$ strain exhibited oxidation levels similar to those of the $\Delta 2665$ strain, and the $\Delta 2665 \Delta 2447::2665$ strain behaved similarly to the $\Delta 2447$ mutant (Fig. 2C). From this, we conclude that the phenotype of the double mutant is due to the in-frame deletions of the two genes.

Phenotypic characterization of the single mutant strains. PputGB1_2447 has homology to *Bacillus* sp. SG-1 MnxG (Table 3), which has been shown to catalyze both the Mn(II)-to-Mn(III) and Mn(III)-to-Mn(IV) steps of Mn(II) oxidation (14). But the presence of two oxidases in *P. putida* GB-1 raises the possibility that each catalyzes just one of the two electron transfer steps. Therefore, we screened the ability of the deletion strains to oxidize

Mn(III) as well as Mn(II). Both the $\Delta 2447$ and $\Delta 2665$ strains were capable of oxidizing Mn(III), while the double mutant failed to do so (Fig. 2D). Thus, each of the enzymes is capable of oxidizing both Mn(II) and Mn(III).

It is clear from Fig. 2 that the $\Delta 2447$ and $\Delta 2665$ strains, while exhibiting decreased Mn(II) oxidation, do not behave identically. In particular, the severity of their oxidation defect varied depending on whether the growth medium was liquid or solid. Therefore, we quantified the levels of Mn(III,IV) oxides produced by the mutant strains in liquid and on solid media. Again, the $\Delta 2447 \Delta 2665$ double mutant failed to produce detectable Mn(III,IV) oxides in either medium. Oxidation by the $\Delta 2447$ strain was at the limit of detection after 1 day of growth in liquid, while the $\Delta 2665$ strain showed significant oxidation (Fig. 3A); at 2 days, the two mutants displayed the same level of oxidation, suggesting that $\Delta 2447$ had delayed onset of Mn(II) oxidation. On plates, the $\Delta 2447$ mutant behaved essentially like the wild type, while the $\Delta 2665$ strain had substantially decreased oxidase activity (Fig.

FIG 3 Quantification of the oxidation defect of the $\Delta 2665$, $\Delta 2447$, and $\Delta 2665 \Delta 2447$ strains in liquid (A) and on plates (B). Results are the averages from two independent cultures; error bars indicate standard deviations.

3B). This difference in oxidation on plates versus in liquid may reflect differences in O_2 concentration, with lower levels in a colony and higher in a shaking liquid culture. In agreement with this, the Mn(II) oxidation defect of $\Delta 2447$ can be suppressed by growth in a chamber containing a CampyPak (data not shown).

DISCUSSION

During its long history as a model organism for the study of bacterial Mn(II) oxidation, *P. putida* GB-1 has resisted many attempts to identify the gene encoding its Mn(II) oxidase. All failed because, as we show here, GB-1 carries two genes—PputGB1_2447 and PputGB1_2665—that each encode an enzyme capable of oxidizing Mn(II). Disrupting either one of these genes individually leads to a moderate, condition-dependent decrease in oxidation; to obtain a complete loss of oxidation phenotype, screened for in previous genetic approaches, both genes must be deleted simultaneously. Based on this new understanding, we conclude that the GB-1 2447 and 2665 proteins function as a partially redundant, complementary MCO pair and are the Mn(II) oxidases of *P. putida* GB-1.

PputGB1_2447 and PputGB1_2665 were initially identified by homology to MnxG and MofA, respectively. However, as the homology between PputGB1_2665 and *mofA* (Table 3) is weak, and the proteins encoded by PputGB1_2665 and *mofA* are very different in size (Fig. 4A), we do not think it is appropriate to name this gene *mofA*. Rather, we propose naming PputGB1_2665 *mcoA* (Mn(II) copper oxidase A). PputGB1_2447 was initially identified as a homolog of *Bacillus* MnxG, which is an unusual MCO in that it has five putative Cu-binding regions (regions A to D and F), with the order of regions being altered such that C and D are switched with A and B compared to other MCOs (Fig. 4A). PputGB1_2447 exhibits a high level of homology to MnxG within these putative Cu-binding domains, although the Cu-binding histidines of region F are not conserved in PputGB1_2447 (Fig. 4B), and the regions are present in the same order in the protein as in *Bacillus* MnxG (Fig. 4A). As a result, we propose naming PputGB1_2447 *mnxG*.

Whereas we conclude that *mnxG* and *mcoA* encode enzymes that each oxidize Mn(II), it is possible that the pair generate a cofactor essential for the actual Mn(II) oxidase to function. We do not believe that this is the case, for the following reasons. (i) A partially purified protein fraction from *P. putida* GB-1, when separated by nondenaturing gel electrophoresis, formed two bands,

each with Mn(II) oxidase activity, visualized by an in-gel Mn(II) oxidation assay. The apparent molecular masses of the two bands, 250 kDa and 180 kDa (28), are not far off from the predicted molecular masses for *P. putida* GB-1 MnxG (209 kDa) and McoA (123 kDa). Indeed, the size discrepancy may be due to potential N-glycosylation of MnxG and McoA, as has been reported for other MCOs in archaea and *Saccharomyces cerevisiae* (41, 42), or due to incomplete denaturation, causing the proteins to run anomalously. (ii) The failure of all the previous genetic experiments to identify the Mn(II) oxidase (21–25) also suggested that there is more than one oxidase gene in *P. putida* GB-1, since it would be extremely difficult to simultaneously disrupt multiple oxidase genes through random mutagenesis. (iii) Addition of Cu to *P. putida* GB-1 cultures stimulates Mn(II) oxidation (20), supporting the idea that the Mn(II) oxidase in this organism is an MCO. (iv) MnxG was directly identified as the *Bacillus* Mn(II) oxidase by mass spectroscopy in a partially purified, Mn(II)-oxidizing protein fraction (10). Thus, it is likely that the GB-1 MnxG is also a Mn(II) oxidase. Purification of the proteins and analyzing their ability to oxidize Mn(II) *in vitro* will provide ultimate confirmation, but given the available evidence, we believe *mnxG* and *mcoA* each encode Mn(II) oxidases.

In the organisms studied previously, there appeared to be just one Mn(II) oxidase present. Why, then, does *P. putida* GB-1 have two? According to the Laccase Engineering Database (<http://www.lccd.uni-stuttgart.de/>) (43), McoA belongs to the bilirubin oxidase MCO superfamily, while GB-1 MnxG has a low level of similarity to the small laccase (SLAC) superfamily. Both are predicted to have signal peptides and be exported, but McoA has a putative transmembrane helix, suggesting that it may be membrane bound (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>). Both are regulated by the transcription factor MnxR (Fig. 1B); however, the high level of *mcoA* transcript in the $\Delta mnxR$ background suggests that an additional level of posttranscriptional regulation may control McoA activity. The two proteins are also very different in size and in the order and number of putative Cu-binding sites (Fig. 4A). Even within the Cu-binding domains, there is little sequence similarity between the two proteins. This suggests that the two proteins are not evolutionarily related to one another nor likely the result of a gene duplication. Along with MofA, *Leptothrix cholodnii* SP-6 has a close homolog of GB-1 MnxG (Lcho_3893; E value = 0) that is located in a putative operon similar to that of PputGB1_2447 (see below). This raises the possibility that pos-

FIG 4 (A) Sizes and Cu-binding site distributions of Mn(II)-oxidizing MCO genes. The MCO genes are shown as arrows, with putative Cu-binding motifs in black. *L. cholodnii* SP-6 is shown because *L. discophora* SS-1 is not fully sequenced. (B) Sequence conservation between *Bacillus* sp. SG-1 and *P. putida* GB-1 MnxG proteins at the putative Cu-binding sites. Putative Cu-binding ligands are in bold.

sessing multiple Mn(II)-oxidizing MCOs is a common theme among oxidizing organisms.

The alignment of 12 pseudomonad genomes, based on the reciprocal smallest-distance (RSD) algorithm comparison (see Table S1 in the supplemental material), supports the conclusion that pseudomonads and perhaps other proteobacterial Mn(II) oxidizers, such as *Leptothrix* spp., employ multiple MCOs for Mn(II) oxidation. Notwithstanding the variations in results that may arise as threshold and sequence divergence parameters are set more or less stringently within the RSD application suite, the alignment indicates five distinct, putative operons (PputGB1_2447–PputGB1_2452, PputGB1_2453–PputGB1_2458, PputGB1_2519–PputGB1_2521, PputGB1_2550–PputGB1_2553, and PputGB1_2665–PputGB1_2667) that differentiate the oxidizing species from those that have never been reported as oxidizers. With few exceptions, the genes in each operon appear in the oxidizing species but not the nonoxidizers, and indeed, the Mn(II) oxidase genes *mnxG* and *mcoA* are the first genes of two of the operons. These two operons are also similar in that they both encode putative electron transport/copper chaperone proteins. The third operon (PputGB1_2519–PputGB1_2521) is comprised of the two sensor kinases and the σ^{54} -dependent response regulator, MnxR, that make up the Mnx two-component regulatory pathway that is essential for Mn(II) oxidation (27). The fourth operon (PputGB1_2550–PputGB1_2553) encodes hypothetical proteins that have not previously been implicated in Mn(II) oxidation. The fifth operon

(PputGB1_2453–PputGB1_2458) includes genes identified as being required for the export of oxidase activity (23). In all the *Pseudomonas* Mn(II)-oxidizing species, these five regions of their genomes confer a specific functional trait, the ability to oxidize Mn(II), that differentiates these species from their relatives within the genus.

What purpose does having multiple Mn(II)-oxidizing enzymes serve for the organism? On solid media, the $\Delta mcoA$ strain has a more severe defect in Mn(II) oxidation than does the $\Delta mnxG$ strain, while in liquid media, the $\Delta mcoA$ strain has a relatively mild defect and the $\Delta mnxG$ strain exhibits delayed oxidation (Fig. 3). Because Mn(II) oxidation occurs in stationary phase (7, 44), one explanation for this delay in oxidation could be delayed entry into stationary phase. However, growth curves obtained with cultures in liquid media indicated that both single mutants and the $\Delta mcoA \Delta mnxG$ double mutant entered stationary phase at the same time as the wild type (data not shown). Furthermore, Mn(II) oxidation by the two mutant strains varies quite a bit depending on growth conditions; O_2 levels and the presence of plasmids or antibiotics also differentially affect Mn(II) oxidation by the deletion strains (data not shown). These characteristics suggest that each enzyme may be optimized for Mn(II) oxidation under a specific set of conditions, expanding the repertoire of environments in which *P. putida* GB-1 is capable of oxidizing Mn(II). For example, MnxG could be primarily responsible for Mn(II) oxidation during planktonic growth, whereas McoA is

active when cells aggregate, become particle attached, or form biofilms. Alternatively, one enzyme may be specific for oxidation of Mn(II) while the other primarily oxidizes other metals or organic compounds but is capable of Mn(II) oxidation in the presence of 100 μ M MnCl₂, the concentration commonly used in our media. However, this seems unlikely, since it is possible to detect Mn(II) oxidase activity for wild-type and both Δ mnxG and Δ mcoA strains at Mn(II) concentrations as low as 1 μ M (data not shown). Having identified the Mn(II) oxidases of this genetically tractable organism, we now have the tools in hand to investigate the mechanisms, functions, and benefits of Mn(II) oxidation and address the question of why *P. putida* GB-1 has redundant Mn(II) oxidases.

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REFERENCES

1. Tebo BM, Bargar JR, Clement BG, Dick GJ, Murray KJ, Parker D, Verity R, Webb SM. 2004. Biogenic manganese oxides: properties and mechanisms of formation. *Annu. Rev. Earth Planet. Sci.* 32:287–328.
2. Tebo BM, Johnson HA, McCarthy JK, Templeton AS. 2005. Geomicrobiology of manganese(II) oxidation. *Trends Microbiol.* 13:421–428.
3. Glenn JK, Akileswaran L, Gold MH. 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.* 251:688–696.
4. Miyata N, Tani Y, Maruo K, Tsuno H, Sakata M, Iwahori K. 2006. Manganese(IV) oxide production by *Acremonium* sp. strain KR21-2 and extracellular Mn(II) oxidase activity. *Appl. Environ. Microbiol.* 72:6467–6473.
5. Santelli CM, Webb SM, Dohnalkova AC, Hansel CM. 2011. Diversity of Mn oxides produced by Mn(II)-oxidizing fungi. *Geochim. Cosmochim. Acta* 75:2762–2776.
6. Saratovsky I, Gurr SJ, Hayward MA. 2009. The structure of manganese oxide formed by the fungus *Acremonium* sp. strain KR21-2. *Geochim. Cosmochim. Acta* 73:3291–3300.
7. Tebo BM, Geszvain K, Lee S. 2010. The molecular geomicrobiology of bacterial manganese(II) oxidation, p 285–308. In Barton LL, Mandl M, Loy A (ed), *Geomicrobiology: molecular and environmental perspective*. Springer, Dordrecht, The Netherlands.
8. Ridge JP, Lin M, Larsen EI, Fegan M, McEwan AG, Sly LI. 2007. A multicopper oxidase is essential for manganese oxidation and laccase-like activity in *Pedomicrobium* sp. *ACM* 3067. *Environ. Microbiol.* 9:944–953.
9. Corstjens PLAM, de Vrind JPM, Goosen T, de Vrind-de Jong EW. 1997. Identification and molecular analysis of the *Leptothrix discophora* SS-1 *mofA* gene, a gene putatively encoding a manganese-oxidizing protein with copper domains. *Geomicrobiol. J.* 14:91–108.
10. Dick GJ, Torpey JW, Beveridge TJ, Tebo BM. 2008. Direct identification of a bacterial manganese(II) oxidase, the multicopper oxidase MnxG, from spores of several different marine *Bacillus* species. *Appl. Environ. Microbiol.* 74:1527–1534.
11. Anderson CR, Johnson HA, Caputo N, Davis RE, Torpey JW, Tebo BM. 2009. Mn(II) oxidation is catalyzed by heme peroxidases in “*Aurantimonas manganoxydans*” strain S185-9A1 and *Erythrobacter* sp. strain SD-21. *Appl. Environ. Microbiol.* 75:4130–4138.
12. Kosman DJ. 2010. Multicopper oxidases: a workshop on copper coordination chemistry, electron transfer, and metallophysiology. *J. Biol. Inorg. Chem.* 15:15–28.
13. Soldatova AV, Butterfield C, Tebo BM, Spiro TG. 15 August 2012. How do bacteria form MnO₂? Multi-copper oxidase involvement in both Mn(II) and Mn(III) oxidation. *J. Biological Inorg. Chem.* doi:10.1007/s00775-012-0928-6.
14. Webb SM, Dick GJ, Bargar JR, Tebo BM. 2005. Evidence for the presence of Mn(III) intermediates in the bacterial oxidation of Mn(II). *Proc. Natl. Acad. Sci. U. S. A.* 102:5558–5563.
15. Schlosser D, Hofer C. 2002. Laccase-catalyzed oxidation of Mn²⁺ in the presence of natural Mn³⁺ chelators as a novel source of extracellular H₂O₂ production and its impact on manganese peroxidase. *Appl. Environ. Microbiol.* 68:3514–3521.
16. Hansard SP, Easter HD, Voelker BM. 2011. Rapid reaction of nanomolar Mn(II) with superoxide radical in seawater and simulated freshwater. *Environ. Sci. Technol.* 45:2811–2817.
17. Learman DR, Voelker BM, Vazquez-Rodriguez AI, Hansel CM. 2011. Formation of manganese oxides by bacterially generated superoxide. *Nat. Geosci.* 4:95–98.
18. Learman DR, Wankel SD, Webb SM, Martinez N, Madden AS, Hansel CM. 2011. Coupled biotic-abiotic Mn(II) oxidation pathway mediates the formation and structural evolution of biogenic Mn oxides. *Geochim. Cosmochim. Acta* 75:6048–6063.
19. Jung WK, Schweisfurth R. 1979. Manganese oxidation by an intracellular protein of a *Pseudomonas* species. *Z. Allg. Mikrobiol.* 19:107–115.
20. Brouwers GJ, de Vrind JP, Corstjens PL, Cornelis P, Baysse C, de Vrind-de Jong EW. 1999. *cumA*, a gene encoding a multicopper oxidase, is involved in Mn²⁺ oxidation in *Pseudomonas putida* GB-1. *Appl. Environ. Microbiol.* 65:1762–1768.
21. Caspi R, Tebo BM, Haygood MG. 1998. *c*-type cytochromes and manganese oxidation in *Pseudomonas putida* MnB1. *Appl. Environ. Microbiol.* 64:3549–3555.
22. de Vrind JP, Brouwers GJ, Corstjens PL, den Dulk J, de Vrind-de Jong EW. 1998. The cytochrome *c* maturation operon is involved in manganese oxidation in *Pseudomonas putida* GB-1. *Appl. Environ. Microbiol.* 64:3556–3562.
23. De Vrind J, De Groot A, Brouwers GJ, Tommassen J, De Vrind-de Jong E. 2003. Identification of a novel Gsp-related pathway required for secretion of the manganese-oxidizing factor of *Pseudomonas putida* strain GB-1. *Mol. Microbiol.* 47:993–1006.
24. Ngo TV. 2006. Results of transposon mutagenesis of *Pseudomonas putida* strain GB-1 and characterization of a *mucA*-homolog mutant. M.S. thesis. University of California, San Diego, San Diego, CA.
25. Yamaguchi A. 2009. Genetic analysis reveals complex Mn(II) oxidation regulation in *Pseudomonas putida* GB-1. M.S. thesis. Oregon Health & Science University, Portland, OR.
26. van Waasbergen LG, Hildebrand M, Tebo BM. 1996. Identification and characterization of a gene cluster involved in manganese oxidation by spores of the marine *Bacillus* sp. strain SG-1. *J. Bacteriol.* 178:3517–3530.
27. Geszvain K, Tebo BM. 2010. Identification of a two-component regulatory pathway essential for Mn(II) oxidation in *Pseudomonas putida* GB-1. *Appl. Environ. Microbiol.* 76:1224–1231.
28. Okazaki M, Sugita T, Shimizu M, Ohode Y, Iwamoto K, de Vrind-de Jong EW, de Vrind JP, Corstjens PL. 1997. Partial purification and characterization of manganese-oxidizing factors of *Pseudomonas fluorescens* GB-1. *Appl. Environ. Microbiol.* 63:4793–4799.
29. Buzzo J. 2011. New Insights in Mn(II) Oxidation in *Pseudomonas putida* GB-1. M.S. thesis. Oregon Health & Science University, Portland, OR.
30. Kovach ME, Elzer PH, Steven Hill D, Robertson GT, Farris MA, Roop RM, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175–176.
31. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77–86.
32. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. U. S. A.* 76:1648–1652.
33. West SE, Schweizer HP, Dall C, Sample AK, Runyen-Janecky LJ. 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* 148:81–86.
34. Boogerd FC, de Vrind JP. 1987. Manganese oxidation by *Leptothrix discophora*. *J. Bacteriol.* 169:489–494.

35. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs *Nucleic Acids Res.* 25:3389–3402.
36. Wall DP, Deluca T. 2007. Ortholog detection using the reciprocal smallest distance algorithm, p 95–110. In Bergman NH (ed), *Comparative genomics*, vol. 396. Springer, New York, NY.
37. Horton RM. 1997. In vitro recombination and mutagenesis of DNA. SOEing together tailor-made genes. *Methods Mol. Biol.* 67:141–149.
38. Francis CA, Tebo BM. 2001. *cumA* multicopper oxidase genes from diverse Mn(II)-oxidizing and non-Mn(II)-oxidizing *Pseudomonas* strains. *Appl. Environ. Microbiol.* 67:4272–4278.
39. Wu X, Monchy S, Taghavi S, Zhu W, Ramos J, van der Lelie D. 2011. Comparative genomics and functional analysis of niche-specific adaptation in *Pseudomonas putida*. *FEMS Microbiol. Rev.* 35:299–323.
40. de Avila e Silva S, Echeverrigaray S, Gerhardt GJL. 2011. BacPP: bacterial promoter prediction—a tool for accurate sigma-factor specific assignment in enterobacteria. *J. Theor. Biol.* 287:92–99.
41. Uthandi S, Saad B, Humbard MA, Maupin-Furlow JA. 2009. LccA, an archaeal laccase secreted as a highly stable glycoprotein into the extracellular medium of *Haloferax volcanii*. *Appl. Environ. Microbiol.* 76:733–743.
42. Ziegler L, Terzulli A, Sedlak E, Kosman DJ. 2010. Core glycan in the yeast multicopper ferroxidase, Fet3p: a case study of N-linked glycosylation, protein maturation and stability. *Protein Sci.* 19:1739–1750.
43. Sirim D, Wagner F, Wang L, Schmid RD, Pleiss J. 2011. The Laccase Engineering Database: a classification and analysis system for laccases and related multicopper oxidases. Database (Oxford) 2011:bar006. doi:10.1093/database/bar006.
44. De Palma SR. 1993. Manganese oxidation by *Pseudomonas putida*. Harvard University, Cambridge, MA.