New Insights in Mn (II) Oxidation in *Pseudomonas putida* GB-1

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Dedicated to My Parents John and Colleen Buzzo,

and to My Grandmother Sally Pantti

who inspires me to reach higher.

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Abstract

Manganese (III, IV) oxides are strong naturally occurring oxidants that possess the ability to control the distribution of many trace metals in the environment. Sequence homology of Mn(II) oxidase enzymes with multicopper oxidases and genetic manipulation suggest that two different multi-copper oxidases are the dominant enzymes capable of Mn(II) oxidation in *Pseudomonas putida* GB-1. However, the functional role and enzymatic mechanism remains unknown.

Complete cellular fractionation of *Pseudomonas putida* GB-1 localized Mn(II) oxidizing activity to the outer membrane. Native PAGE demonstrated that the Mn(II) oxidase activity has an apparent molecular weight of >250kDa. Fast protein liquid chromatography using hydrophobic interaction and size exclusion resulted in a partially purified fraction of 130kDa. Tandem MS/MS of the partially pure fraction identified a type two multi-copper oxidase (Mco). However, when *mco* is deleted, Mn(II) oxidation still occurs, consistent with the notion that more than one enzyme is responsible for Mn(II) oxidation.

In vitro experiments with active membrane fractions demonstrate that reactive oxygen species have a role in Mn(II) oxidation. In particular, superoxide dismutase and copper greatly inhibit Mn(II) oxidation, suggesting that superoxide radicals are involved. Hydrogen peroxide also appears to be part of the enzymatic mechanism based on the ability of catalase to inhibit Mn(II) oxidation. These results suggest that the enzymatic oxidation of manganese requires superoxide radical formation and the utilization of hydrogen peroxide for proper catalysis.

Further analysis of MS/MS data identified hypothetical proteins 2552 and 2553 in the partially purified active fraction. Over expression of these hypothetical proteins and the in-frame deletion of 2552 demonstrate that these proteins have an essential role in Mn(II) oxidation in *P.putida* GB-1. Transformation studies suggest an association of the multi-copper oxidase, MnxG with both hypothetical proteins. The fact that 2553 is transcriptionally regulated by the response regulator MnxR provides further evidence that these hypothetical proteins are important for Mn (II) oxidation. However, the exact role and function of these proteins remain unknown. This is the first indication that other outer membrane proteins besides the Mn(II) oxidase(s) are involved with Mn(II) oxidation in *Pseudomonas putida* GB-1.

Chapter 1. Introduction to Manganese Oxidation

Manganese Oxidation

Manganese is the second most abundant transition metal in the Earth's crust and plays a significant role in biogeochemical cycling in the environment. Similar to iron, manganese exists in multiple biologically significant oxidation states. The three most common oxidation states of manganese found in nature are II, III, and IV (Tebo et al., 2004). Manganese cycles between the reduced soluble Mn(II) form to the oxidized insoluble Mn(III) and Mn(IV) forms based on redox conditions in the environment (Nealson et al., 1988). The Mn(II) state is favored in the absence of oxygen and low pH, while the Mn(III) and Mn(IV) oxidation states are more favored in the presence of oxygen and at higher pH (Figure 1.1) (Tebo et al., 2004). Manganese oxidation proceeds via a Mn(III) transient intermediate to Mn(IV), where the Mn(II) to Mn(III) oxidation is the rate-limiting step (Webb et al., 2005).

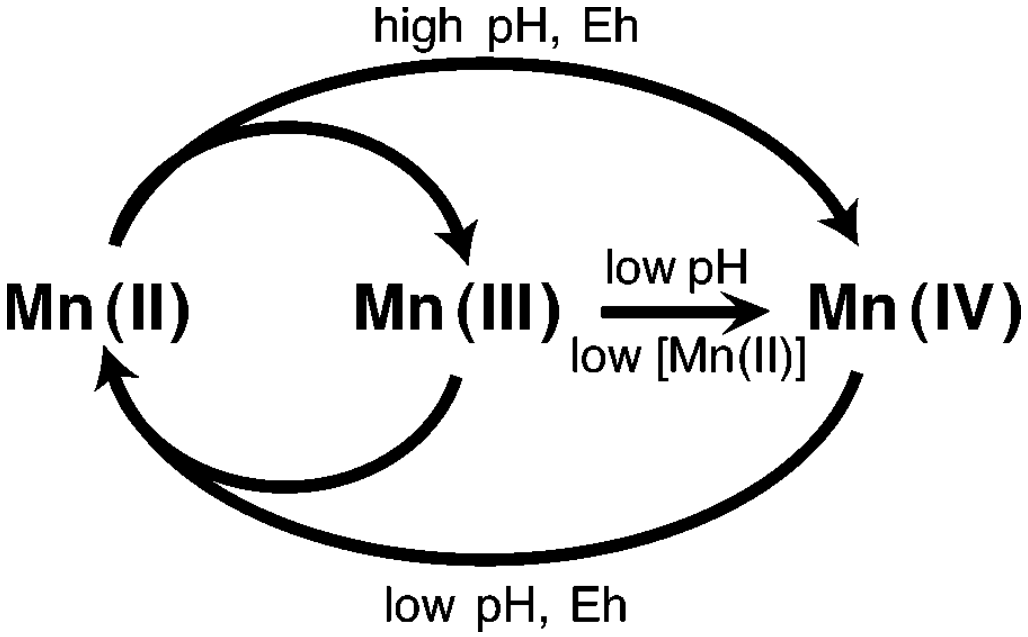

Figure 1.1. The cycling of manganese oxidation states in nature (Tebo et al., 2004).

Manganese oxidation occurs ubiquitously throughout the environment, particularly when there are sufficient amounts of soluble reduced Mn(II). The release of Mn(II) is due to weathering of igneous and metamorphic rock which is then oxidized to form numerous Mn(III), Mn(IV), or mixed Mn(III,IV) oxide/hydroxide minerals (Tebo et al., 2004). Specific environments that manganese oxidation is likely to occur are weathered surfaces such as basalt glasses, hydrothermal vents, metalliferous sediments, ferromanganese nodules and concretions (oceans, lakes, and soils), oxic-anoxic interfaces where redox cycling can occur, desert varnish, hyporheic zones (river and stream sediments), and water pipes (Tebo et al., 2005).

Biogeochemical cycling of manganese in the environment is similar to the cycling of iron and is driven by redox chemistry at the anoxic and oxic transition

zones. Under oxic conditions manganese oxides are produced while under anoxic conditions, reductive dissolution of the oxides produce Mn(II). Cycling can occur by homogeneous, abiotic catalysis on mineral surfaces, photochemical, and enzymatically mediated electron-transfer reactions (van Cappellen et al., 1998)

Manganese (III, IV) oxides are strong naturally occurring oxidants in the environment. They possess the ability to control the distribution of trace metals and other toxic elements due to their redox and sorptive properties (Tebo et al., 2005). Mn (III, IV) oxides can adsorb substantial quantities of metals such as Cu, Ca, Co, Cd, Zn, Fe, Hg, U, Pu, As, and Se, thus controlling the distribution of both toxic and essential trace elements by oxidative precipitation or solubilization (Tebo et al., 2004).

Microbially driven Mn(II) oxidation

Microbially catalyzed oxidation of Mn(II) to Mn(IV) is four to five orders of magnitude faster than abiotic oxidation from mineral surface catalysis or aqueous homogenous oxygenation (Tebo et al., 2004). The ability of microbes to oxidize manganese is a very widespread process and encompasses both fungi and bacterial lineages. Of particular interest is bacterially driven Mn oxidation which is present in Firmicutes, Actinobacteria, and α, ß, and γ Proteobacteria (Figure 1.2). This broad range of bacteria exemplifies the diverse spectrum of manganese oxidizing species. Model manganese oxidizers such as *Leptothrix discophora, Pedomicrobium, Pseudomonas putida,* marine *Bacillus*, *Aurantimons manganoxydans,* and *Erythrobacter* have been characterized and well studied in the laboratory in order

to elucidate the enzymatic mechanisms and determine the functional role of manganese oxidation in the cell (Webb et al., 2005).

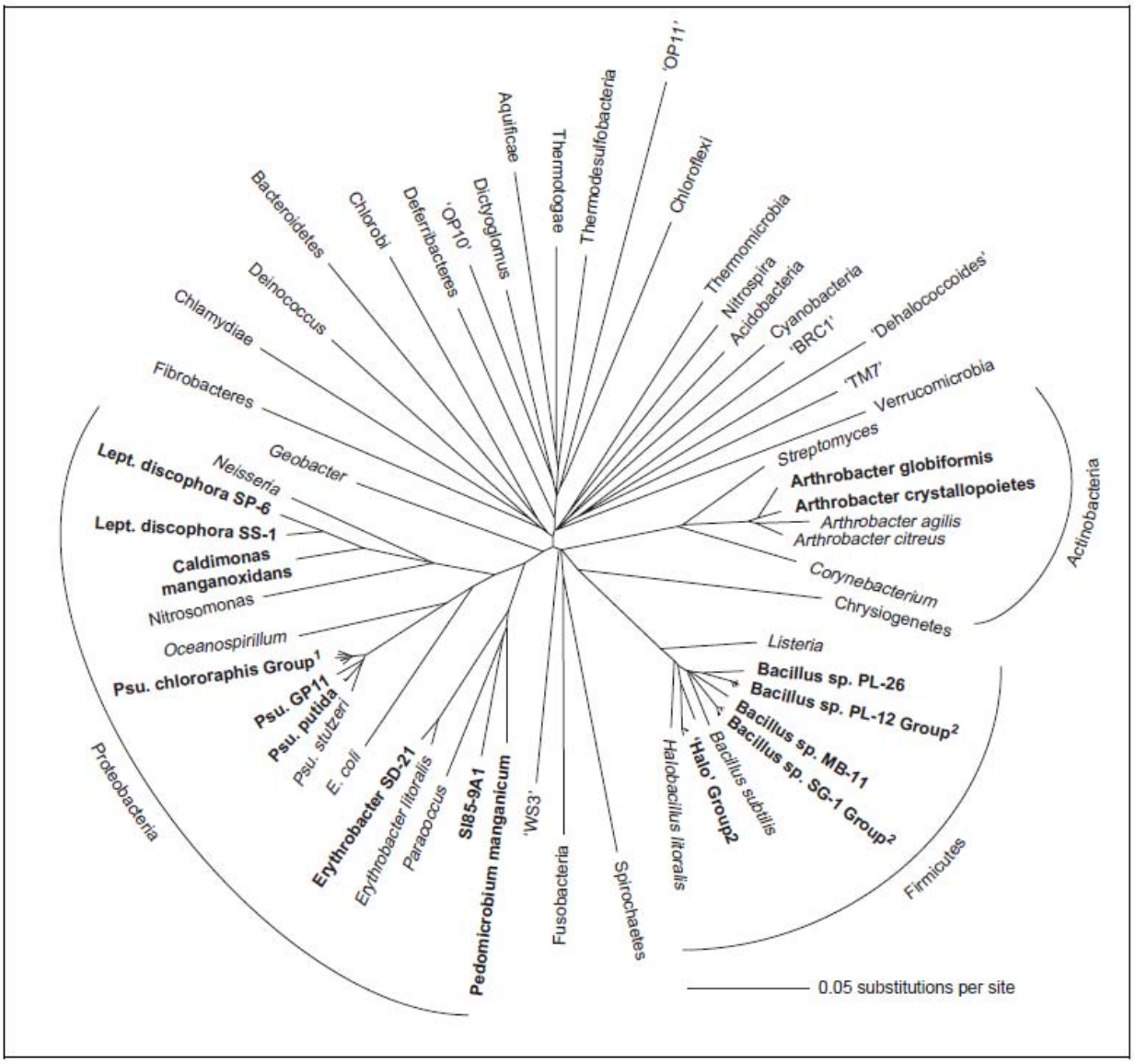

Figure 1.2. Neighbor-joining, unrooted phylogram of the Domain Bacteria indicating representative Mn (II)-oxidizing bacteria (Tebo et al., 2005).

Why do bacteria oxidize manganese?

The biological purpose for bacterial manganese oxidation remains unknown. Bacteria catalyze the oxidation of Mn(II) by direct and indirect processes (Tebo et

al., 2004). Oxidation of Mn(II) to Mn(IV) is thermodynamically favorable with a standard free energy of -70kJ mol^{-1}, which theoretically could sustain bacterial growth. It has been proposed that microbes could potentially utilize the energy of this reaction for ATP formation by shuttling electrons through an electron chain (Brouwers et al., 2000). The involvement and importance of proteins containing cytochrome c (see below) may further indicate that energy utilization and storage are possible functions of manganese oxidation (Caspi et al., 1998 and De Vrind et al., 1998).

Bacteria possibly utilize Mn(III, IV) oxides for a more protective function rather than as an energy source from the oxidation of Mn(II) to Mn(IV). Reactive oxygen species such as superoxide and peroxides, pose a threat to living organisms and thus bacteria might utilize manganese to alleviate this stress. It has been shown that intracellular Mn (II) can act as an antioxidant, protecting cells from superoxide much like the enzyme superoxide dismutase (Archibald and Fridovich, 1981). Manganese complexes such as manganous phosphate, reacts with superoxide to produce a MnO_2^+ transient species which rapidly disproportionates into manganous phosphate, dioxygen, and hydrogen peroxide (Barnese et al., 2008). In *Deinococcus radiodurans*, accumulation of soluble Mn (II), scavenges superoxide radicals that protect the bacteria from high levels of ionizing gamma radiation (Daly et al., 2004). Bacteria can also indirectly oxidize soluble Mn (II) through the enzymatic generation of extracellular superoxide radicals (Learman et al., 2011). Protection from UV radiation, predation, metal toxicity, and viral attack has also

been proposed as a possible function of Mn(II) oxidation by coating and accumulation of Mn(III, IV) oxides on the bacterial cell (Tebo et al., 2005).

Mn(III, IV) oxides are capable of oxidizing complex humic substances, breaking them down into lower molecular mass organic compounds, which can be utilized as a substrate for growth (Sunda and Kieber, 1994). If this is true of bacterial manganese oxidation, it could allow bacteria to access large carbon pools that otherwise would be inaccessible in natural waters, sediments, and soils (Sunda and Kieber, 1994). Thus Mn(II) oxidation could be an important component of carbon biogeochemical cycling in the environment (Tebo et al., 2005).

Manganese oxidation in fungi

The mechanism of oxidation in fungi provides insight into the types of enzymes and substrates that may be utilized in bacteria as well as the possible functional role of Mn (II) oxidation. *Phanerochaete chrysosporium* utilizes a heme containing Manganese Peroxidase (MnPs), the active site of which is oxidized by one H_2O_2 molecule to produce two Mn(III) equivalents (Wariishi et al., 1992). The Mn(III) is then chelated by organic acids and used as a strong oxidant to break down lignin (Perez and Jeffries, 1992). Other litter-decaying basidiomycetes such as *Stropharia rugosannulata* employ laccases to catalyze manganese oxidation. Laccases belong to the multi-copper oxidase (MCO) family of enzymes and oxidizes Mn(II) to Mn(III) to produce H_2O_2, which in turn oxidizes the MnPs for extracellular oxidation and a cooperative biodegradation of lignin and xenobiotics (Schlosser and Hofer, 2002). The utilization of these two types of enzymes and the known abilities

of cooperation between multi-copper oxidases and heme containing peroxides in fungal systems could directly provide insight into the complicated and interesting mechanism of Mn(II) oxidation in bacteria.

Enzymes that catalyze the oxidation of soluble manganese in bacteria

Heme Peroxidases

As discussed in the previous section, Eukaryotic enzymes that are capable of the oxidation of Mn(II) to Mn(III) are heme-containing manganese peroxidases (MnPs), specifically found in lignin degrading fungi. MnPs have Mn(II) bound at a single binding site in close proximity to a heme group. The resting enzyme containing reduced Mn(II) is oxidized by H_2O_2 in a two electron transfer reaction to produce two Mn(III) equivalents to one H_2O_2 and one H_2O molecule (Wariishi et al., 1992).

Until recently, there were no known peroxidases used in bacterial catalyzed Mn (II) oxidation. In both *Aurantimons manganoxydans* Strain SI85-9A1 and *Erythrobacter* sp. Strain SD-21, the Mn(II) oxidase activity was localized and the protein identified by MS/MS spectroscopy as hemolysin-type Ca^{2+} binding animal heme peroxidase, termed MopA for manganese oxidizing protein (Anderson et al., 2009). MnP catalyzed Mn(II) oxidation in bacteria is not fully understood and it is uncertain as to whether these enzymes work in concert with MCOs.

Multi-copper oxidases

Multi-copper oxidases (MCOs) are diverse and ubiquitous, and utilize multiple copper atoms as cofactors to couple the oxidation of numerous substrates such as organics like phenolics, and metals such as Fe^{2+} and Mn^{2+} to the subsequent reduction of O_2 to H_2O (Solomon et al., 1996, Brouwers et al., 2000, Dick et al., 2008). MCOs such as human ceroplasmin and Fet3p are known to directly catalyze Fe^{2+} oxidation (Brouwers et al.. 2000), while *mnxG* in *Bacillus* is the only MCO in bacteria directly linked both biochemically and genetically to Mn(II) oxidation (Dick et al., 2008).

Genetic identification of other putative MCOs hypothesized to be responsible for Mn(II) oxidation in bacteria are as follows; *moxA* in *Pedomicrobium* sp. ACM 3067 (Ridge et al., 2007), *mofA* in *Leptothrix discophera* (Corstjens et al., 1997), and *mco* or *mnxG* in *Pseudomonas putida* strain GB-1 (Geszvain, personal communication), not *cumA* as previously reported (Geszvain and Tebo, 2010). Although many MCOs are redox active and catalyze the oxidation of various substrates, some enzymes, such as CopA are not redox active (Brouwers et al., 2000). MCOs appear to play an integral and substantial role in manganese oxidation, but other enzymes such as MopA might provide a crucial component either by direct or indirect association with MCOs.

What is known about manganese oxidation in *P. putida* GB-1?

Pseudomonas putida GB-1 is a freshwater, gram negative bacterium capable of Mn(II) oxidation in stationary phase (Corstjens et al., 1992 and Okazaki et al.,

1997). Its relative accessibility to genetic manipulation and ease of growth under standard laboratory conditions makes it an ideal and adaptable model organism to study the functional and mechanistic aspects of Mn(II) oxidation (Geszvain and Tebo, 2010). Transposon mutagenesis studies and targeted in-frame gene deletions have been applied to identify genes essential for Mn(II) oxidation by isolating both defective and increased Mn(II) oxidizing mutants (Brouwers et al., 2000, Geszvain and Tebo, 2010, and Geszvain et al., 2011). These screens have identified numerous gene categories that result in defective Mn (II) oxidation, particularly genes involved with the cytochrome c maturation operon (de Vrind et al., 1998 and Caspi et al., 1998), general secretion pathway genes (de Vrind et al., 2003 and Brouwers et al., 1998), genes encoding TCA cycle and tryptophan biosynthesis components (Caspi et al., 1998), genes comprising a two component regulatory pathway essential for Mn(II) oxidation (Geszvain and Tebo, 2010), and genes involved with flagella (Geszvain et al., 2011).

The cytochrome c maturation operon (*ccm*) consists of eight membrane proteins that are involved with heme trafficking and proper positioning of heme to apocytochrome c (Shulz et al., 1999). This operon is an essential component of Mn(II) oxidation, although the exact functional role is not fully understood. Transposon mutagenesis screens resulted in mutants with insertions in the *ccmF*, *ccmA*, and *ccmE* genes which resulted in the complete abolishment of Mn(II) oxidation (Caspi et al., 1998 and De Vrind et al., 1998). Not only were these mutants defective in Mn(II) oxidation, but they were all cytochrome c oxidase

negative and had no spectrophotometrically detectible c-type cytochromes (Caspi et al., 1998). A possible link of the *ccm* operon to Mn(II) oxidation is the integration of cytochrome c to the transfer of electrons from the putative Mn(II) oxidase(s) to terminal electron acceptor oxygen as an intermediate in an electron transport chain (De Vrind et al., 1998). Other possible explanations are that c-type cytochromes are directly associated with the Mn(II) oxidase in a protein complex (De Vrind et al., 1998 and Caspi et al., 1998).

Pseudomonas putida GB-1 catalyzes the oxidation of Mn(II) extracellularly by an enzyme predicted to be located on the outer membrane (Okazaki et al., 1997). Thus, specific cellular machinery and processes are required for proper exoprotein translocation, folding, and positioning on the outer membrane of the bacterium (Brouwers et al., 1998). Transposon mutagenesis studies have implicated the requirement of the general secretion pathway, also known as the type II secretion pathway in transporting the manganese oxidase to the outer membrane. These studies mapped transposon insertions in *xcpA* (prepilin peptidase), *xcpT* (pseudopilin), and *xcmX* (pseudopilin related protein) resulting in transport mutants that could no longer oxidize Mn(II) extracellularly. (De Vrind et al.,2003 and Brouwers et al.,1998).

Mn(II) oxidation in *Pseudomonas putida* GB-1 requires a two component regulatory (TCR) pathway called the MnxR TCR Pathway. TCR pathways generally consist of two main components. A sensor histidine protein kinase responds to environmental stimuli by autophosphorylation at a particular histidine residue,

followed by transfer of the phosphoryl group to an aspartate residue of a response regulator, resulting in a conformational change. This activated response regulator then generally modifies the transcription of associated downstream genes as a result from environmental stimuli (Stock et al., 2000). In this case, the environmental stimuli might be soluble Mn (II) or other unknown factors.

The Mnx TCR pathway is composed of two associated sensor histidine kinases, *mnxS1* and *mnxS2*, and the response regulator *mnx.* In-frame deletions of each gene completely abolished Mn (II) oxidation. *MnxR* is predicted to be a transcription factor that interacts with σ^{-54} containing RNA polymerase and thus activation of this two component regulatory pathway results in the expression of σ^{-54} promoter dependent genes. It is plausible that these genes include those that encode for the putative Mn (II) oxidase(s) or other associated proteins (Geszvain and Tebo, 2010). The discovery of this regulatory pathway could lead to identifying the enzyme(s) involved with Mn (II) oxidation.

Other genes identified by transposon mutagenesis studies that promote Mn (II) oxidation are those associated with components of the tricarboxylic acid cycle. Transposon insertions in genes encoding various subunits of succinate dehydrogenase (*sdh*), lipoate acetyltransferase subunit of the pyruvate dehydrogenase complex (*aceA*), and two isocitrate dehydrogenase encoding genes caused mutants that grew poorly and colony sizes that were smaller than wild type. These mutants were also cytochrome c oxidase negative and had no detectable c-

type cytochromes. Thus they likely affect Mn (II) oxidation by the same mechanism as the *ccm* operon mutants (Caspi et al., 1998).

Transposon mutagenesis studies tentatively determined that the Mn (II) oxidase was encoded by *cumA*, which encodes a putative multi-copper oxidase. When the *cumA*::*Tn5* mutant was screened for Mn(II) oxidation activity, it resulted in a no oxidation (Brouwers et al. 1999). However, when an in-frame deletion of *cumA* was generated, this strain was fully able to oxidize Mn (II) and was still stimulated by the addition of copper (Geszvain and Tebo 2010). These results indicate that *cumA* is not part of Mn (II) oxidation.

Mn(II) oxidation begins at early stationary phase and is directly dependent on oxygen levels. Mn(II) oxidation occurs above fifteen percent oxygen saturation and is optimal at twenty six percent (Okazaki et al., 1997). Previous attempts to purify the manganese oxidase in *P. putida* GB-1 reported purified active preparations containing proteins ranging from apparent molecular weights of 130kDa (Corstjens, 1993) to 180kDa and 250kDa as determined by polacrylamide gel electrophoresis (Okazake et al., 1997). Previous characterization attempts of the Mn(II) oxidizing factor indicated that it has a pH optimum (~7), temperature dependence (optimal at 35° C), and is inhibited by various compounds such as NaN_3 (inhibitor of redox enzymes) and $HgCl_2$ (interacts with sulfhydral groups of proteins), which suggest that the Mn(II) oxidizing factor is in fact an enzyme (Okazaki et al.,1997).

Aim of Research

The purpose of my thesis research was to identify and purify the putative Mn(II) oxidase(s) in *P. putida* GB-1. Identifying the Mn(II) oxidase(s) is crucial to further our understanding of the mechanism of Mn(II) oxidation. Chapter 2 describes the localization of Mn(II) oxidation activity by cellular fractionation, partial protein purification of the active fractions by hydrophobic interaction and size exclusion chromatography FPLC, and protein identification by MS/MS spectroscopy analysis.

To determine whether MCOs or MnPs were the dominant player in Mn (II) oxidation, an in-vitro biochemical approach was used to test substrates specific to these enzymes such as the effects of copper or hydrogen peroxide. Chapter 3 describes these assays and the suggested role of reactive oxygen species in Mn(II) oxidation. Two novel hypothetical proteins were co-identified with Mco in MS/MS Spectroscopy data. Chapter 4 introduces and describes the over expression and in-frame deletion of these genes. These results suggest that these proteins are important for Mn(II) oxidation.

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Chapter 2. Cellular Localization and Partial Purification

Objective

Previous attempts to localize the Mn(II) oxidase in *P. putida* GB-1 determined that the enzyme was associated with the outer membrane and consisted of two active oxidizing factors with apparent molecular weights of 250kDa and 180kDa (Okazaki et al., 1997). Despite partial purification the identity of the Mn(II) oxidase has not yet been confirmed The main objective of the research described in this chapter was to develop a complete cellular fractionation protocol in order to verify manganese oxidizing activity localization and to use Fast Protein Liquid Chromotography along with tandem MS/MS spectroscopy to purify and identify the putative Mn (II) oxidase.

Materials and Methods

Bacterial Strain and Culture Conditions

Pseudomonas putida strain GB-1 (Corstjens et al. 1992) was plated onto LB (Luria-Bertani) medium and grown overnight at room temperature. Single colonies were inoculated in 5mL LB medium and allowed to incubate overnight at room temperature. A 0.1% final volume (e.g. 500ul into 500mL) was subcultured into *Leptothrix* medium (Lept) supplemented with 100μM $MnCl_2$ and grown with continuous shaking for two days at room temperature using multiple 1L Fernbach flasks.

Complete Cellular Fractionation and Localization of Oxidase Activity

Complete cellular fractionation was accomplished by harvesting two liters of Mn(II) oxidizing *P. putida* GB-1 bacterial cells by centrifugation after removal of manganese oxides by the addition of 1-10mM ascorbate using a protocol modified from Anderson et al., (2009). The medium was collected, pre-filtered through 47mm Whatman filter paper, filtered through a 0.2 micron Millipore stericup filter, and ultra-concentrated using a 400mL Amicon stirring filtration system containing a 47mm 10kDa cellulose membrane. The retentate (secreted proteins) were dialyzed overnight in 10mM HEPES buffer (pH 7.5) using 6,000 – 8000 molecular weight cutoff dialysis tubing at 4° C.

The pellet was resuspended in 100mM Tris-HCl, 0.1mM DTE, 0.25mM KCl and stirred vigorously for 2 hours at 4° C. Cells were harvested by centrifugation

and supernatant (loosely bound proteins) was concentrated using the Amicon filtration system containing a 10kDa 47mm cellulose membrane. The resulting pellet was then resuspended in STE buffer for 10 minutes and supplemented with 10mM $MgSO_4$ to remove excess EDTA from solution. The pellet was then harvested by centrifugation and the supernatant decanted. The pellet was then resuspended in cold ddH_2O and incubated on ice for 10 minutes. The resulting pellet was centrifuged and the supernatant was collected containing the periplasmic proteins. The cell pellet was resuspended in cold 5mL 10mM Tris-HCl buffer at pH 8.1 with added 50mM $CaCl_2$ and 0.1mM PMSF followed by three passes through French Press pressure cell (20,000lbs/in^2). The homogenate was centrifuged at 75,000 rpm for one hour, while the supernatant (cytoplasmic proteins) and precipitate were collected. The precipitate was resuspended in 10mM HEPES buffer at pH 7.5 containing 1% Triton-X-100 to solubilize membrane proteins and incubated at room temperature for twenty minutes. The membrane fraction was centrifuged at 50,000 rpm for 2 hours and the resulting supernatant (inner membrane proteins) and precipitate was collected. The resulting pellet was resuspended in 10mM HEPES (pH 7.5) containing 1/10 volume of lysozyme and allowed to react for 15 minutes followed by incubation of 2% N-laurosylsarcosine in ddH_20 for another 15 minutes. The pellet was harvested by centrifugation at 50,000 rpm for one hour. The resulting supernatant (outer membrane proteins) was collected. All collected fractions were then dialyzed overnight in 10mM HEPES buffer (pH 7.5) using 6,000 – 8000 molecular weight cutoff dialysis tubing at 4°C. The fractions were tested for

manganese oxidation activity by adding normalized protein fractions into 10mM HEPES buffer (pH 7.5) supplemented with 100μM $MnCl_2$ allowing the mixture to react overnight at room temperature. Activity was quantified spectrophotometrically using the leucoberblin blue (LBB) method measuring the absorbance at 618 nm according to (Krumbein and Altman, 1973). When reduced LBB reacts with Mn(III/IV) oxides, the LBB becomes oxidized and turns blue. Subsequent fractionation did not include removal of loosely bound proteins or separation of the periplasmic and cytoplasmic proteins and were omitted from the protocol (see results section).

Simplified protocol to isolate secreted and membrane bound proteins

Two liters of oxidizing *P. putida* GB-1 bacterial cells were harvested by centrifugation after removal of manganese oxides by the addition of 1-10mM ascorbate. The medium was collected and concentrated as described above.

The cell pellet was resuspended in 5mL cold 10mM Tris-HCl buffer at pH 8.1 with added 50mM $CaCl_2$ and 0.1mM PMSF followed by three passes through a French Press pressure cell (20,000 lbs/in^2) for cell lysis. The homogenate was centrifuged at 75,000 rpm for one hour to collect the supernatant and precipitate. The precipitate was resuspended in 10mM HEPES buffer at pH 7.5 containing 1% Triton-X-100 and incubated at room temperature for twenty minutes to solubilize membrane proteins. The membrane fraction was centrifuged at 50,000 rpm for two hours. The resulting supernatant was collected and dialyzed overnight in 10mM HEPES buffer (pH 7.5) at 4°C using 6,000- 8,000 molecular weight cut off dialysis

tubing. Localization of manganese oxidase activity was determined by adding the normalized protein fractions to 10mM HEPES buffer pH 7.5 supplemented with 100μM $MnCl_2$ and incubating overnight at room temperature. Activity was quantified spectrophotometrically using the leucoberblin blue method as described above (Krumbein and Altman, 1973).

FPLC partial protein purification of oxidase activity

Membrane fractions were extracted from 2 liters of oxidizing *P. putida* GB-1cultures. Partial purification was performed with Amersham Biosciences AKTA Fast Protein Liquid Chromatography (FPLC) using chromatography columns from GE Healthcare at 4°C. Two milliliters of active membrane fraction were concentrated using a 10kDa molecular weight cutoff Falcon tube and equilibrated with 3M $(NH_4)_2SO_4$ to a total concentration of 1.7M $(NH_4)_2SO_4$. The initial purification step post equilibration used a 5mL HiTrap phenyl high-performance hydrophobic interaction column in 20mM HEPES (pH 7.5) with a decreasing linear gradient of 1.7M $(NH_4)_2SO_4$. After hydrophobic interaction chromatography, further partial purification used size exclusion with a Hi Prep 16sixty S306 GF002 chromatography column to separate out active proteins compared to standard protein elutions (Table 2.1) using the MWGF1000 kit (Sigma-Aldrich). Cation and anion exchange chromatography were attempted targeting the pI of ~5 based on probable manganese oxidase targets (MnxG and Mco). For cation exchange, a 50mM formic acid buffer (pH 4.0) was used with a linear elution gradient of 0 to 1.0M NaCl using the Hi Trap (X) chromatography column. For anion exchange, a HiTrap

Q Fast Flow column with 20mM bis-Tris buffer (pH 6.0) supplemented with 1% triton-x-100 and 2mM EDTA was used with a 0 to 1M NaCl linear elution gradient. After each particular purification step, manganese oxidase activity was tested by adding 90µl aliquots of eluted fractions to 10µl of 1mM $MnCl_2$ for a total concentration of 100µM $MnCl_2$ with a total reaction volume of 100µl in a 96 well plate. The reaction was incubated overnight at room temperature and a 1:1 ratio of 0.4% LBB was added to determine if manganese oxides were produced. Active eluted fractions were pooled and concentrated using a 10kDa molecular weight cut-off spin filter for use in further purification and analysis using SDS PAGE, Native PAGE, and MS/MS spectroscopy.

Table 2.1. Protein standards used for size exclusion chromatography FPLC

Protein Standard	MW
Albumin, bovine serum	66kDa
Alcohol Dehydrogenease, yeast	150kDa
β-Amylase, sweet potato	200kDa
Apoferritin, horse spleen	443kDa
Blue dextran	2000kDa
Carbonic Anhydrase, bovine erythrocytes	29kDa
Thyroglobulin, bovine	669kDa

SDS-PAGE and Native-PAGE of Active Protein Fractions

Native-PAGE was used to separate active and non active crude fractions and FPLC partially purified proteins using a 4% stacking gel (0.5 M Tris-HCl Buffer pH 6.8) and 10% resolving gel (1.5M Tris-HCl Buffer pH 8.8). Protein concentrations were estimated using a Nano Drop ND-1000 spectrophotometer (A280 of 1 = 1 $mgml^{-1}$) or the Lowry method. Twenty micrograms of total protein samples were

loaded and run in duplicate by electrophoresis and divided into two sections. The first section was silver stained (Pierce) or Coomassie blue stained according to standard protocols. The second section was assayed for manganese oxidation activity. In-gel manganese oxidation activity assay was prepared by incubating the Native-PAGE gel in pre-wash solution containing 10% glycerol and 0.05% triton-X-100 for 15 minutes. After the pre-wash solution was removed, it was replaced with 10mM HEPES buffer (pH 7.5) and incubated for 10 minutes to rid the gel of the pre-wash solution. After rinsing, a 10mM HEPES buffer (pH 7.5) containing 100μM $MnCl_2$ was added to the gel and allowed to incubate overnight at room temperature to test for oxidase activity. The silver stained gel was then compared to the band containing manganese oxides. The gel band stained with Coomassie blue pertaining to the active manganese oxidase band in the in-gel assay was removed for MS/MS analysis by the Shared Protein Resource facility at Oregon Health & Science University (OHSU) as described (Anderson et al. 2009). Alternatively, post FPLC partially purified protein solutions containing ~ 20μg total protein were also analyzed by MS/MS spectroscopy.

SDS-PAGE was used to separate active and non-active crude protein fractions and FPLC partially purified proteins according to standard protocols (BioRad). SDS and ß-mercaptoethanol were added to each particular fraction and boiled for 10 minutes before running on the gel. Precast 4-15% Tris-HCl SDS-PAGE gels (BioRad) were used to determine protein purity and relative molecular weights of associated proteins.

Results

Localization of manganese oxidase activity

In order to determine the localization of the manganese oxidase cellular fractionation was performed. After fractioning to separate the soluble proteins (periplasmic and cytoplasmic) and the membrane associated proteins (outer membrane and inner membrane) each fraction was tested for the ability to oxidize soluble Mn(II) by visible formation of Mn(IV) oxides and for the presence of LBB positive activity. The location of oxidation is associated with the outer membrane fraction and is not within the cell (Figure 2.1). The activity was routinely found in the outer membrane fraction throughout the development of this protocol. However, activity was periodically present in the secreted medium, the loosely bound fraction, and even the periplasmic fraction demonstrating the difficulty in enzyme localization by cellular fractionation (data not shown). Using a simpler cellular fractionation method (See materials and methods), the majority of activity is localized to the triton-X-100 solubilized membrane fraction, but the soluble fraction also contains some activity (data not shown). This may be indicative of more than one enzyme being utilized for manganese oxidation.

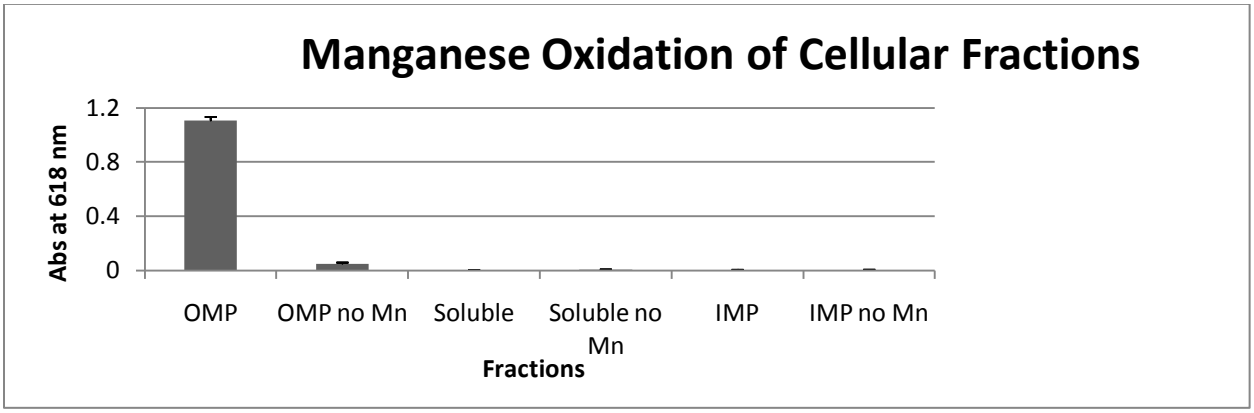

Figure 2.1. Localization of Mn(II) oxidase activity to the outer membrane. Forty microgram total protein from cellular fractions were added to 10mM HEPES buffer (pH 7.5) supplemented with 100μM $MnCl_2$. Mn(II) oxidation was determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

Fast protein liquid chromatography partial protein purification of oxidase activity from outer membrane

In order to identify the manganese oxidase by MS/MS spectroscopy, fast protein liquid chromatography was performed using a Amersham Biosciences AKTA Fast Protein Liquid Chromatography (FPLC) system on the outer membrane active fraction using hydrophobic interaction chromatography followed by size exclusion chromatography (Figure 2.2 and 2.3). The resulting active fraction post size exclusion eluted at roughly 130 kDa when compared to the standard proteins, indicating the active manganese oxidase is roughly 130kDa in size (Figure 2.3).

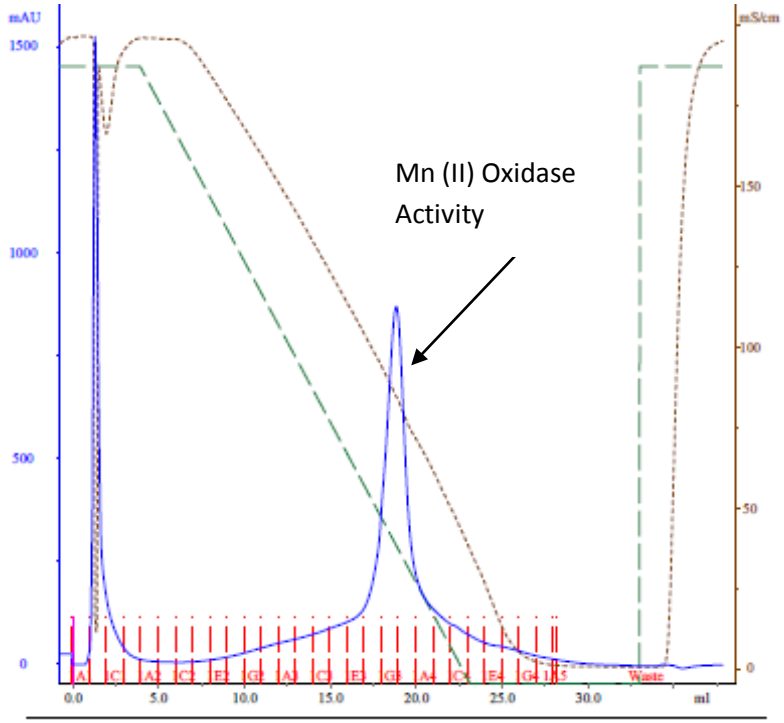

Figure 2.2. Hydrophobic interaction chromatography of 1mL crude active outer membrane fraction equilibrated with 1.7M $(NH_4)_2SO_4$.

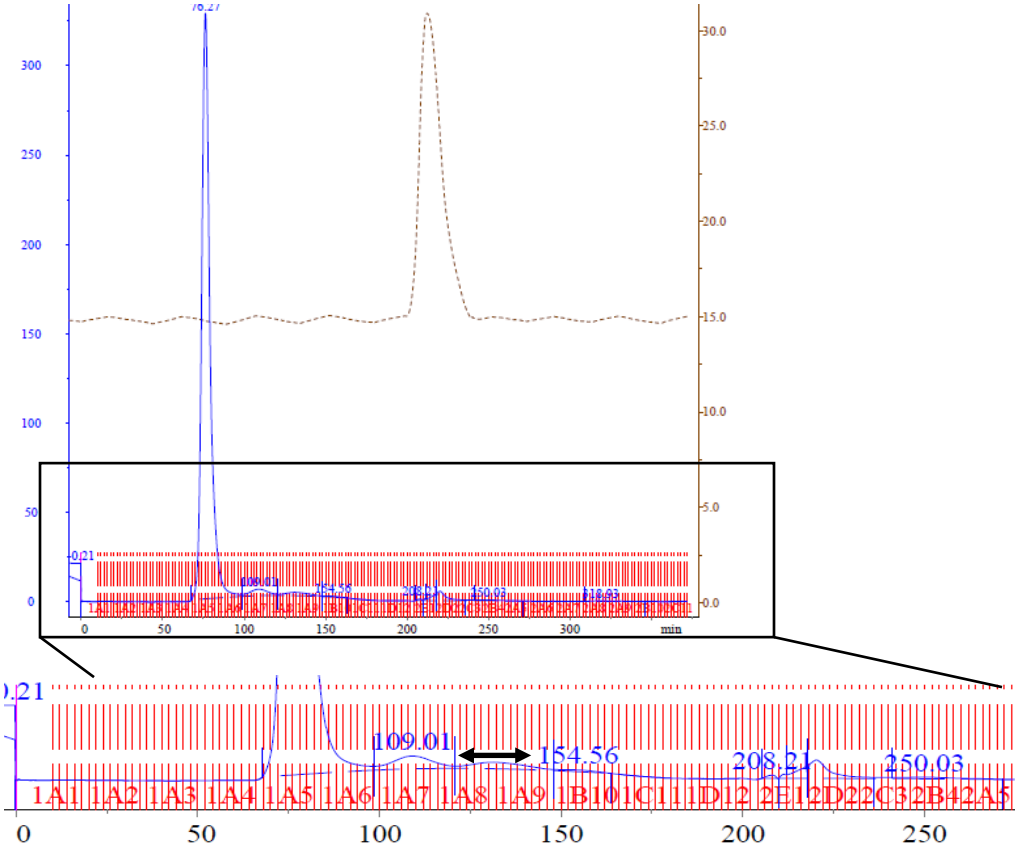

Figure 2.3. Size exclusion chromatography of post concentrated hydrophobic interaction chromatography fractions. Mn(II) oxidase activity eluted at 130kDa compared to loaded protein standards elution positions (Table 2.1).

Fast protein liquid chromatography partial protein purification of oxidase activity

In order to identify the manganese oxidase using the simpler cellular fractioning protocol, hydrophobic interaction chromatography was used. Using the larger column resulted in much better separation and an extremely active fraction indicated by overnight manganese oxidation assays (data not shown). The fraction elutes late in the column indicating it binds tightly to the hydrophobic column as expected due to its membrane association. The signature is very different when compared to the previous sections (Figure 2.2). Cation and anion exchange chromatography was performed numerous times using post hydrophobic interaction active fractions and crude active membrane fractions. Each attempt resulted in either no protein eluted from the column or no initial binding, resulting in no manganese oxidase activity.

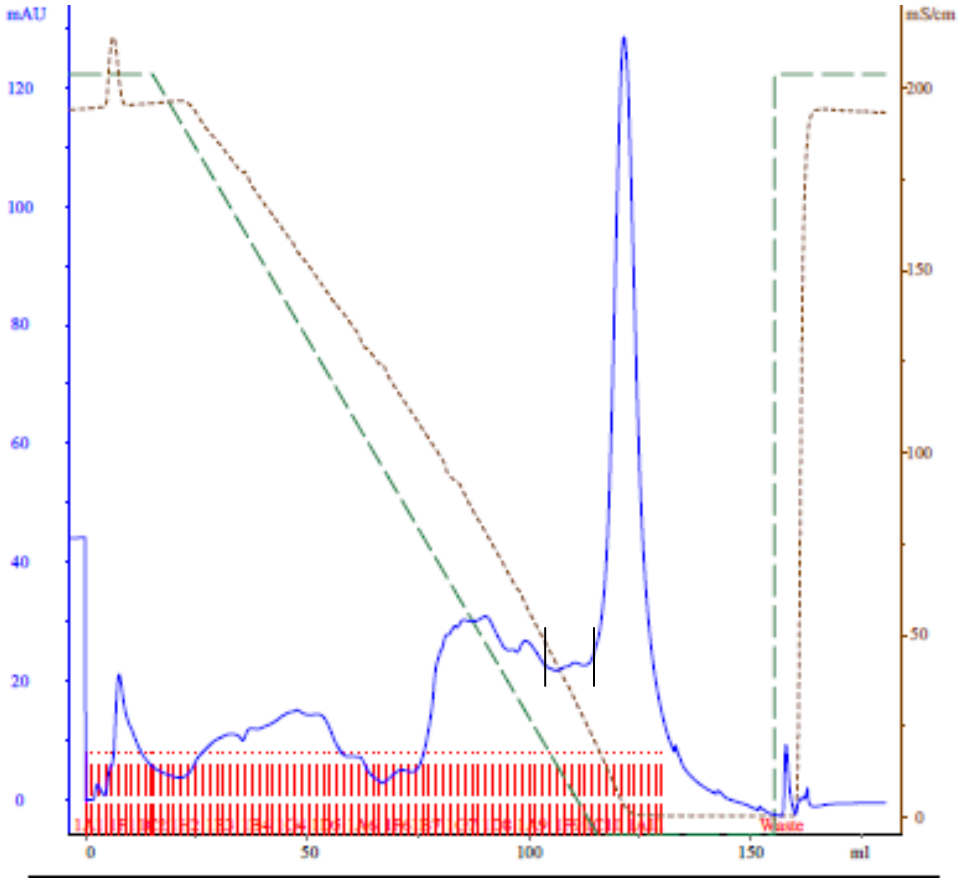

Figure 2.4. Hydrophobic interaction chromatography of active membrane associated fraction using 5mL HiTrap Phenyl column. Active fractions indicated by vertical black lines.

SDS-PAGE and Native-PAGE of Active Protein Fractions

Native PAGE was performed in order to determine the apparent size of the proteins responsible for the manganese oxidizing activity. Activity assays of the associated membrane fraction in Native PAGE gels suggest that the protein or multi-protein complex is larger than 250kDa (Figure 2.5). Properties of the protein or protein complex might not allow proper gel migration in the native conformation, allowing for a smaller complex to appear larger so the actual size of the protein complex is uncertain.

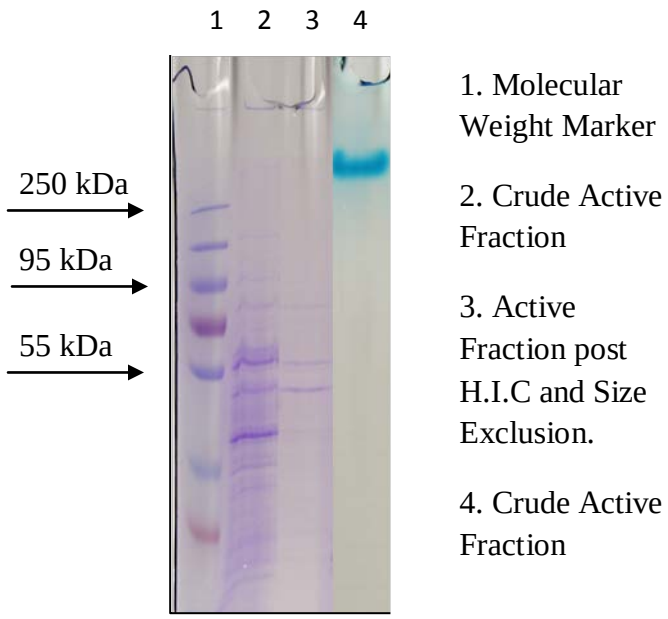

Figure 2.5. Molecular weight marker (1). Coomassie stain of SDS PAGE crude active fraction (2). Coomassie strain of SDS PAGE post H.I.C and Size Exclusion (3). Native PAGE in gel Mn (II) Oxidation assay with LBB activity indicating the apparent size Mn oxidase(s) (4).

To assess purification of the manganese oxidase and to determine the size of the manganese oxidase, SDS-PAGE was performed the using post hydrophobic interaction chromatography and size exclusion chromatography active fractions. Two distinct protein gel bands were visualized at 50kDa and 60kDa using SDS-PAGE and were excised for MS/MS spectroscopy analysis (Figure 2.6).

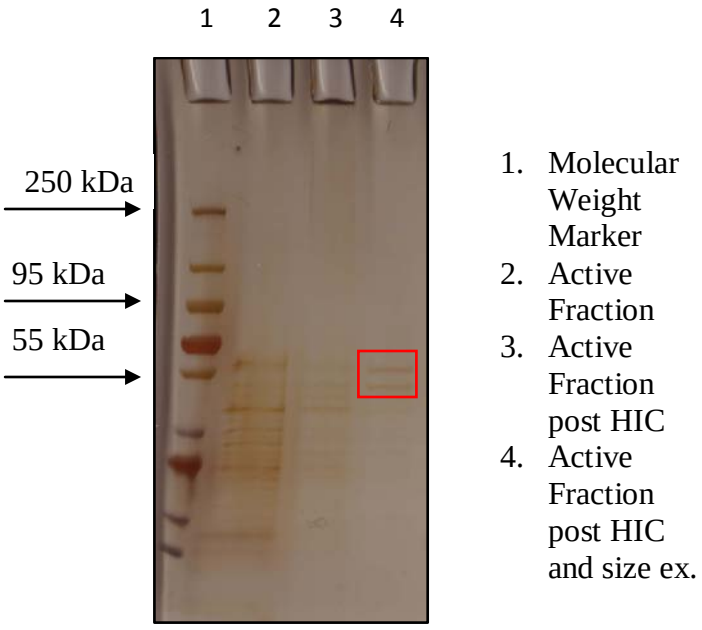

Figure 2.6. SDS PAGE showing Partial purification of the manganese oxidase activity using fast protein liquid chromatography. The 60kDa and 50kDa protein bands were excised for MS/MS spectroscopy.

MS/MS Spectroscopy Analysis

In order to identify the manganese oxidase by MS/MS spectroscopy, two SDS-PAGE bands were excised at 50kDa and 60kDa (Figure 2.6). The 50kDa band was identified as arginine deiminase while the 60kDa band was identified as dihydrolipoamide dehydrogenase. These two proteins are annotated and are not likely the putative manganese oxidase. The solution of partially purified active fraction post size exclusion chromatography was also analyzed by MS/MS spectroscopy. The three proteins that received the most hits were arginine deiminase, dihydrolipoamide dehydrogenase, and catalase peroxidase HPI (Appendix A). After pooling multiple MS/MS data sets (See Appendix C), three low abundant proteins of interest were identified that consistently were co-localized.

The proteins of interest were multi-copper oxidase type 2 (PputGB1_2665) and two hypothetical proteins (PputGB1_2552 and PputGB1_2553).

Discussion

In this chapter, Mn(II) oxidizing activity was confirmed to be localized to the outer membrane fraction (Figure 2.1). Although the outer membrane or membrane associated fractions were the dominant fraction in which the activity was localized, activity was also located in the soluble fraction in some preparations. These findings suggest that the fractioning protocol needs more optimization or more likely, there may be multiple enzymes that are capable of oxidizing Mn(II). Previous studies have shown that Mn(II) oxidizing activity was associated with the outer membrane in *P. putida* GB-1 (Okazaki et al., 1997), and also intracellularly in *P. putida* MnB1 (Jung and Schweisfurth, 1978), which might explain why activity is difficult to ascertain. Localization of proteins to the outer membrane and in the supernatant is typical of type II secretion system (Mehta et al. 2006); similar to what is found in *P. putida* GB-1.

Optimizing culture conditions for maximal Mn (II) oxidation is necessary for increased expression of the Mn(II) oxidase(s). This will allow for higher protein yield for use in subsequent protein purification. Mn(II) oxidation in *P. putida* GB-1 is extremely dependent on the type of flask and growth conditions. For optimal oxide formation, a 0.1% final volume (e.g. 500 µl into 500ml) of an overnight culture was subcultured into Lept medium supplemented with 100 µM $MnCl_2$ into a 1L Fernbach flask. This ratio of culture to head space is essential for optimal oxide

formation, which is most likely due to the dependence on oxygen concentration and availability (Okazaki et al., 1997).

Hydrophobic interaction chromatography of active membrane associated fractions followed by subsequent size exclusion FPLC eluted a partially purified active fraction at 130kDa. A Mn(II) oxidizing factor in *P. putida* GB-1 was shown previously to be 130kDa in size (Corstjens, 1993 and Brouwers et al., 1999). MS/MS spectroscopic analysis of this partially pure protein fraction identified among other things a type 2 multi-copper oxidase (PputGB1_2665) which might indicate that the Mn(II) oxidase under this particular growth conditions is an Mco with a predicted molecular weight of 123kDa. MS/MS spectroscopy of the partially pure fraction also co-identified two hypothetical proteins PputGB1_2552 and PputGB1_2553 that have a combined molecular weight of 123kDa and are hypothesized to form a protein complex. The homologue of MnxG, a multi-copper oxidase known to oxidize Mn (II) in *Bacillus* SG1 (Dick et al., 2008) (Discussed in Chapter 3) is predicted to have a molecular weight of 208 kDa in *P. putida* GB-1 and thus far has not been identified by MS/MS spectroscopy in any active fraction or excised gel band. This could be due to the lack of the ability of trypsin to digest MnxG (Dick et al., 2008) or that MnxG is not the Mn(II) oxidase utilized under the specific growth conditions used.

Difficulties in identifying the Mn (II) oxidase could be attributed to its extremely low abundance as indicated by low protein concentrations post size exclusion chromatography (Figure 2.3) and lack of visual protein bands on SDS-

PAGE showing the predicted molecular weights of the putative Mn(II) oxidase(s). Even with the extremely low abundance, the protein or protein complex is highly active in Mn(II) oxidation assays.

Future Direction

Since the putative Mn(II) oxidases have been genetically identified in *P. putida* GB-1 as MnxG, Mco, and MopA (Geszvain, personal communication), His-tagged recombinant expression of these proteins in *E. coli* can be used for higher protein yield and protein purification. Highly purified and active proteins can then be used for kinetic and mechanistic studies of the Mn(II) oxidase(s). If in fact *P. putida* GB-1 uses different enzymes under different environmental conditions, the protein localization and FPLC purification methods used in this chapter might not be suitable. Protein labeling, such as the green fluorescent labeling, would be better suited for localizing the Mn(II) oxidase and to study the protein localization under different environmental conditions such as low oxygen or liquid vs. solid medium.

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Chapter 3. In-Vitro Studies of Membrane Associated Fraction

Objective

The purpose of this research was to use crude active cellular fractions to gain insight into the Mn(II) oxidase mechanism. An in-vitro based biochemical approach was used to identify the types of enzymes and the role of reactive oxygen species (ROS) based on the effects of enzymes, metals, and cofactors.

Genomic screening of *P. putida* GB-1 indicates that its genome carries homologues to the putative Mn(II) oxidase-encoding genes of other organisms, including the manganese oxidizing heme peroxidase, *mopA* (*Erythrobacter* sp. Strain SD-21and *A. manganoxydans* strain SI85-9A1) and the multi-copper oxidase, *mnxG* (*Bacillus* SG-1). When the homologue of *mnxG* in *P. putida* GB-1 was deleted, the strain is still able to oxidize Mn (II) at a significantly reduced rate compared to wild type GB-1. Similarly, when *mco* or both *mopA* and *mco* are deleted, oxidation is impaired but not completely absent. However, Δ*mnxG*Δ*mopA* is a severely decreased oxidizer. Interestingly, when *mopA* is deleted there is a stimulation of Mn(II) oxidation. Two mutant strains, Δ*mnxG*Δ*mopA*Δ*mco* triple mutant and the Δ*mnxG*Δ*mco* double mutant resulted in complete abolishment of Mn (II) oxidation (Geszvain, personal communication). The genetic approach has thus identified key enzymes that are involved with Mn(II) oxidation, but a biochemical approach is useful for characterizing the oxidase activity and supporting these findings.

In oxidizing cultures, the addition of copper to the medium stimulates oxidation, which is suggestive of a role for MCOs in Mn (II) oxidation (Brouwers et

al. 1999). To further test the hypothesis that an MCO plays a central role in Mn(II) oxidation in *P. putida* GB-1, the ability of Cu^{2+} to stimulate oxidation in cell extracts was screened. In contrast, if a Ca^{2+} heme-peroxidase like MopA is required for oxidation in *P. putida* GB-1, the addition of Ca^{2+} or hydrogen peroxide to cell extracts would be predicted to stimulate oxidation. Thus, through in vitro experiments with cell extracts, I hoped to determine the nature of the Mn(II) oxidase: MCO, heme-peroxidase or both?

Reactive oxygen species (ROS) have also been implicated to be involved Mn (II) oxidation. Recently, it was shown that superoxide radicals mediate Mn (II) oxidation in *Roseobacter*, while other ROS species had no effect (Learman et al. 2011). To determine whether and which ROS variant mediate Mn(II) oxidation in *P. putida* GB-1, *in vitro* assays were developed using superoxide dismutase (SOD), which catalyzes the dismutation of superoxide radicals to produce hydrogen peroxide and oxygen (McCord and Fridovich, 1969); Catalase, which catalyzes the decomposition of hydrogen peroxide to oxygen and water (Jakopitsch et al., 2007); and the hydroxyl radical scavenger dimethyl sulfoxide (DMSO) (Reilly et al., 1991). These enzymes each involve different ROS, thus if a particular variant were involved Mn(II) oxidation would be inhibited. Together these biochemical approaches provide further insight into the mechanism of Mn (II) oxidation in *P. putida* GB-1 although no definitive conclusions can be made.

Materials and Methods

Role of reactive oxygen species in manganese oxidation

Active membrane fractions were used with distinct enzymes to elucidate the possible role reactive oxygen species have in manganese oxidation. In-vitro experiments were developed to test the effects of superoxide dismutase and catalase. Superoxide dismutase (bovine erythrocytes, Sigma) (0.05, 0.5, 5.0, and 10μM) or catalase (bovine liver, Sigma) (5, 10, 20, and 50 μM) were added to 10mM HEPES buffer (pH 7.5) containing 20-100μg total protein of active membrane fraction depending on particular assay. Hydrogen peroxide (10, 20, 40, and 100μM) was added to 10mM HEPES buffer (pH 7.5) containing 20-100μg total protein of active membrane fraction depending on particular assay. DMSO (0.5, 1, and 10mM) was added to 10mM HEPES buffer (pH 7.5) containing 20-100μg total protein of active membrane fraction. Each well was supplemented with 100μM $MnCl_2$ and incubated overnight at room temperature in 96 well plates and brought to a total volume of 100μl of 10mM HEPES buffer pH 7.5. A 1:1 ratio of 0.4% LBB was added to the oxidation assays and measured using the spectrophotometer at 618nm to spectrophotometrically determine oxide formation.

The effects of metal on manganese oxidation

Various metals such as calcium, molybdenum, cobalt, and copper were assayed in a 96 well plate format to determine whether any of them affect manganese oxidation. To test this, $CaCl_2$ (10μM), $CoCl_2$ (50μM), and $CuSO_4$ (10μM)

were assayed along with a no metal addition control in to10mM HEPES buffer (pH 7.5) containing normalized active membrane protein fractions supplemented with 100μM $MnCl_2$. MoO4 (0.05, 0.25, 0.5, 1, 5, and 20 μM) was also tested using the same assay method. A more detailed range of $CuSO_4$ (0.025, 0.05, 0.5, 1, and 10 μM) and (10, 25, and 50μM) were also tested using the same protocol. All experiments were conducted overnight at room temperature and tested for manganese oxides by the LBB assay.

Oxidation of soluble Mn(II) and chelated Mn(III)

Experiments were conducted testing the ability of active membrane fractions to oxidize both prepared Mn(III)- Deferoxamine (DFO) and Mn(III)-pyrophosphate. Mn(III)-DFO was prepared by adding 100mM DFO to 90mM $MnCl_2$ in a 15mL Falcon tube with overnight shaking at 10 °C (Duckworth and Sposito, 2005). Mn(III) pyrophosphate was prepared by adding 10mM Mn(III) acetate dihydrate to 40mM sodium pyrophosphate pH 8.1 and stirred rapidly at room temperature (Webb et al., 2005). Normalized cell fractions were added to 10mM HEPES buffer (pH 7.5) supplemented with 100μM Mn(III) - DFO and incubated overnight at room temperature in a 96 well plate, and assayed for Mn oxides using LBB. Mn (III) - DFO is not LBB reactive but Mn(III) -pyrophosphate is so it is difficult to distinguish Mn(III) and Mn(IV) oxides while using Mn (III) - pyrophosphate other than visually. Cellular fractions were tested with Mn(III)- pyrophosphate but no data were collected.

Results

Role of reactive oxygen species in manganese oxidation

To elucidate the potential role that reactive oxygen species (ROS) play in manganese oxidation, cell extracts were incubated in the presence of Mn(II) and various enzymes or other additives that would be predicted to affect concentrations of distinct ROS species and the resulting levels of Mn(IV) oxide production quantified. For example, superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals to produce hydrogen peroxide and oxygen and thus would result in increased H_2O_2 levels and decreased levels of superoxide (McCord and Fridovich, 1969). Catalase catalyzes the decomposition of hydrogen peroxide to oxygen and water so its addition would decrease H_2O_2 concentration (Jakopitsch et al., 2007); and levels of hydroxyl radicals would be reduced by the addition of the hydroxyl radical scavenger dimethyl sulfoxide (DMSO) (Reilly et al., 1991).

Superoxide dismutase and catalase both inhibited manganese oxidation in-vitro at pH 7.5 and completely abolished oxidation at μM concentrations (Figures 3.1 and 3.2). In particular, SOD inhibits the oxidation of Mn(II) and reduced the oxidation of Mn(III)-DFO (data not shown). When hydrogen peroxide, an oxidant and product of superoxide dismutation was tested in a similar manner, there were no significant effects at any μM concentration tested (Figure 3.3). A no protein control was also tested containing 100μM hydrogen peroxide which resulted in no abiotic manganese oxidation. DMSO, a known scavenger of hydroxyl radicals also

exhibited no significant effects up to mM concentrations (Figure 3.4).

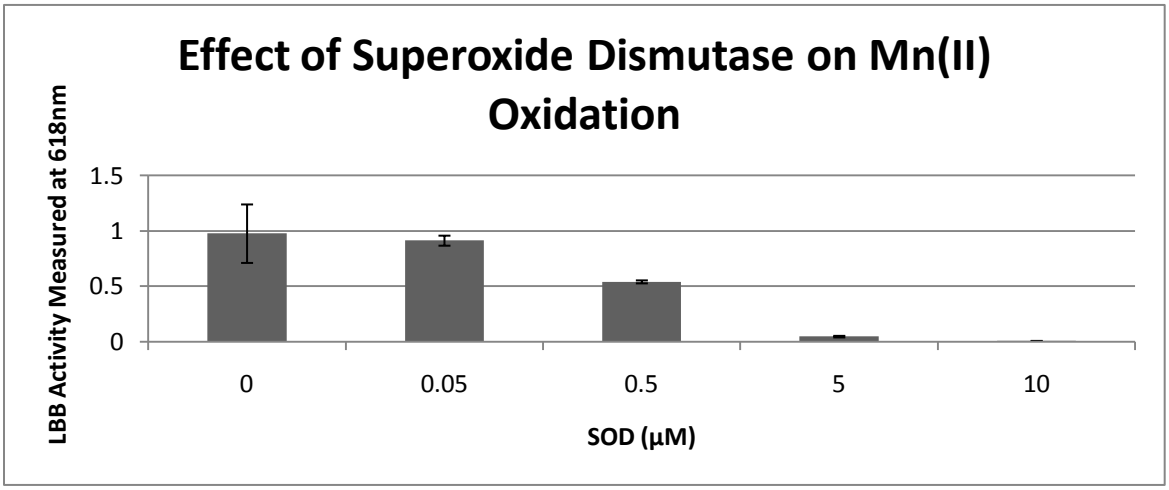

Figure 3.1. Inhibition of Mn(II) oxidation by SOD. One hundred micrograms total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100µM $MnCl_2$ and SOD (0, 0.05, 0.5, 5, and 10 µM). Mn(II) oxidation was determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

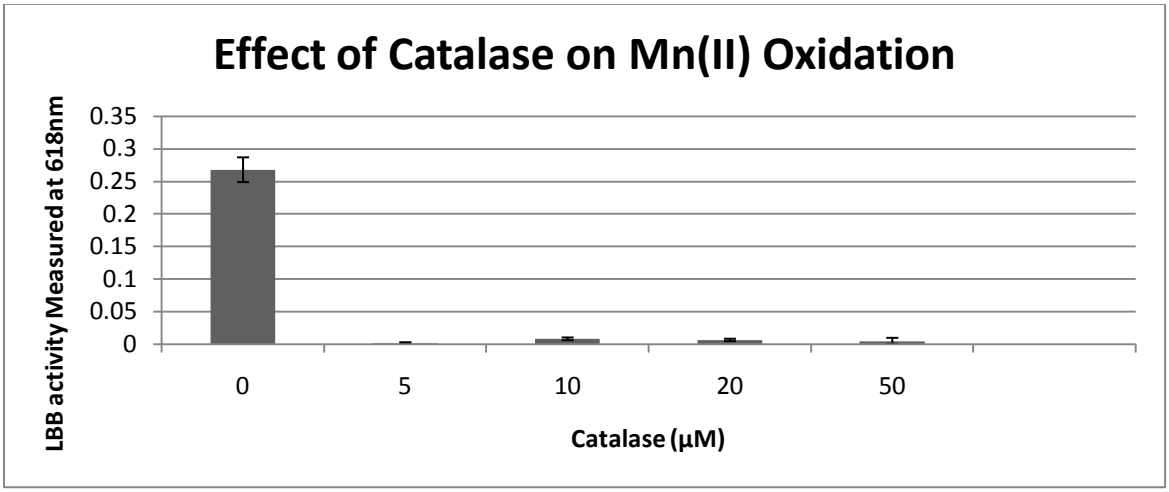

Figure 3.2. Inhibition of Mn(II) oxidation by catalase. Forty micrograms total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100µM $MnCl_2$ and catalase (0, 5, 10, 20, and 50 µM). Mn(II) oxidation was determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

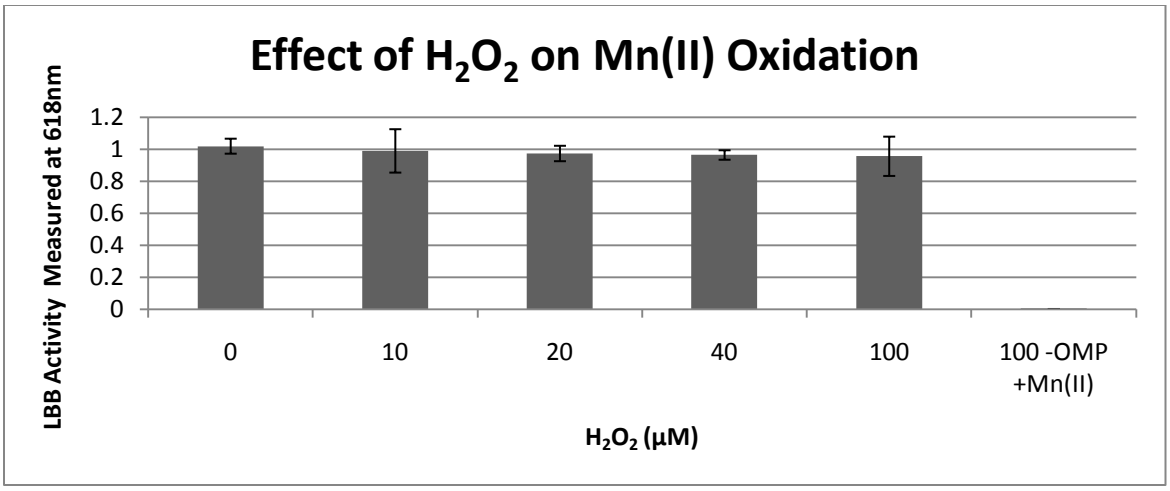

Figure 3.3. No effect of hydrogen peroxide on Mn(II) oxidation. One hundred micrograms total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100μM $MnCl_2$ and H_2O_2 (0, 10, 20, 40, and 100 μM). No non-enzymatic oxidation of Mn(II) by 100 μM H_2O_2 was observed. Mn(II) oxidation was determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

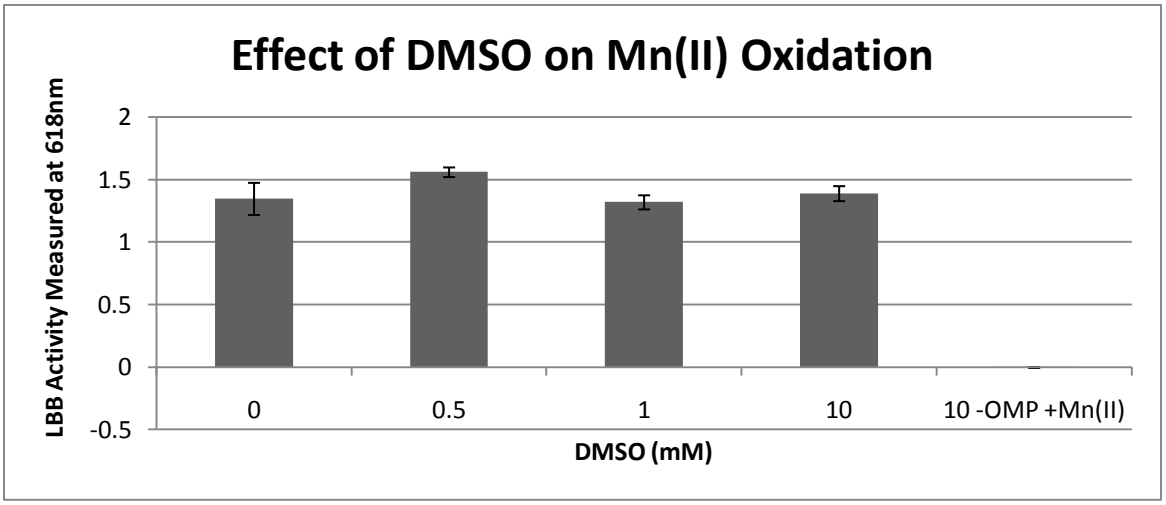

Figure 3.4. No effect of the hydroxyl radical scavenger DMSO on Mn(II) oxidation. One hundred micrograms total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100μM $MnCl_2$ and DMSO (0, 0.5, 1, and 10mM). DMSO had no non-enzymatic effect on Mn(II) oxidation. Mn(II) oxidation was determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

The effects of metal on manganese oxidation

Various divalent metals were tested such as copper, calcium, cobalt, and molybdenum in overnight assays to determine what effect they may have in manganese oxidation. It is known that copper stimulates manganese oxidation in whole cell cultures of *P. putida* GB-1 (Brouwers et al., 1999), which supports the involvement of multi-copper oxidases. When cell free extracts were tested, copper had a severe inhibitory effect on manganese oxidation (Figures 3.5 and 3.6). Other metals such as calcium, cobalt, and molybdenum do not have stimulatory or inhibitory effects on manganese oxidation (Figures 3.7 and 3.8).

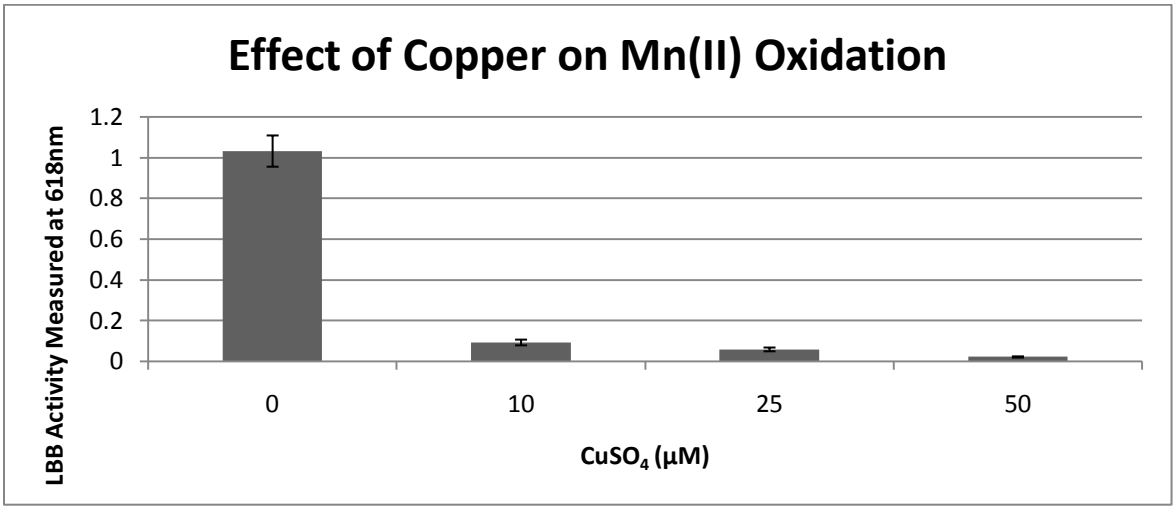

Figure 3.5. Inhibition of Mn(II) oxidation by Cu^{2+}. One hundred micrograms total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100 µM $MnCl_2$ and $CuSO_4$ (0, 10, 25, and 50 µM). Mn(II) oxidation was determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

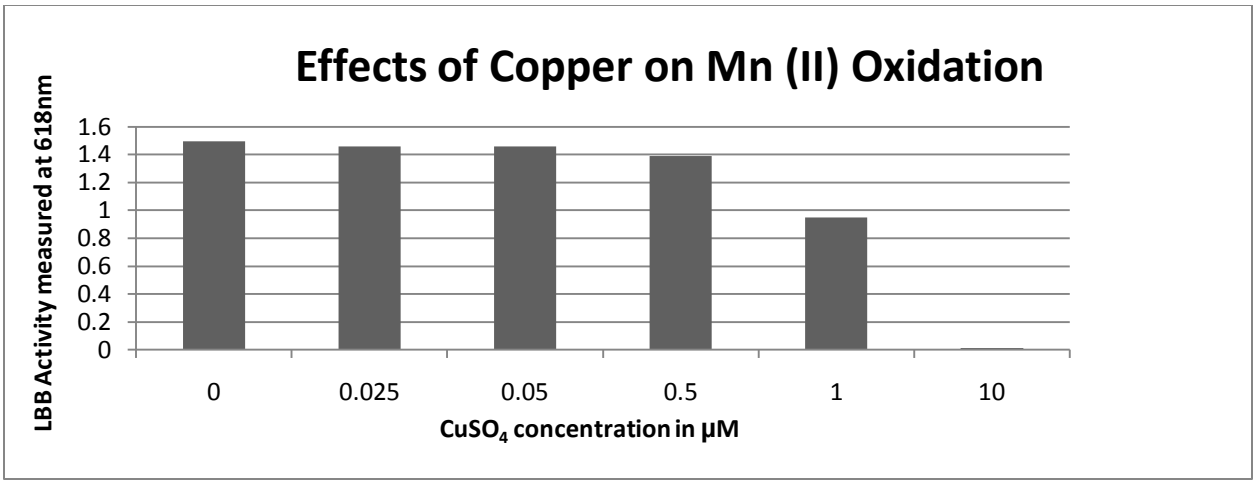

Figure 3.6 Inhibition of Mn(II) oxidation by Cu^{2+}. One hundred micrograms total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100 µM $MnCl_2$ and $CuSO_4$ (0, 0.025, 0.05, 0.5, 1, and 10 µM). Mn(II) oxidation was determined by LBB activity measured at 618nm.

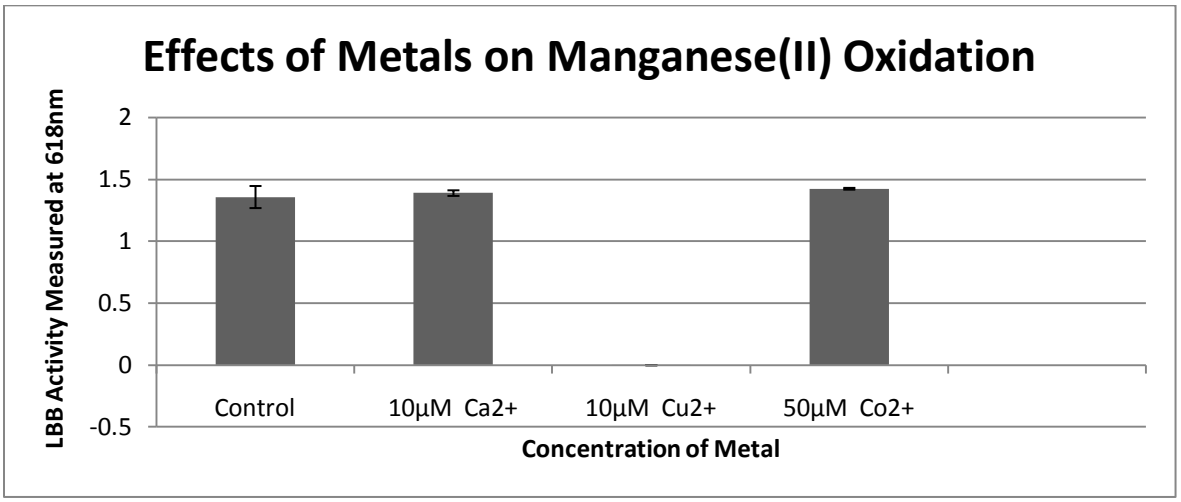

Figure 3.7. Effects of various divalent metals on Mn (II) oxidation. One hundred micrograms total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100 µM $MnCl_2$ and respective metal concentrations. Mn (II) oxidation was determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

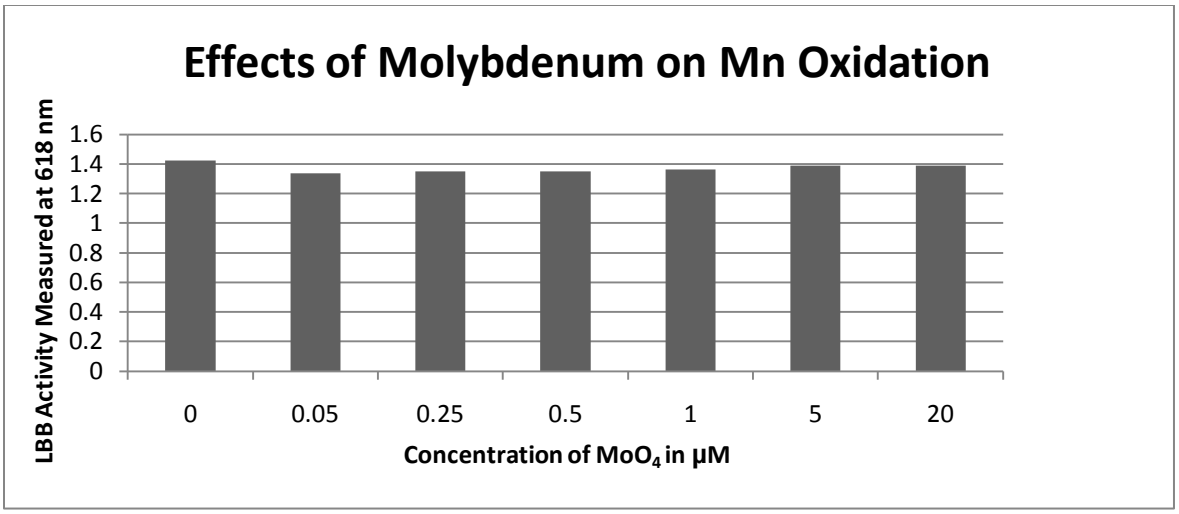

Figure 3.8. No effect of Molybdenum on Mn (II) oxidation. One hundred micrograms total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100 µM $MnCl_2$ and MoO_4 (0, 0.05, 0.25, 0.5, 1, 5, and 20 µM). Mn (II) oxidation was determined by measuring LBB activity at 618nm.

Oxidation of soluble Mn(II) and chelated Mn(III)

To determine whether manganese oxidation proceeds via a Mn(III) intermediate similar to other bacteria, active membrane fraction were assayed with both soluble Mn(II) and chelated Mn(III)-DFO. The results indicate that both soluble Mn(II) and chelated Mn(III)-DFO are oxidized to Mn(IV) which suggests manganese oxidation proceeds through a Mn(III) intermediate. This indicates that the oxidation reaction is also localized to the outer membrane fraction (Figure 3.9). Mn(III)-DFO is strongly chelated which reduces the amount of Mn(IV) that is produced when compared to soluble Mn(II). Mn(III)-pyrophosphate can also be oxidized to Mn(IV) by the active membrane fractions which was visually observed but not graphically represented due to its ability to oxidize LBB and produce a LBB positive result.

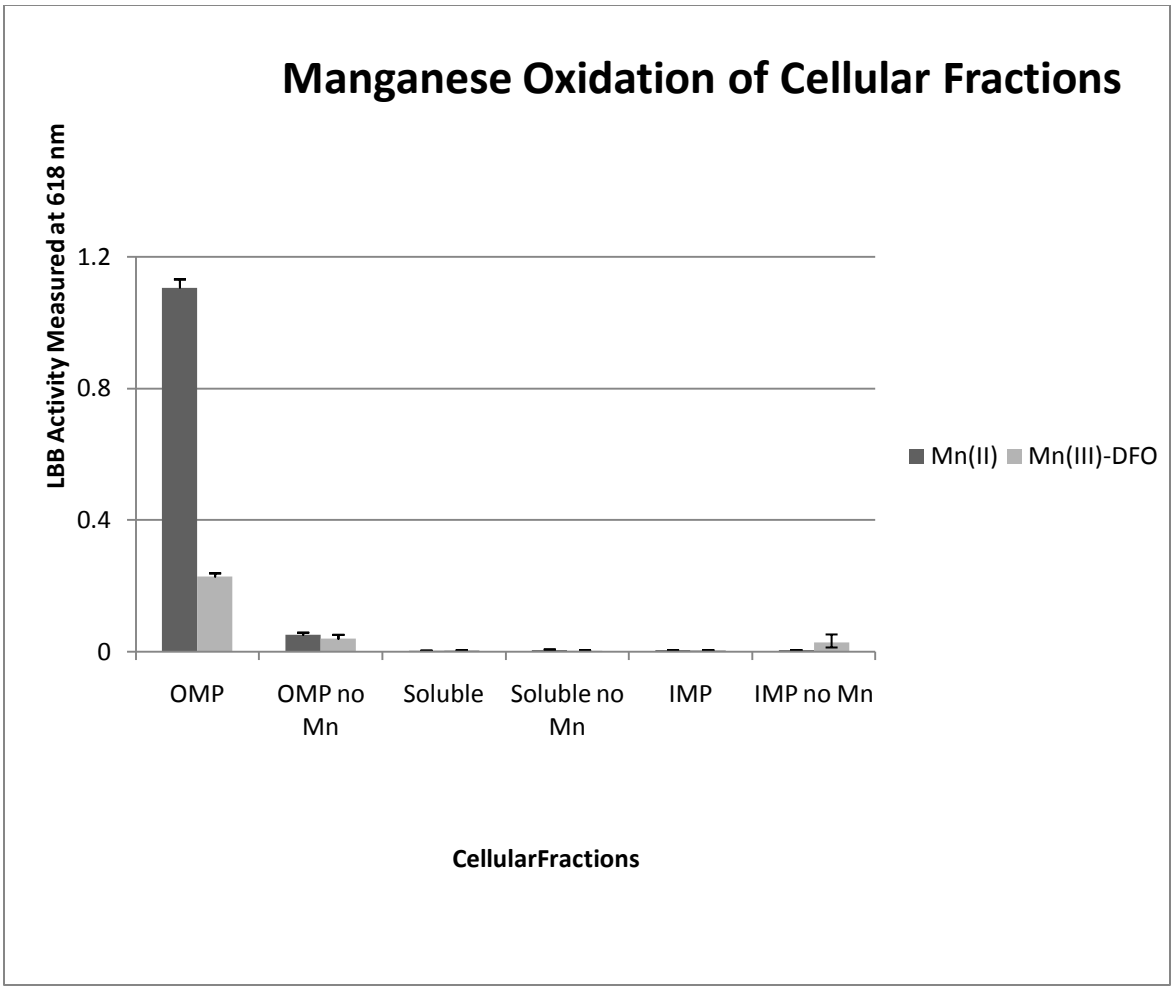

Figure 3.9. The outer membrane fraction oxidizes both soluble Mn (II) and chelated Mn (III)-DFO. Forty micrograms total protein of normalized cellular fractions were added to 10mM HEPES buffer (pH 7.5) and supplemented with 100 μM $MnCl_2$ and allowed to react overnight at room temperature. Mn (II) and Mn (III) oxidation were determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

Discussion

The main intent of the *in vitro* based biochemical studies of active membrane fractions was to gain insight into the mechanism of Mn(II) oxidation and to distinguish which type of enzyme, a MCO or a heme containing MnP is the dominant enzyme in *P. putida* GB-1. These experiments do not demonstrate the prevalence of a MCO or MnP Mn(II) oxidase, but do implicate the involvement of superoxide radicals in the oxidation of manganese.

It is not clear whether the Mn(II) oxidase in *P.putida* GB-1 is a MnP such as MopA, or MCOs such as MnxG or Mco. To determine whether a MCO is the dominant Mn(II) oxidase, the ability of Cu^{2+} to stimulate oxidation in cell extracts was screened since it was shown previously that the addition Cu^{2+} to bacterial cultures resulted in increased Mn(II) oxidation (Brouwers et al. 1999). In contrast, if heme-peroxidase like MopA is required for the oxidation of Mn(II) utilizing H_2O_2 as a substrate, the addition of Ca^{2+} or hydrogen peroxide to cell extracts would be predicted to stimulate oxidation. As the results indicate, the addition of Ca^{2+} or H_2O_2 has no effect and suggests that MopA is not directly involved. However, the inhibition of Mn(II) oxidation by the addition of Cu^{2+} does not directly support the idea that a MCO enzyme is responsible for the majority of Mn(II) oxidase activity. However, the inhibition of Mn(II) oxidation by the addition of Cu^{2+} could be due to some other factors such as its ability to scavenge superoxide radicals by catalyzing superoxide dismutation (Voelker et al., 2000).

Reactive oxygen species have been implicated in Mn(II) oxidation in *Roseobacter* AzwK-3b by the production of extracellular superoxide radicals by a currently unknown enzyme. In this model, superoxide radicals oxidize soluble Mn(II) to form a Mn(III) intermediate. Experiments based on Mn(III) - pyrophosphate trapping, the production of H_2O_2 by the concomitant dismutation of superoxide radicals, and the inhibitory effects of superoxide dismutase and copper further indicate that superoxide radicals are involved in Mn(II) oxidation in this species (Learman et al. 2011). When superoxide dismutase, which catalyzes the dismutation of superoxide radicals to produce hydrogen peroxide and oxygen was added to cell extracts of *P. putida* GB-1 Mn(II) oxidation was completely abolished. However, the addition of superoxide dismutase to cultures of *P. putida* GB-1 resulted in no inhibition of Mn(II) oxidation (Sung-Woo Lee, personal communication). When Cu^{2+} a known superoxide radical scavenger (Voelker et al. 2000) was added it resulted in severe inhibition. However, copper also binds sulfhydryl and amino groups of proteins irreversibly and non-specifically (Letelier et al., 2010; Song et al., 2009; Koch et al., 1997). Thus copper may be affecting the Mn(II) oxidase by either structurally or functional modifications. Other divalent metals such as Ca^{2+}, Co^{2+} or Mo^{2+} had no effect on Mn(II) oxidation. While the data suggests superoxide radicals are required for Mn(II) oxidation in *P. putida* GB-1, the specific role they play is unclear. Soluble Mn(II) could be oxidized by extracellularly generated superoxide radicals as in *Roseobacter* (Learman et al., 2011); however, it is not clear where the superoxide radicals are produced in *P.*

putida GB-1or whether Mn(II) or the Mn(III) intermediate are the substrate of oxidation.

To determine whether hydrogen peroxide was a substrate for Mn (II) oxidation, the enzyme catalase was added to cell extracts of *P. putida* GB-1. Interestingly, catalase significantly inhibits Mn(II) oxidation despite the fact that the addition of hydrogen peroxide has no effect. However, the addition of hydrogen peroxide above basal levels could have saturated the system, resulting in the apparent lack of stimulation or inhibition. Superoxide radicals generated by enzymatic sources such as xanthine oxidase or pulse radiolysis have been shown to inhibit catalase (Kono and Fridovich, 1982; Shimizu et al., 1984). Other ROS such as hydroxyl radicals are not involved in Mn(II) oxidation. When DMSO, a hydroxyl radical scavenger was added, there was no effect on Mn(II) oxidation. This further indicates that the reaction is specific for superoxide radicals.

Mn(II) oxidation to Mn(IV) proceeds via a Mn(III) intermediate in *Bacillus* SG1 using the multi-copper oxidase MnxG. This implies that Mn(II) oxidation occurs as a sequence of two enzymatically mediated one-electron transfer reactions (Webb et al., 2005). To test whether Mn(II) oxidation in *P. putida* GB-1 proceeds via a Mn(III) intermediate, both soluble Mn(II) and chelated Mn(III)-DFO was added to the outer membrane cellular fractions. It was shown that both Mn(II) and Mn(III)-DFO are oxidized, suggesting that oxidation proceeds via an enzyme bound Mn(III) intermediate. The localization of both Mn(II) and Mn(III) oxidizing activity to the outer membrane also implies that both reaction steps occur in the same part of the

cell. Whether the same enzyme oxidizes both Mn(II) and Mn(III) as it appears to in *Bacillus* SG-1 (Dick et al., 2008) remains unknown.

Future Direction

To fully elucidate the mechanism of Mn(II) oxidation in *P. putida* GB-1, pure and fully functional proteins need to be available. Once active proteins from recombinant expression in *E. coli* are produced, kinetic and mechanistic studies to determine both substrates and reactants of Mn(II) oxidation can be studied.

Methods for superoxide generation need to be explored in more detail such as the xanthine oxidase method or pulse radiolysis. This will determine whether superoxide can oxidize soluble Mn(II) or Mn(III) solely through superoxide production or whether an actual Mn(II) oxidase is required. Superoxide quantification using chemiluminescent probe MCLA of the actual Mn(II) oxidation reaction over time is also necessary to support the idea of superoxide radicals being involved in the reaction. Once optimal conditions to quantify superoxide production are available, the use of transposon mutagenesis could be used to determine the enzyme(s) responsible for the extracellular production of superoxide radicals.

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Chapter 4. Identification of two novel proteins important for manganese oxidation

Introduction

While the partial purification and mass spectroscopy described in Chapter 2 did not identify a likely Mn(II) oxidase enzyme, it did identify two hypothetical proteins encoded by the loci PputGB1_2552 and PputGB1_2553 from post hydrophobic interaction and size exclusion chromatography and excised native PAGE gel bands. Further analysis, including prediction of protein localization and both DNA and protein sequence analyses, made it more evident that these two hypothetical proteins might have an important role in Mn (II) oxidation and should be explored in more detail.

Sequence analysis of PputGB1_2552 and PputGB1_2553 (JGI Integrated Microbial Genomes http://img.jgi.doe.gov/cgi-bin/pub/main.cgi, Markowitz and Kyrpides, 2007) indicate that hypothetical protein 2552 contains a transmembrane helix domain and a signal peptide sequence while hypothetical protein 2553 contains a signal peptide sequence. Subcellular protein localization of 2552 is predicted to be located in the outer membrane and 2553 is unknown (Psort v3.0 http://www.psort.org, Yu et al., 2010). Protein sequence searches of each hypothetical protein indicate that they have associated fibronectin domains which are postulated to facilitate the adhesion of the protein to polysaccharides or cell receptors, or these domains participate in maintaining proper conformation of binding or catalytic sites of enzymes (Kataeva et al., 2002 and Watanabe et al.,

1994). Hypothetical protein 2553 also contains two EXXE motifs which are putative metal binding sites (Severance et al., 2004). Since Mn (II) oxidation in *P. putida* GB-1 is thought to occur extracellularly and on the outer membrane (Okazaki et al., 1997), the predicted protein localization of the hypothetical proteins, and the proposed function of fibronectin domains suggest these two proteins might have a role in the Mn (II) oxidase mechanism.

Genes encoding factors required for Mn(II) oxidation would be predicted to be conserved in organisms that oxidize Mn(II) but absent from non-oxidizers. Genome comparisons of ten *Pseudomonas* species indicate that PputGB1_2552 and PputGB1_2553 are only found in oxidizing or putative oxidizing strains while they are absent in non-oxidizing strains (Table 4.1). Other known manganese oxidizers such as *Leptothrix* and *Bacillus* SG-1 also contain homologues to 2552 indicating that these proteins might have a functional role in Mn(II) oxidation other than in *Pseudomonas putida* GB-1.

Table 4.1. Genomic comparisons of *Pseudomonas* species. The blue highlighted species contain both hypothetical proteins PputGB1_2552 and PputGB1_2553. Black highlighted species do not contain either hypothetical proteins. Genome comparisons were completed by James K. McCarthy (Personal communication).

Oxidizer	Putative Oxidizer	Non- oxidizer	Putative Non-oxidizer
P. putida GB1	*P. putida* W619	*P. stutzeri* A1501	*P. mendocina* ymp
P. putida F1	*P. entomophila* L48	*P. Aeruginosa* PAO1	PF5
P. putida KT2440		*P. syringae* Tomato	
P. Fluorescens Pfo-1			

As discussed previously, Mn (II) oxidation is regulated by a two component regulatory system comprised of the sensor histidine kinases MnxS1 and MnxS2, and the response regulator MnxR, which is predicted to be a transcription factor that interacts with a σ^{-54} containing RNA polymerase. Activation of this two component regulatory pathway is predicted to result in the expression of σ^{-54} promoter dependent genes (Geszvain and Tebo 2010).

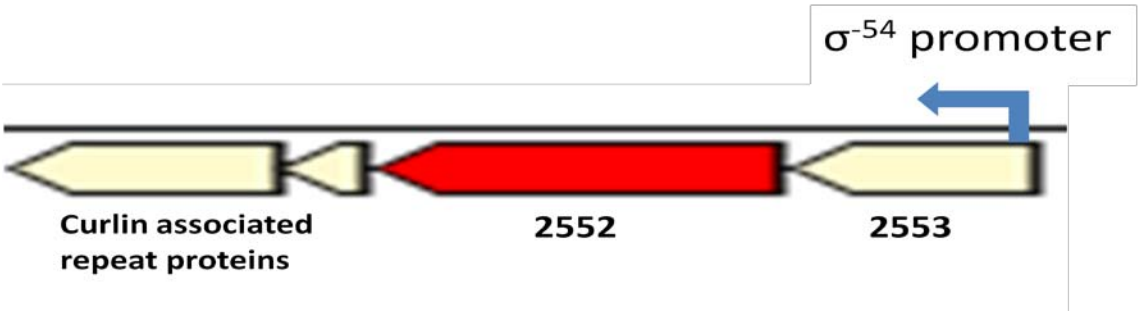

Figure 4.1. Putative operon of hypothetical proteins PputGB1_2552 and PputGB1_2553

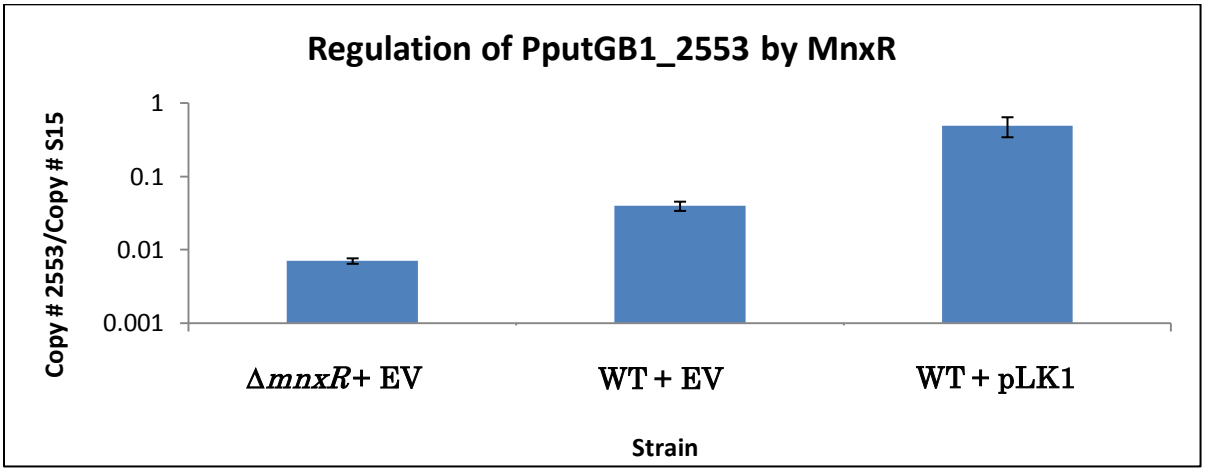

Figure 4.2. Regulation by the Mn(II) oxidation regulator MnxR. Total RNA was isolated from cultures of wild-type bacteria carrying empty vector or a plasmid encoding an increased activity allele of mnxR (pLK1) and the mnxR in-frame deletion strain carrying empty vector and cDNA generated. The transcript copy number was determined for PputGB1_2553 by Q-PCR and normalized to the copy number of the housekeeping gene S15. Each strain was analyzed in triplicate. Error bars represent the standard deviation (Geszvain, personal communication).

PputGB1_2552 and PputGB1_2553 loci are organized on the chromosome such that the putative operon is predicted to contain a σ^{-54} promoter (PromScan http://molbiol-tools.ca/promscan, Studholme and Dixon 2003) (Figure 4.1). The putative operons of *mnxG* and *mco* are also predicted to have σ^{-54} promoters and thus might be involved with MnxR. Recent quantitative PCR (Geszvain, personal communication) results clearly show that *mco, mnxG,* and PputGB1_2553 are regulated by the response regulator *mnxR* (Figure 4.2), while previous semi-quantitative PCR indicated that *mopA* was not under the same control (data not shown). The Q-PCR results suggest that the two hypothetical proteins are intricately linked to Mn (II) oxidation.

Objective

The main purpose of this section is to determine the role hypothetical proteins PputGB1_2552 and PputGB1_2553 have in Mn (II) oxidation and how they correlate with the putative Mn (II) oxidase(s), Mco and MnxG. To address these questions, I employed genetic techniques including multi-copy expression of PputGB1_2553 and 2552 and in-frame deletions.

Materials and Methods

Over expression of genes 2552 (pJB3), 2553 (pJB1), and both 2553 and 2552 (pJB2)

Strains and media used

Escherichia coli strains were grown on LB medium containing antibiotics when necessary to keep plasmid transformed. *Pseudomonas putida* GB-1was grown on LB medium or Lept medium supplemented with 100 μM $MnCl_2$ and contained antibiotics when needed to keep plasmid transformed. *E. coli* cultures were grown at 37 °C while *P. putida* GB-1 cultures were grown either at room temperature or 30 °C. Antibiotics were added to the medium in the following concentrations: 100 μg/mL ampicillin (Ap), 30 μg/mL kanamicin (km), and 50 μg/mL gentamicin (Gm) in LB or 2.5μg/mL in Lept.

Primer design and cloning

The PputGB1_2553 gene was PCR amplified with primers JB-2553-F' and JB- 2553-R-DWN followed by ligation into pJet 1.2/blunt vector. It was then digested with BglII and the resulting fragment was subcloned into pUCP22 followed by restriction digest with XbaI and SacI. The plasmid was sequence verified for proper orientation and digested with XbaI and SacI. The resulting fragment was further subcloned into pBBR-MCS-5. The PputGB1_2552 gene was PCR amplified with primers JB-OE 2552-F and JB-2552-R and cloned described above. The genes PputGB1_2552 and PputGB1_2553 were PCR amplified together using primers JB 2553-F' and JB 2552-R and cloned as described above (See table 4.3 for primers).

DNA from all pBBR1-MCS-5 plasmids containing genes of interest was extracted and transformed into wild type *P. putida* GB-1.

Making *Pseudomonas putida* GB-1 competent for transformation of plasmid DNA

P. putida GB-1 was made competent by washing 1ml overnight culture twice with 1ml cold sterile 0.1M $CaCl_2$, followed by incubation on ice for 10 minutes. The cells were pelleted and resuspended in 200µl of cold sterile $CaCl_2$. The cells were transformed by adding ~3µl of plasmid DNA followed by incubation on ice for 30 minutes. The cells were then heat shocked for 90 seconds at 42□ C followed by incubation on ice two minutes. Then, 800 µl of LB was added to the cell mixture and allowed to incubate for 1 hour at 30 □ C. The cell mixture was pelleted, plated on Gm containing LB plates and incubated at 30 °C overnight. The resulting transformants were struck out onto Gm containing Lept plates for screening of phenotype. After two days of incubation at room temperature, the plates were photographed to determine the effect on manganese oxidation.

Transformation of pJB2 into mutant strains

Mutant strains of *P.putida* GB-1 Δ*mnxR*, Δ*mnxG*, Δ*mco*, Δ*mopA*, and *ΔmnxGΔmopA* were inoculated and grown in LB medium overnight at room temperature. The cells were made competent as previously described. The resulting transformants were plated onto Gm containing Lept plates to determine manganese oxidation phenotype. After two days of incubation at room temperature, the plates

were photographed to determine the effect on manganese oxidation and spot tested with LBB for validation.

Reconstitution of activity in *ΔmnxR* background

The *ΔmnxR* strain of *P. putida* GB-1 was inoculated and grown overnight in LB medium. The cells were made competent as previously described above. Single colonies of the resulting transformants were inoculated in 5ml LB medium containing Gm and grown overnight at room temperature. The transformants were subcultured into Lept medium supplemented with 100µM $MnCl_2$ and 2.5µg/µl Gm to an O.D. of 0.2. The transformants were allowed to grow until the pMnxR + *ΔmnxR* control started to oxidize and then were treated with 10mM ascorbate to remove oxides. Four and a half milliliters of all cultures were harvested by centrifugation and resuspended in 5ml 10mM HEPES buffer (pH 7.5). The pellet was harvested by centrifugation and resuspended in 2.5ml 10mM HEPES buffer (pH 7.5). The cell suspension was sonicated for 20 seconds on and 20 seconds for three times on ice. The supernatant was collected after the cell suspension was harvested by centrifugation and concentrated using a 10kDa cut-off tube. The resulting supernatants were tested for manganese oxidation activity in the standard 96 well plate assay and allowed to react for 48 hours at room temperature. 10µl of each pMnxR, pBBR1- MCS-5 empty vector, pMco, pMnxG, and pJB2 supernatants were added to 90µl 10mM HEPES (pH 7.5) containing 100µM $MnCl_2$ for a total volume of 100µl. Various combinations of pMco, pMnxG, and pJB2

supernatants were assayed in a similar manner to determine whether oxidation could be restored in the ΔMnxR background.

The remaining 500µl of each *ΔmnxR* + pMco, pMnxG, and pJB2, *ΔmnxR*+pMnxR (positive control), and *ΔmnxR* +pBBR MCS-5 empty vector (negative control) cultures were spotted onto Lept medium containing Gm and 100 µM $MnCl_2$ and allowed to grow for two days at room temperature.

Transformation of pJB2 into non-oxidizing and poor oxidizing species of *Pseudomonas*

P. putida KT2440 and *P. fluorescens* were grown in LB medium overnight at room temperature. The cells were made competent as previously described and transformed with pJB2. Single transformant colonies were plated onto Lept medium supplemented with 100 µM $MnCl_2$ and Gm to determine the manganese oxidation phenotype.

In-Frame deletion of PputGB1_2552

Generation of in-frame deletion

The deletion construct was prepared according to the method of Geszvain and Tebo (2010). Forward (del-2552-UP-F) and reverse (del 2552-DWN-R) primers ~500 bp upstream and downstream from the gene of interest were designed in conjunction with forward (Junct 2552-F') and reverse (Junct 2552-R') junction

primers to fuse the 5' and 3' ends of the target gene sequence to the upstream and downstream primers by PCR (See Table 4.3 for primers).

The deletion construct was brought into *P. putida* GB-1 cells by triparental mating using pRK2013 helper plasmid. Overnight cultures of *P. putida* GB-1(recipient), pRK2013 (helper), and pEX18Gm Δ2552 (donor) were inoculated in fresh medium containing necessary antibiotics, until mid-exponential phase and were mixed in a 3:1:2 ratios respectively. Once pelleted, the cells were resuspended and plated on LB medium for 24 hours at room temperature. The cells were removed from the LB plate and resuspended in 1ml of LB medium followed by plating on *Pseudomonas* isolation agar (Difco) containing Gm to select for conjugates. Conjugates were screened for Gm resistance and sucrose sensitivity which corresponds to colonies that resulted in integration of the plasmid into the chromosome (first recombination event). Next, the colonies were screened for sucrose resistance and Gm sensitivity indicating that the plasmid backbone from the chromosome was removed (second recombination event). The colonies were also streaked out onto Lept medium supplemented with 100 μM $MnCl_2$ to screen for oxidation phenotype. Of the sucrose resistant and Gm sensitive candidate strains, five oxidizing and five non-oxidizing patches were struck out onto LB medium to obtain single colonies. Single colonies of each candidate strain were inoculated and grown overnight in LB medium for isolation of genomic DNA (Wizard® Genomic DNA Purification Kit, Promega). PCR was performed on the isolated genomic DNA using upstream (del-2552-UP-F) and downstream (JB-2552-R) primers. The gene

deletion was determined by the absence of the gene product in the non-oxidizing candidates compared to oxidizing candidate strains. The deletion strains were inoculated and grown overnight in LB medium. One hundred microliters of overnight culture was subcultured in liquid Lept medium supplemented with 100 μM $MnCl_2$ or 100 μM Mn(III)-pyrophosphate to test the ability to oxidize both Mn (II) and Mn (III) respectively.

Manganese oxidation assay with Δ2552 cellular extracts

Overnight cultures of *P. putida* GB-1 wild type, Δ2552-1, and Δ2552-5 were grown in Lept medium supplemented with 100μM $MnCl_2$ at room temperature until wild type oxidized manganese. One milliliter of each strain was washed with 10mM ascorbate for manganese oxide removal and pelleted by centrifugation. Each pellet was resuspended in 500μl 10mM HEPES buffer (pH 7.5) to rinse spent medium. Each suspension was sonicated for a few second burst on ice followed by centrifugation and retrieval of supernatant. Fifty microliters of each supernatant (~60ng/μl protein A280 using NanoDrop) was added to 10mM HEPES buffer (pH 7.5) containing 100μM $MnCl_2$ and allowed to react overnight at room temperature in a 96 well plate. Manganese oxide formation was determined by the addition of LBB in a 1:1 ratio to each well and measured the absorbance at 618nm.

Table 4.2. Strains and plasmids used in this chapter

Strains or plasmid	Genotype, characteristics, or construction[a]	Source or reference
Strains		
E. coli TAM1	*mrcA* Δ*(mrr-hsdRMS-mcrBC)* φ*80lacZ*ΔM15 Δ*lacX74 recA1 araD139 (ara-leu) 7697 galU galK rspL endA1 nupG*	active motif
P. putida		
GB-1	Wild Type	Corstjens et al., 1992
KG51	GB-1 *ΔmnxG*	Geszvain, personal communication
KG65	GB-1 *ΔmopA*	Geszvain, personal communication
KG70	GB-1 *ΔmnxGΔmopA*	Geszvain, personal communication
KG127	GB-1 *ΔmnxR*	Geszvain, personal communication
JB1	GB-1 *Δ2552-1*	This study
Plasmids		
pEX18Gm	Gene replacement vector; Gm^r; *oriT sacB*	Hoang et al., 1998
pJet1.2/blunt	Commercial cloning vector	Fermentas
pRK2013	Helper plasmid for conjugation ; km^r	Figurski and Helinski, 1979
pUCP22	Broad-host-range vector; Gm^r ; ColE1	West et al., 1994
pBBR1-MCS-5	Broad-host-range vector; Gm^r ; IncP, IncW, IncQ, ColE1 and p15A	Kovach et al. 1994
pKG 207	pUCP22 carrying *mnxR*	Geszvain, personal communication
pKG 196	pBBR1-MCS-5 carrying *mco*	Geszvain, personal communication
pKG 193	pBBR1-MCS-5 carrying *mnxG*	Geszvain, personal communication
pJB1	pBBR1-MCS-5 carrying 2553	This study
pJB2	pBBR1-MCS-5 carrying 2552 and 2553	This study
pJB3	pBBR1-MCS-5 carrying 2552	This study
pJB4	The ~500bp upstream of PputGB1_2552 fused to the ~500bp downstream and cloned into the pEX18Gm, for generating an in-frame deletion	This study

Table 4.3. Primers used in this chapter

Primer	Sequence (5' - 3')
JB 2553-F’	CAA GTC CAT GGG CCC GTT TG
JB 2553-R-DWN	GCC TCC TTG AAG GCT GAG CC
JB-OE 2552-F	CGT GGT GCT GGT GCC ATG
JB 2552-R	GCC AGG CTG AAC AGC AGT G
del 2552-UP-F	CGC CTG CCA GCG TAA CC
del 2552-DWN-R	TGG GAC GAG GTG CTG TGA C
Junct 2552-F’	CGC CGG TTT TTC CCT CAC CCG CAC CGT CAA CGT GGT TG
Junct 2552-R’	CAA CCA CGT TGA CGG TGC GGG TGA GGG AAA AAC CGG CG

Results

Over expression of genes 2552 (pJB3), 2553 (pJB1), and both 2553 and 2552 (pJB2)

To determine which of the hypothetical proteins were important for manganese oxidation, each gene was over expressed individually and then together. The target genes PputGB1_2552, PputGB1_2553, and both PputGB1_2553 and PputGB1_2552 combined were cloned into pBBR1-MCS-5 vector (Table 4.2) and transformed into wild type *P. putida* GB1 along with an empty vector control. Each transformant was screened on Lept plate or liquid medium supplemented with 100μM $MnCl_2$ and 2.5μM Gm to determine the effects on manganese oxidation phenotype. There was no visual growth defect between the different transformants although in general, the addition of Gm slowed down manganese oxidation, probably due to interactions with the outer membrane. Multi-copy expression of PputGB1_2553 had a slight increase in manganese oxidation while PputGB1_2552 had little or no effect compared to the empty vector control. Over expression of both genes induced a very drastic increase compared to the empty vector control demonstrating the importance both proteins have on manganese oxidation (Figure 4.3). In liquid, the effect of the 2553 plasmid on manganese oxidation was similar to the empty vector control, although the culture appeared more diffuse. The 2552 plasmid resulted in manganese oxidation being more concentrated in the biofilm than is seen in the empty vector control and the culture appeared less diffuse. Similar to what is seen on solid medium the plasmid expressing both genes induced

a drastic increase in liquid, further demonstrating the importance of these two proteins in manganese oxidation (Figure 4.3).

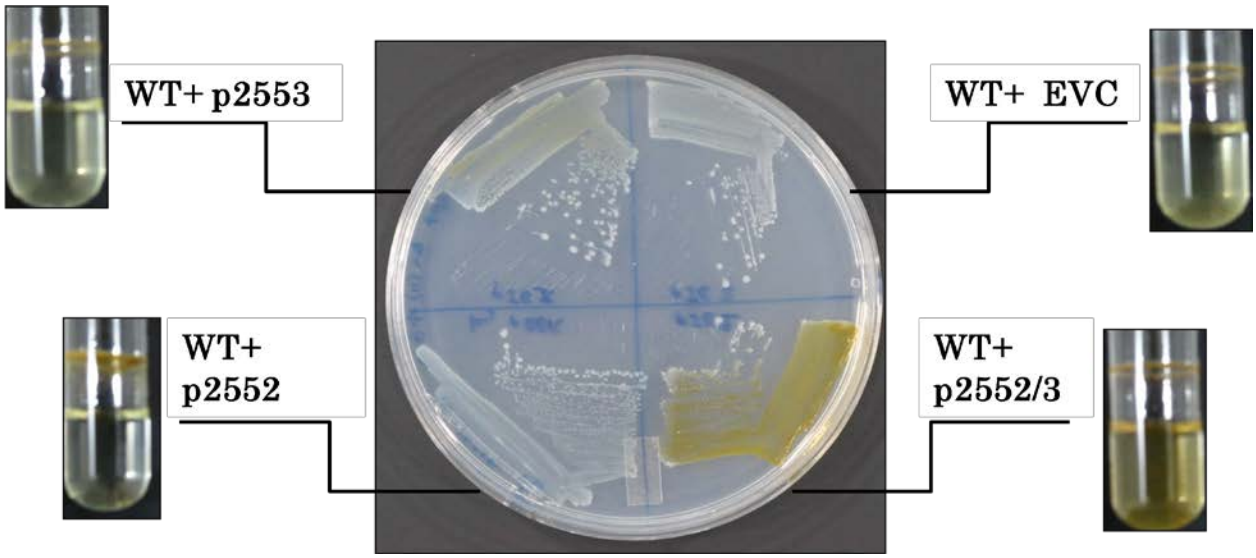

Figure 4.3. Over expression of hypothetical proteins 2552 and 2553. Empty vector pBBR1-MCS-5 and the plasmid carrying hypothetical protein genes 2552 (pJB3), 2553 (pJB1), and both 2552 and 2552 (pJB2) were transformed into WT *P. putida* GB-1. Transformants were plated onto solid Lept medium supplemented with 100μM $MnCl_2$ and 2.5μM Gm and incubated for 48hrs at room temperature. Transformants were inoculated into liquid Lept medium supplemented with 100μM $MnCl_2$ and 2.5μM Gm and incubated at room temperature for 48 hours. (EVC = Empty vector control)

Transformation of pJB2 into mutant strains

To determine which, if any, proteins were required for the stimulation of Mn(II) oxidation by PputGB1_2553/2552, a plasmid expressing both genes or empty vector pBBR1-MCS-5 as a control were transformed into the deletion strains *ΔmnxG, ΔmnxGΔmopA, Δmco*, Δ*mopA* , and in wild type *P. putida* GB1

backgrounds. Each transformant was plated onto solid Lept medium supplemented with 100μM $MnCl_2$ to assess the effect on manganese oxidation. When pJB2 was transformed into Δ*mopA* or Δ*mco* strains, a dramatic increase in manganese oxidation was observed when compared to the respective empty vector controls (Figure 4.4b). These results are similar to when pJB2 is transformed into wild type *P. putida* GB1. In contrast, when pJB2 was transformed into the Δ*mnxG* or Δ*mnxG*Δ*mopA* strains there was no increase in manganese oxidation (Figure 4.4a). These results indicate that the enhancement of Mn (II) oxidation activity requires the presence of MnxG.

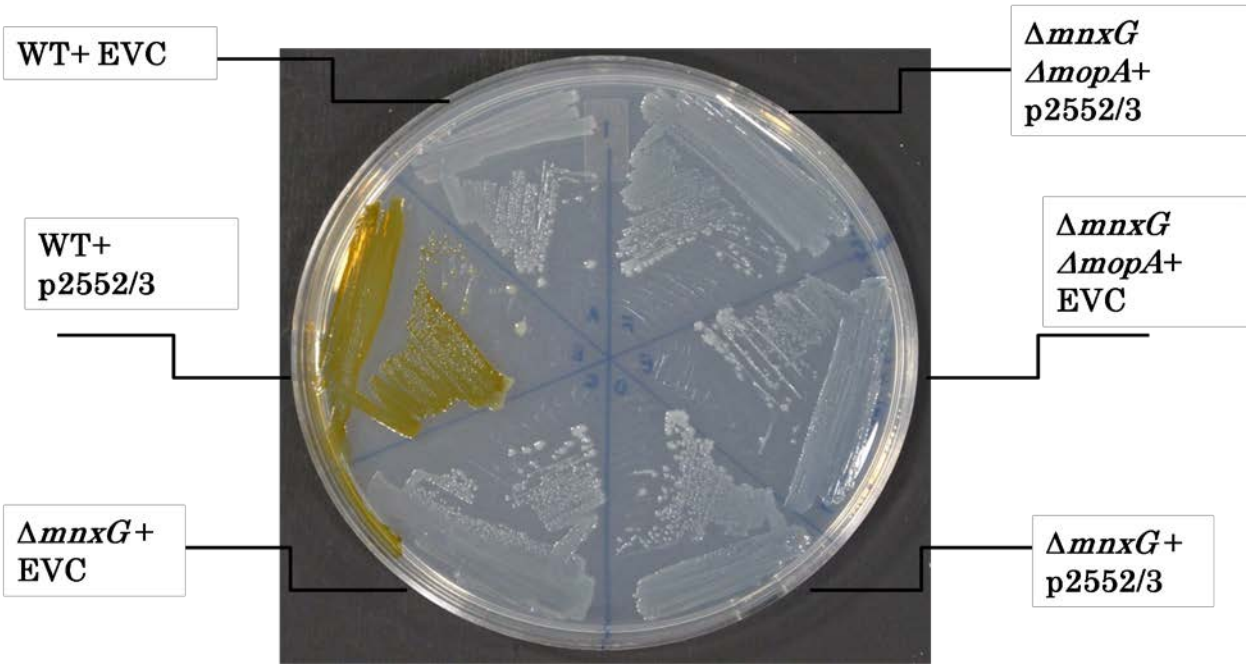

Figure 4.4a. Transformation of hypothetical proteins into various mutants. Empty vector pBBR-MCS-5 and pBBR1-MCS-5 carrying hypothetical protein genes 2552 and 2553 (pJB2) were transformed into WT *P. putida* GB-1 and various mutant strains (*ΔmnxGΔmopA* and *ΔmnxG*). Transformants were plated onto Lept medium supplemented with 100 μM $MnCl_2$ and 2.5μM Gm and incubated at room temperature for 48 hours. (EVC = Empty vector control).

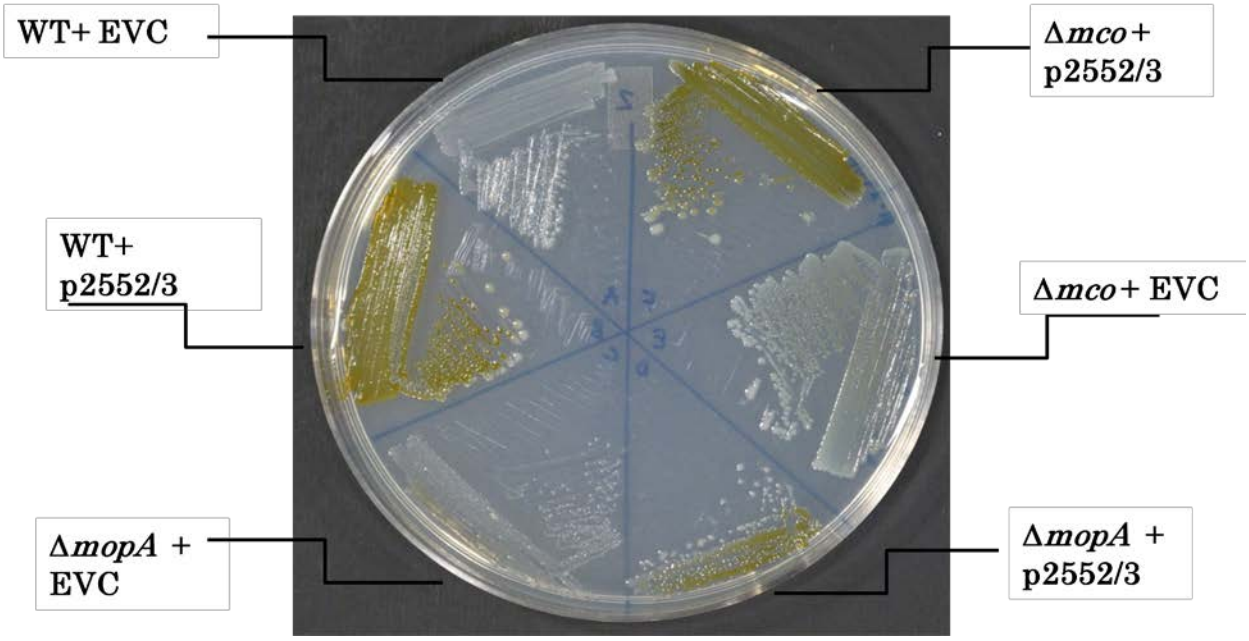

Figure 4.4b. Transformation of hypothetical proteins into various mutants. Empty vector pBBR1-MCS-5 and pBBR-MCS-5 carrying hypothetical protein genes 2552 and 2553 (pJB2) were transformed into WT *P. putida* GB-1 and various mutant strains (*ΔmopA* and *Δmco*). Transformants were plated onto Lept medium supplemented with 100 µM $MnCl_2$ and 2.5µM Gm and incubated at room temperature for 48 hours. (EVC = Empty vector control).

The *ΔmnxR* strain is not able to oxidize manganese under any conditions tested thus far. To determine if 2553 and 2552 are sufficient to induce oxidation in the absence of any other MnxR regulated gene products, a plasmid expressing both genes and empty vector pBBR1- MCS-5 were transformed into the *ΔmnxR* background. The transformants were plated onto solid Lept medium supplemented

with 100μM $MnCl_2$ and 2.5μM Gm to assess whether manganese oxidation could be restored. The plasmid expressing both 2552 and 2553 genes was not able to restore the oxidation defect in the *ΔmnxR* background as expected (Figure 4.5).

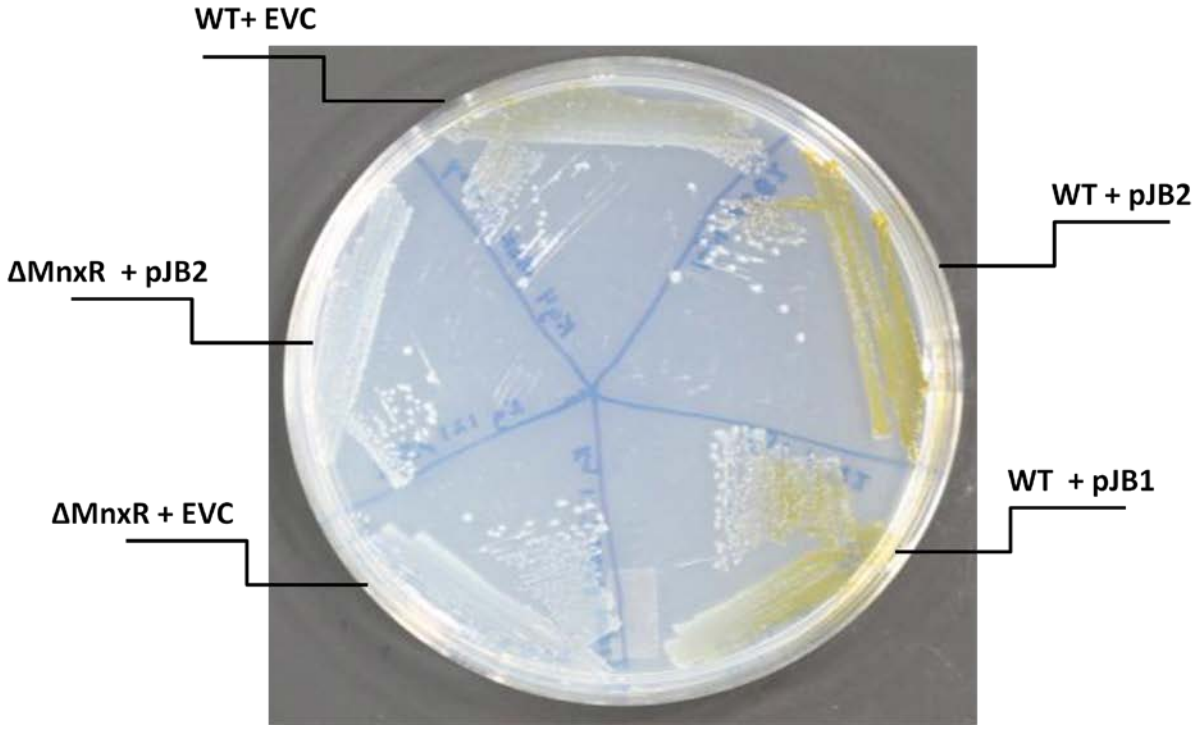

Figure 4.5 Transformation of hypothetical protein genes into Δ*mnxR* mutant strain. Empty vector pBBR1-MCS-5 and the plasmid carrying hypothetical protein genes 2553 (pJB1), and both 2552 and 2553 (pJB2), were transformed into WT *P. putida* GB-1 and *ΔmnxR* strains. Transformants were plated onto solid Lept medium containing 100μM $MnCl_2$ and 2.5μM Gm and incubated at room temperature for 48 hours. (EVC = Empty vector control).

Reconstitution of activity in *ΔmnxR* background

The Δ*mnxR* strain is not able to oxidize manganese under any conditions tested. Experiments were developed to reconstitute oxidase activity by transforming

plasmids containing genes thought to be utilized in manganese oxidation into the Δ*mnxR* background strain, followed by the collection of cellular extracts. The cellular extracts were then assayed as previously described, in various combinations to determine which protein(s) are responsible for manganese oxidation and whether the *mnxR* strain can be restored by reconstitution. Only the pMnxR plasmid control extract was able to restore activity in Δ*mnxR* (Figure 4.6). To determine whether combining whole cell cultures could reconstitute activity in the Δ*mnxR* strain, overnight cultures of various transformants were combined and spotted onto Lept medium supplemented with 100 μM $MnCl_2$ and 2.5μM Gm. Again, only pMnxR was able to restore manganese oxidation in the Δ*mnxR* background (Figure 4.7).

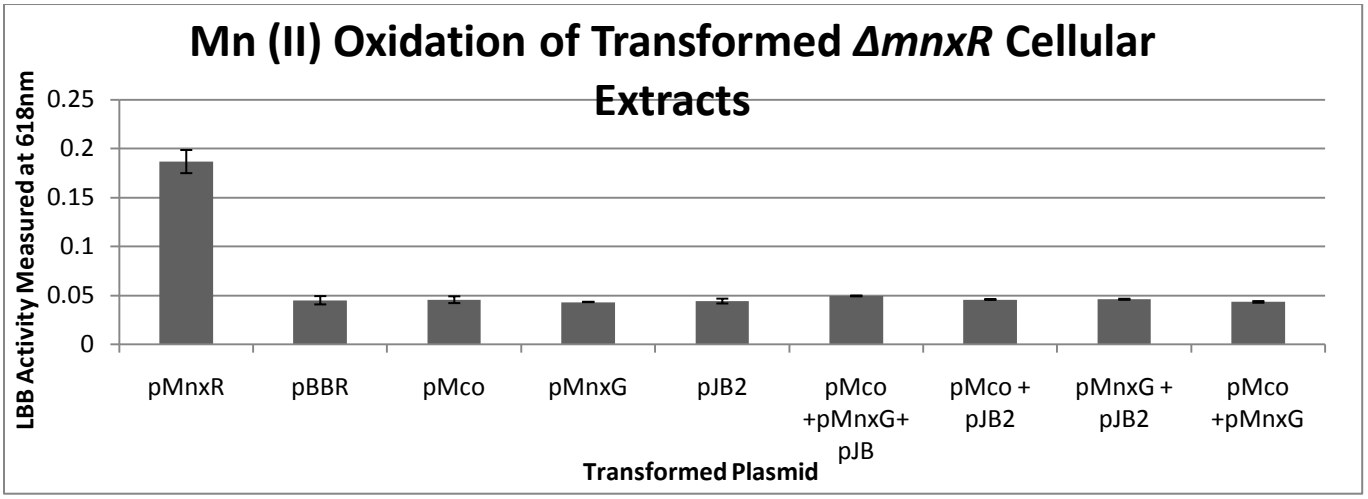

Figure 4.6. Reconstitution of Mn (II) oxidation activity of *ΔmnxR* strains transformed with plasmids pMnxR (positive control), pBBR-MCS-5 (empty vector control), pMco, pMnxG, and pJB2. Cellular extracts of transformants were assayed in various combinations overnight in 10mM HEPES buffer (pH 7.5) and tested for LBB activity. Treatments were assayed in triplicate with error bars representing standard deviations.

A. Δ*mnxR* + pMco, pMnxG, and pJB2 cultures

B. Δ*mnxR* + pMnxR

C. Δ*mnxR* + pBBR-MCS-5 empty vector

Figure 4.7. Reconstitution of Mn (II) oxidation activity of *ΔmnxR* strains transformed with plasmids pMnxR (positive control), pBBR-MCS-5 (empty vector control), and a combination of pMco, pMnxG, and pJB2 transformed cultures.

Generation of in-frame deletion

Over expression of both PputGB1_2552 and PputGB1_2553 hypothetical proteins results in a dramatic enhancement in Mn (II) oxidation. In order to further distinguish which of these two proteins were important for manganese oxidation, five independent strains of *P. putida* GB-1 carrying an in-frame deletion of the hypothetical protein gene PputGB1_2552 were generated. To assess the ability of the Δ2552 strains to oxidize manganese, the strains were plated with wild type onto solid Lept medium supplemented with 100μM $MnCl_2$. The Δ2552 strains resulted in a complete lack of manganese oxidation on solid medium (Figure 4.8). To determine whether this defect in manganese oxidation is due to an artifact caused by growth on solid medium, Δ2552 and wild type were inoculated in liquid Lept medium supplemented with 100 μM $MnCl_2$ or 100 μM Mn(III)-pyrophosphate. The Δ2552 strains were also unable to oxidize Mn (II) or Mn (III) in liquid Lept medium (Figure 4.9). These results indicate that 2552 is an essential component of manganese oxidation in *P. putida* GB-1.

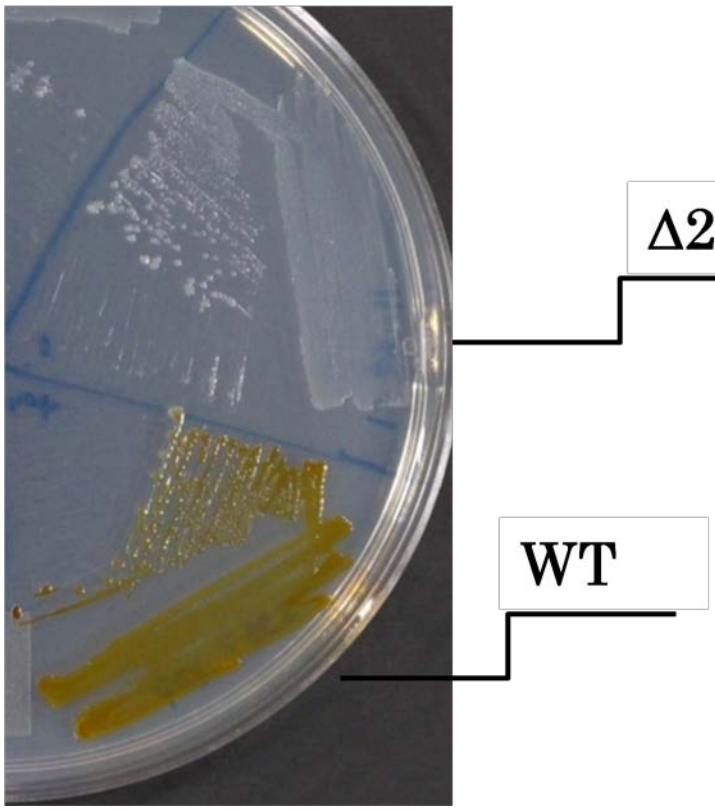

Figure 4.8. The in-frame deletion of 2552 completely abolishes Mn (II) oxidation. Independent strains of Δ2552 and wild type *P. putida* GB-1 were screened on solid Lept medium supplemented with 100μM $MnCl_2$ and incubated at room temperature overnight.

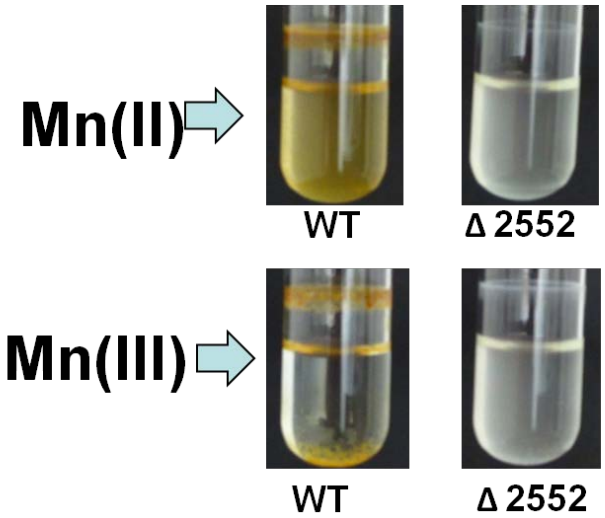

Figure 4.9. The in-frame deletion of 2552 completely abolishes Mn (II) and Mn (III) oxidation. The Δ2552 strain and wild type *P. putida* GB-1 were inoculated into liquid Lept medium supplemented with 100μM $MnCl_2$ or 100μM Mn(III)-pyrophosphate as indicated. Each culture was incubated at room temperature overnight.

Manganese oxidation assay with Δ2552 cellular extracts

As indicated in the previous section, the Δ2552 strain is completely defective at oxidizing manganese on solid and liquid medium. To assess whether this lack of oxidation is due to a failure to transport the manganese oxidase to the outer membrane of the cell, cellular extracts from both wild type *P. putida* GB-1 and two independent Δ2552 strains were assayed. Only wild type cellular extracts were able to oxidize Mn (II), while the strains Δ2552-1 and Δ2552-1 were not able to oxidize Mn (II) (Figure 4.10) or Mn (III) - pyrophosphate (data not shown). This result indicates that the complete lack of manganese oxidation in the Δ2552 strains is not due to a transport defect.

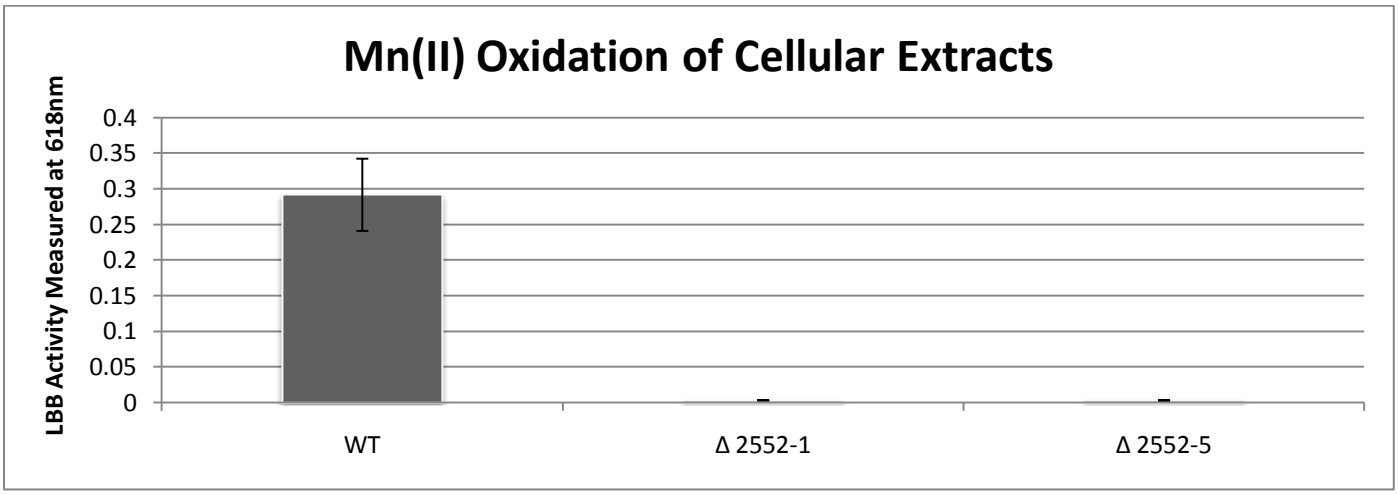

Figure 4.10. Cellular extracts of Δ2552 strains are not capable of Mn(II) oxidation compared to the wild type control. Three micrograms total protein from each cellular extract was added to 10mM HEPES buffer (pH 7.5) supplemented with 100 μM $MnCl_2$ and allowed to react overnight at room temperature. Mn (II) oxidation was determined by LBB activity measured at 618nm. All assays were complete in triplicate with error bars representing standard deviations.

Discussion

Protein sequence analysis comparing other species of bacteria indicate that 2552 and 2553 has sequence homology to Fibronectin type III protein domains such as found in *Leptothrix cholodnii* and sequence homology to cell surface receptor proteins containing IPT-TIG domain such as found in *Leptothrix cholodnii*, *Bacillus* SG1, and *Geobacter sulfurreducens* (BLAST http://blast.ncbi.nlm.nih.gov, Altschul et al., 1990).

Fibronectin type III domains are postulated to facilitate the adhesion of the protein to polysaccharides or cell receptors, or these domains participate in maintaining proper conformation of binding or catalytic sites of enzymes (Kataeva et al., 2002 and Watanabe et al., 1994). In *Clostridium thermocellum,* fibronectin domains promote hydrolysis of cellulose by surface modifications (Kataeva et al., 2002). Thus, these domains might allow for surface modifications or attachment to the polysaccharide matrix or the Mn(III/IV) oxides of *P. putida* GB-1. The OmpB protein of *Geobacter sulfurreducens* contains both fibronectin type III and MCO domains and is required for Fe(III) and Mn(IV) reduction in this organism (Mehta et al., 2006). OmpC, which is similar to OmpB are both required for reduction of Fe(III) oxides (Holmes et al., 2008).

Previous studies have suggested that outer membrane c-type cytochromes participate in electron transfer to Mn(IV) and Fe(III) in *Geobacter* (Lovley et al., 2004 and Qian et al., 2007). The outer membrane associated cytochrome OmcB is

necessary for optimal reduction of soluble, chelated Fe(III) in *G. sulfurreducens* (Kim et al., 2006 and Leang et al., 2003). The *ccm* operon, which is responsible for c-type cytochrome biosynthesis is also required for Mn(II) oxidation in *P. putida* GB-1 (de Vrind et al., 1998 and Caspi et al., 1998). Other types of outer membrane proteins are important in Fe(III) reduction, such as MtrB, a non-heme containing outer membrane protein required in *Shewanella putrefaciens* (Beliaev and Saffarini, 1998).

Hypothetical protein 2553 also contains two putative EXXE motifs, which are potential Fe-binding sites, which is known to bind Fe(III) in the transporter FTR1 (Severance et al., 2004), and is also found in OmpB (Mehta et al., 2006). Since Fe and Mn chemistry are similar, it is possible for the EXXE metal binding site to bind Mn. The secondary structure of the two EXXE motifs in 2553 are predicted to be coiled and surface exposed, which could potentially bind metals such as manganese. However, the EXXE motif such as found in OmpB most likely has an alpha helical structure. (http://www.cbs.dtu.dk/services/NetSurfP, Petersen et al., 2009).

The hypothetical proteins do not share sequence homology with OmpB or OmpC other than possessing Fibronectin type III protein genes but it is plausible that the functional role might be similar. Thus I propose a model in which the hypothetical proteins allow for proper positioning of an outer membrane c-type cytochrome or proper binding and positioning of soluble Mn(II) into close proximity to the catalytic site of the Mn(II) oxidase, such as MnxG or Mco.

The proposed association of MnxG (Figure 4.11) with the two hypothetical proteins is supported by the transformation results where over expression of both hypothetical proteins could not stimulate Mn(II) oxidation in the Δ*mnxG,* or the *ΔmnxGΔmopA* double mutant*,* but could greatly enhance Mn(II) oxidation in the *Δmco* or *ΔmopA* mutant strains. This association could be similar to the OmpB and OmpC proteins, which has both multi-copper oxidase domains and fibronectin type III protein domains.

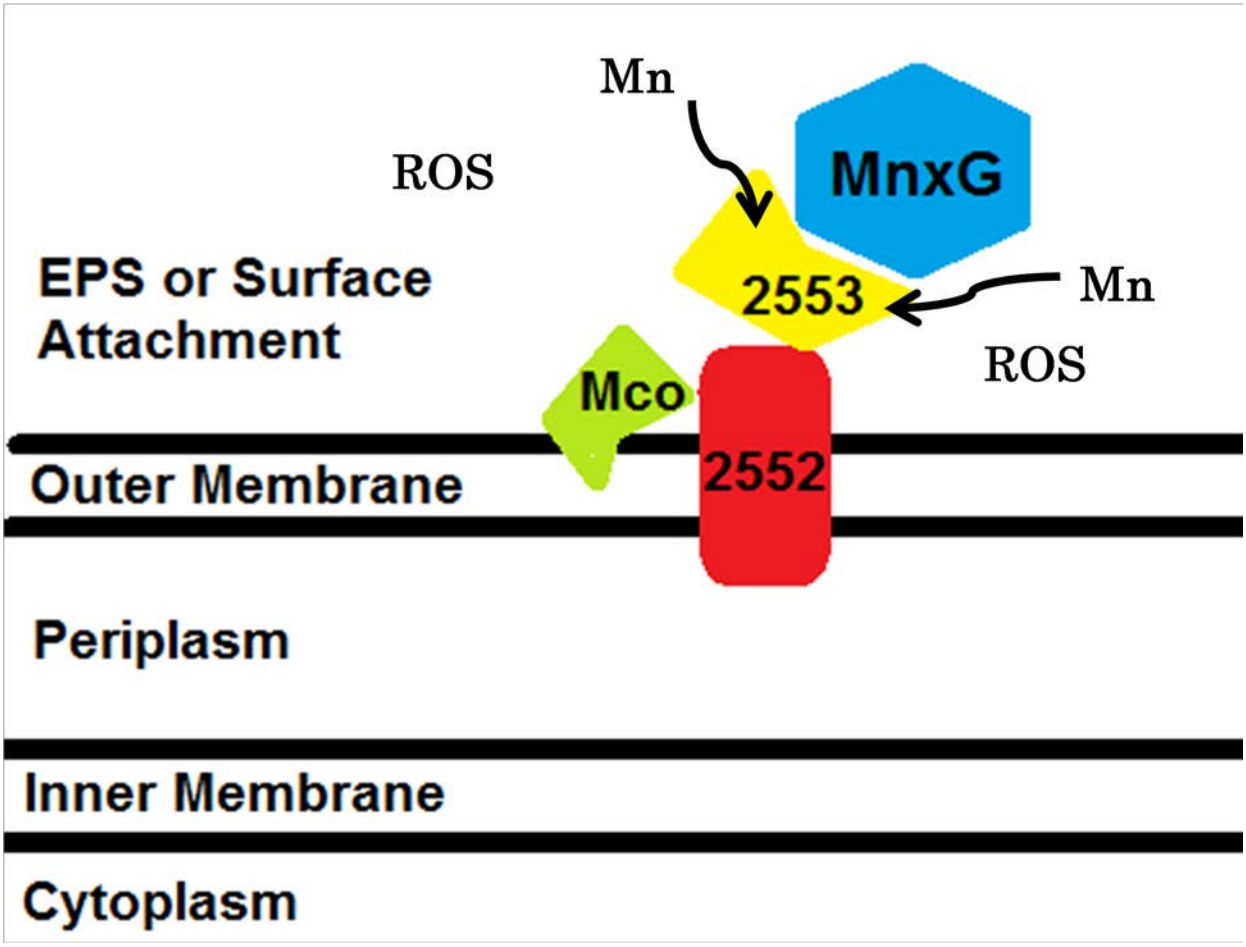

Figure 4.11. Proposed model of hypothetical proteins 2552 and 2553 and their association with multi-copper oxidases MnxG or MCO predicted to be localized to the outer membrane of *P. putida* GB-1.

Over expression of hypothetical proteins 2552 and 2553 and the in-frame deletion of 2552 demonstrates that these proteins have an essential role in Mn (II) oxidation in *P.putida* GB-1. However, complementation of Δ2552 with plasmids containing 2552 and both 2552 and 2553 was not possible. Polar effects on downstream genes could cause the lack of complementation, but it remains unknown why complementation was not possible.

Transformation studies suggest an association of the multi-copper oxidase MnxG with both hypothetical proteins. The fact that *mnxG, mco,* and 2553 are under transcriptional regulation by the response regulator MnxR provides further evidence that these hypothetical proteins are important. However, the exact role and function of these proteins remain unknown and will be the topic of further investigations. Hypothetical proteins 2552 and 2553 may act to attach MnxG to the cell surface or EPS, allowing proper positioning of both Mn(II) or Mn(III) to the active site of the Mn(II) oxidase, possibly providing a mode of electron transfer to surface exposed c-type cytochromes. Mco may also be attached to the outer membrane near the MnxG/2552/3 complex, providing some component of Mn(II) oxidation.

Future Direction

To determine which hypothetical protein is required for Mn(II) oxidation more in-frame deletions need to be generated. Specifically the deletion of 2553 separately and both 2552/2553 together will complement the previous deletion of 2552. Then the Mn(II) oxidation phenotype can be assessed. The use of GFP labeling and microscopy could be used to determine the actual protein localization. To determine what proteins or protein complex is associated with the hypothetical proteins, specific antibodies could be developed and used for immunoprecipitation and western blot analysis. Recombinant protein expression of each hypothetical protein is important to determine whether the proteins are catalytic and to determine if 2553 can bind a metal ion. Together these techniques will help determine what role hypothetical proteins 2552 and 2553 play in Mn(II) oxidation.

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Chapter 5. Conclusion

Previous attempts to localize the Mn(II) oxidase in *P. putida* GB-1 determined that the enzyme is associated with the outer membrane (Okazaki et al., 1997) or found intracellularly in *P. putida* MnB1 (Jung and Schweisfurth, 1978). The work described in this thesis confirmed that Mn(II) oxidizing activity is localized to the outer membrane fraction, although activity was also confirmed to be in the soluble fraction in some preparations. It is likely that there may be multiple enzymes that are capable of oxidizing Mn(II) in *P. putida* GB-1 such as MCO and MnxG. This could explain why activity localization is somewhat sporadic and difficult to determine. Hydrophobic interaction chromatography and size exclusion FPLC of active membrane fractions eluted a partially purified active fraction at 130kDa, roughly the predicted size of type 2 MCO (123kDa). Difficulties in identifying the Mn(II) oxidase(s) could be attributed to its low abundance. However, the protein or protein complex is highly active.

To determine whether a MCO such as MnxG or Mco is the dominant Mn(II) oxidase, the ability of Cu^{2+} to stimulate oxidation in cell extracts was tested, which resulted in a complete inhibition of Mn(II) oxidizing activity. In contrast, if heme-peroxidase like MopA is required for the oxidation of Mn(II) utilizing H_2O_2 as a substrate, the addition of Ca^{2+} or hydrogen peroxide to cell extracts would be predicted to stimulate oxidation. As the results indicate, the addition of Ca^{2+} or H_2O_2 had no effect and suggests that MopA is not directly involved. Thus, based on

these results it is uncertain which type of enzyme is the dominant player in Mn(II) oxidation.

When superoxide dismutase, which catalyzes the dismutation of superoxide radicals to produce hydrogen peroxide and oxygen (McChord and Fridovich, 1969) was added to cell extracts of *P. putida* GB-1 Mn(II) oxidation was completely abolished. This effect was similar to when Cu^{2+}, a known superoxide radical scavenger (Voelker et al., 2000) was added, while other metals such as Ca^{2+}, Co^{2+} or Mo^{2+} had no effect. Other reactive oxygen species such as hydroxyl radicals and H_2O_2 also had no effect on Mn(II) oxidation under the conditions tested. However, catalase was shown to greatly inhibit Mn(II) oxidation. Since the addition of H_2O_2 had no effect, it is unknown why catalase can inhibit the reaction. While the data suggest superoxide radicals are required for Mn(II) oxidation in *P. putida* GB-1, the specific role they play is unclear and it is unknown as to where the superoxide radicals are produced or whether manganese is the substrate for oxidation.

Two hypothetical proteins encoded by the loci PputGB1_2552 and PputGB1_2553 were identified from MS/MS spectroscopy analysis of partially purified active fractions. Over expression of both 2552 and 2552 induces a drastic enhancement of Mn(II) oxidation while an in-frame deletion of 2552 completely abolishes activity. Transformation studies also suggest an association of the multi-copper oxidase MnxG with both hypothetical proteins. These results demonstrate that the two hypothetical proteins are essential for Mn(II) oxidation in *P.putida* GB-1. Recent quantitative PCR (Geszvain, personal communication) results clearly

show that *mco, mnxG,* and PputGB1_2553 are regulated by the response regulator MnxR, which is part of the Mnx TCR pathway that is essential for Mn(II) oxidation (Geszvain and Tebo, 2010). These results suggest that the two hypothetical proteins are intricately linked to Mn(II) oxidation. However, the exact role and function of these proteins remain unknown. I propose that hypothetical proteins 2552 and 2553 are involved with surface attachment of an MCO which allows proper positioning of either Mn(II) or Mn(III) to the active site of the Mn(II) oxidase.

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Appendix A. Catalase Peroxidase HPI

Introduction

The solution of partially purified active fraction post size exclusion chromatography and multiple excised Native PAGE gel bands of active membrane fractions were analyzed by MS/MS spectroscopy (Chapter 2). After pooling multiple data sets, catalase peroxidase HPI (KatG) was identified as a candidate for the Mn(II) oxidase.

Catalase peroxidases are multifunctional enzymes ubiquitously found throughout bacteria, archaea, and even fungi (Klotz and Loewen, 2003). KatGs are homodimers or homotetramers of ~80kDa subunits, each containing two sequence related domains likely as a result of a gene duplication event (Singh et al, 2008). These enzymes contain both peroxidase and catalase domains, which are responsible for the dismutation of hydrogen peroxide to form water and oxygen (Jakopitsch et al., 2007). The peroxidase domain has an optimal pH of 5 while the catalase domain has an optimal ph of 6.5. However, it has been shown that KatGs produced by *B. pseudomallei*, *E. coli*, and *M. tuberculosis* exhibit NADH oxidase activity. These enzymes oxidize NADH to form NAD+ and either hydrogen peroxide or superoxide radicals depending on pH (Singh et al., 2004). The KatG of *M. smegmantis* has been shown to possess Mn(II) peroxidase activity, measured by production of Mn(III)-malonate formation (Magliozzo and Marcinkeviciene, 1997). Based on MS/MS spectroscopy identification and the capability of known KatGs to

oxidize Mn(II) made this protein the target for *in vitro* studies and genetic manipulations. The main objective was to generate an in-frame deletion of PputGB1_2231 (*katG*) in *P. putida* GB-1.

Materials and Methods

The effect of NADH on Mn(II) oxidation

Freshly prepared solution of NADH (10, 50, and 100μM) was added to 10mM HEPES buffer (pH 7.5) containing 20-40μg total protein of active membrane fractions depending on the particular assay. Each well was supplemented with 100μM $MnCl_2$ and incubated overnight at room temperature in 96 well plates and brought to a total volume of 100μl. A 1:1 ratio of LBB was added to the oxidation assays and read on the spectrophotometer at 618nm to spectrophotometrically determine oxide formation.

In-frame deletion of *katG*

Escherichia coli strains were grown on LB medium containing antibiotics when necessary. *Pseudomonas putida* GB-1were grown on LB medium *E. coli* were grown at 37 □ C while *P. putida* GB-1 was grown either at room temperature or 30 □ C. Antibiotics were added to the medium in the following concentrations: 100 μg/mL ampicillin (Ap), 30 μg/mL kanamicin (km), and 50 μg/mL gentamicin (Gm) in LB.

The deletion construct was prepared according to the method of Geszvain and Tebo (2010). Forward (2231-F) and reverse (2231-R) primers ~500 bp upstream and downstream from the gene of interest were designed in conjunction with forward (Junct 2231-F) and reverse (Junct 2231-R) junction primers to fuse the 5' and 3' ends of the target gene sequence to the upstream and downstream primers by PCR (See Table A.1 for primers).

The deletion construct was brought into *P. putida* GB-1 cells by triparental mating using pRK2013 helper plasmid. Overnight cultures of *P. putida* GB-1(recipient), pRK2013 (helper), and pEX18Gm Δ2552 (donor) were inoculated in fresh medium containing necessary antibiotics, until mid-exponential phase and were mixed in a 3:1:2 ratios respectively. Once pelleted, the cells were resuspended and plated on LB medium for 24 hours at room temperature. The cells were then removed from the LB plate and resuspended in 1ml of LB medium followed by plating on LB agar containing Gm and Amp to select for conjugates. Conjugates were screened for Gm resistance and sucrose sensitivity which corresponds to colonies that resulted in integration of the plasmid into the chromosome (first recombination event).

Table A.1. Primers used in this chapter

Primer	Sequence (5' - 3')
JB-2231-F	TCA GCA ACG GGT CGT CGA AC
JB-2231-R	GCT GCC CGC CAT CGA TG
JB-Junct 2231-F	GTC GCG GCT TGG CAG AAG
JB-Junct 2231-R	GTC GCG GCT TGG CAG AAG

Results

The effect of NADH on Mn(II) oxidation

The addition of NADH to active outer membrane protein fractions resulted in inconsistencies and variations between fractionation preparations. Either it was shown to have no significant change in Mn(II) oxidation (Figure A.1) or it was shown to stimulate based on each particular fractioning preparation (Figure A.2).

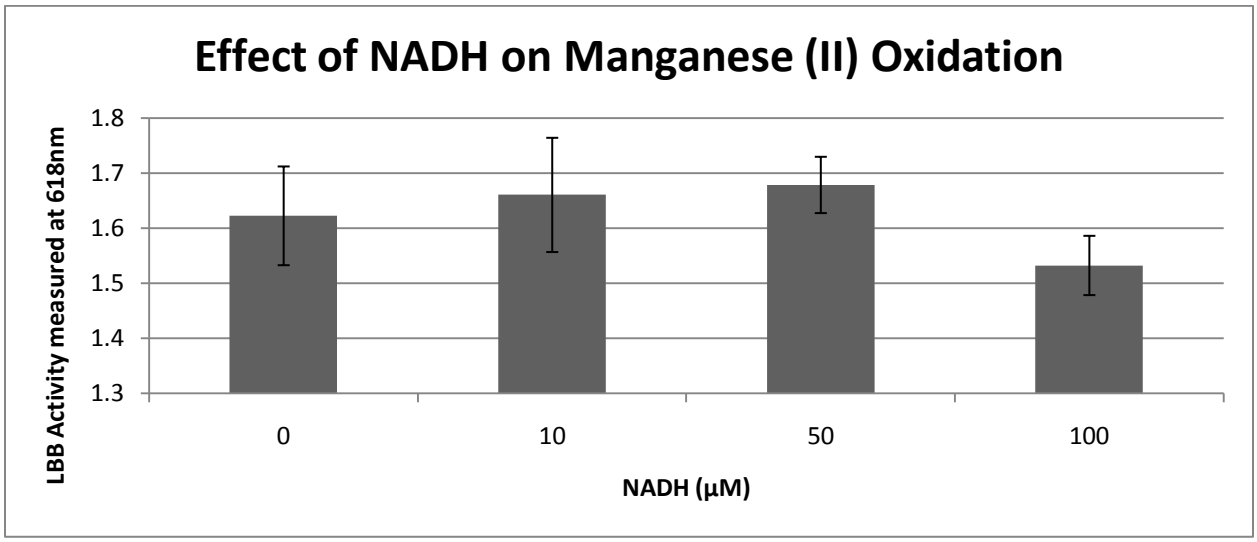

Figure A.1. No stimulation of Mn(II) oxidation. 20µg total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100µM $MnCl_2$ and NADH (0, 10, 50, and 100 µM). Mn (II) oxidation was determined by LBB activity measured at 618nm. Treatments were assayed in triplicate with error bars representing standard deviations.

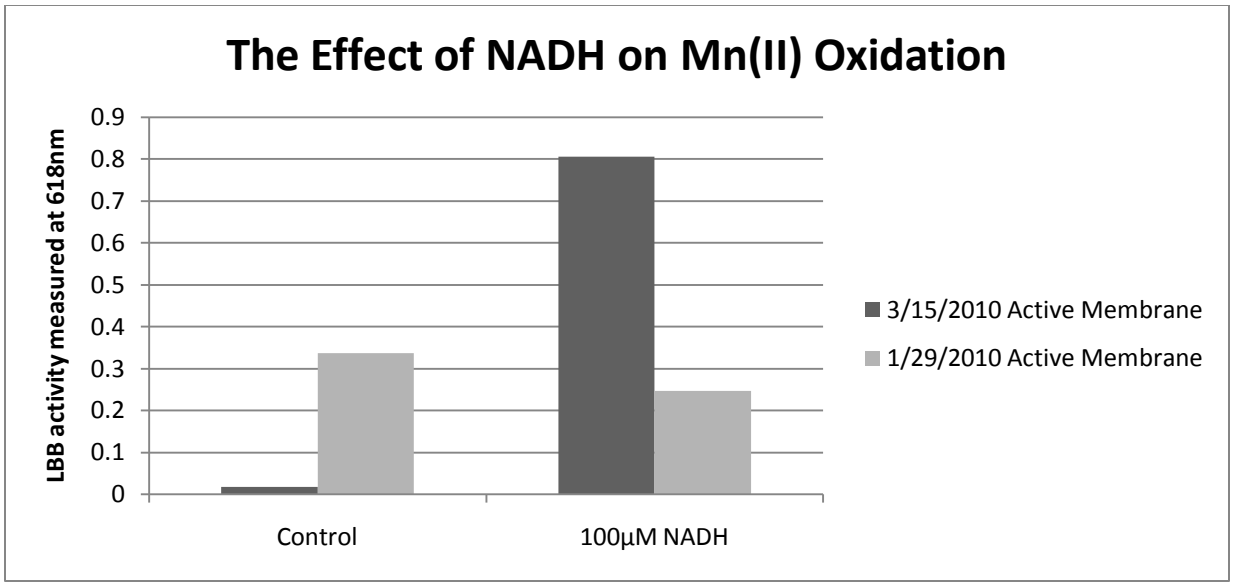

Figure A.2. The NADH and of Mn(II) oxidation. 20µg total active membrane proteins were added to 10mM HEPES buffer (pH 7.5) supplemented with 100µM $MnCl_2$ and NADH (100 µM). Mn (II) oxidation was determined by LBB activity measured at 618nm.

In-frame deletion of *katG*

The in-frame deletion of PputGB1_2231 (*katG*) was not able to be generated after multiple attempts. Over 500 colonies were screened after the first recombination event (Gm resistance and sucrose sensitivity) in each attempt.

Discussion

The ability for NADH to stimulate Mn(II) oxidation has been shown in *Roseobacter*, while NAD+ has no effect. This stimulatory affect can be completely negated in the presence of superoxide dismutase, which removes superoxide radicals (Learman et al., 2011). This is suggestive that NADH might act as an enzymatic substrate for superoxide radical generation since there is a link between NADH oxidoreductases and bacterial extracellular superoxide production (Huycke et al., 2001). The inconsistent results of the effect of NADH addition to cellular fractions of *P. putida* GB-1 could be attributed to the variation of Mn(II) oxidase localization. It is plausible that the enzyme responsible for NADH oxidase activity and thus superoxide radical production might not have been in proximity to the Mn(II) oxidase. This would result in the lack of NADH stimulation of Mn(II) oxidation in certain preparations.

Attempts to generate an in-frame deletion of KatG in *P. putida* GB-1 were not successful. This is most likely due to the essentiality of *katG* ((http://tubic.tju.edu.cn/deg, Zhang and Lin, 2009; Zhang et al., 2004) because the first recombination event screen was not possible. All colonies screened did not appear to be *P. putida* GB-1, suggesting the deletion of *katG* is either highly difficult or is lethal. Based on the apparent essentiality of *katG*, it does not seem likely that it is the Mn(II) oxidase since Mn(II) oxidation is not essential (http://tubic.tju.edu.cn/deg, Zhang and Lin, 2009; Zhang et al., 2004). However, it

might have some function in Mn(II) oxidation based on its role with reactive oxygen species.

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Appendix B. Decreased oxidation mutants

Objective

The ability to genetically manipulate *P. putida* GB-1 by targeted in-frame deletions and transposon mutagenesis provides a good method to study Mn(II) oxidation. Previous studies have utilized transposon mutagenesis to find Mn(II) oxidizing deficient mutants (See Chapter 1). This work used transposon mutagenesis to select for Mn(III) oxidizing deficient mutants and determine the point of transposon insertion.

Materials and Methods

Strains and media used

Escherichia coli strains were grown on LB medium containing antibiotics when necessary. *Pseudomonas putida* GB-1was grown on LB medium containing antibiotics when necessary. *E. coli* were grown at 37 □ C while *P. putida* GB-1 was grown either at room temperature or 30 □ C. Antibiotics were added to the medium in the following concentrations: 100 μg/mL ampicillin (Ap) and 30 μg/mL kanamicin (Km).

Transposon mutagenesis was initiated by Sung-Woo Lee according to Geszvain et al. (2011). Overnight cultures of *P. putida* GB-1(recipient), pRK2013 (helper), and pRL27 (donor) were inoculated in fresh medium containing necessary antibiotics, grown until mid-exponential phase and were mixed in a 2:1:2 ratios

respectively. Once pelleted, the cells were resuspended and plated on LB medium for 24 hours at room temperature. The cells were then removed from the LB plate and resuspended in 1ml of LB medium followed by spreading on LB solid medium containing Km and Ap. After 20 hours, the colonies were replica plated on solid Lept medium supplemented with 100 μM Mn(III)-DFO (Duckworth and Sposito, 2005). Colonies from the replica plate that exhibited decreased Mn(III) oxidization Km resistance and those and failure to grow at 37 □ C were chosen for further screening. Genomic DNA was extracted and sequenced to determine the transposon insertion.

Results and Discussion

The mapped Tn5 insertion in JB-DMO-1 is in PputGB1_4729, which encodes for the heat shock protein GrpE a molecular chaperone (JGI Integrated Microbial Genomes http://img.jgi.doe.gov/cgi-bin/pub/main.cgi, Markowitz and Kyrpides, 2007). This strain is severely deficient in both Mn(II) and Mn(III) oxidation (Figures B.1 and B.2). This suggests that the manganese oxidase requires GrpE for proper protein folding and stability. The Tn5 insertion in JB-DMO-2 has two inserts. One is in PputGB1_1879 a hypothetical protein and the other into PputGB1_3248, which is a metal dependent phosphohydrolase (JGI Integrated Microbial Genomes http://img.jgi.doe.gov/cgi-bin/pub/main.cgi, Markowitz and Kyrpides, 2007). Both of these genes are found in both oxidizing and non-oxidizing species of *Pseudomonas*. However, they are not ubiquitously found. Neither gene appears to be under control of the σ-54 dependent promoter (PromScan http://molbiol-tools.ca/promscan,

Studholme and Dixon, 2003). JB-DMO-2 has a defect in Mn(II) and Mn(III) oxidation, but eventually will oxidize (Figure B.1 and B.2). This suggests that the defect is not directly related to the manganese oxidase but some form of indirect result.

Figure B.1. Transposon mutagenesis screen of *P. putida* GB-1 screened on solid Lept medium supplemented with 100 μM Mn(III)-DFO and grown for three days at room temperature.

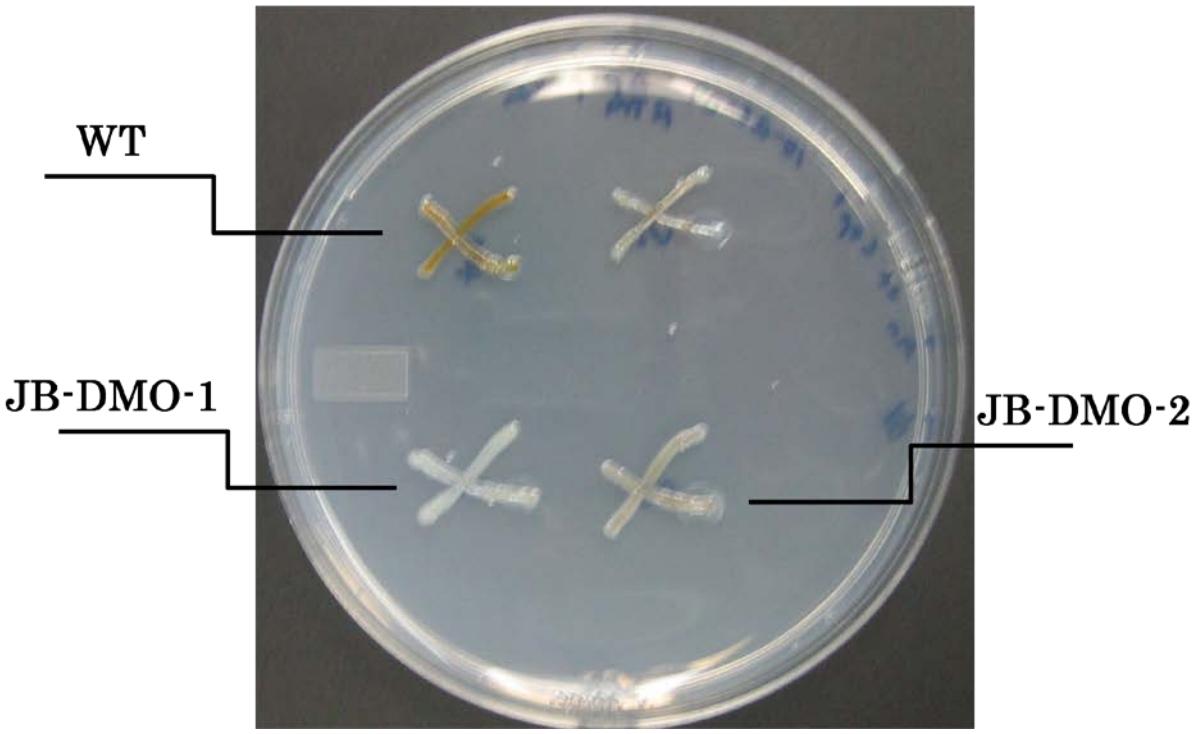

Figure B.2. Transposon mutagenesis of *P. putida* GB-1 screened on solid Lept medium supplemented with 100 μM $MnCl_2$ and grown for three days at room temperature.

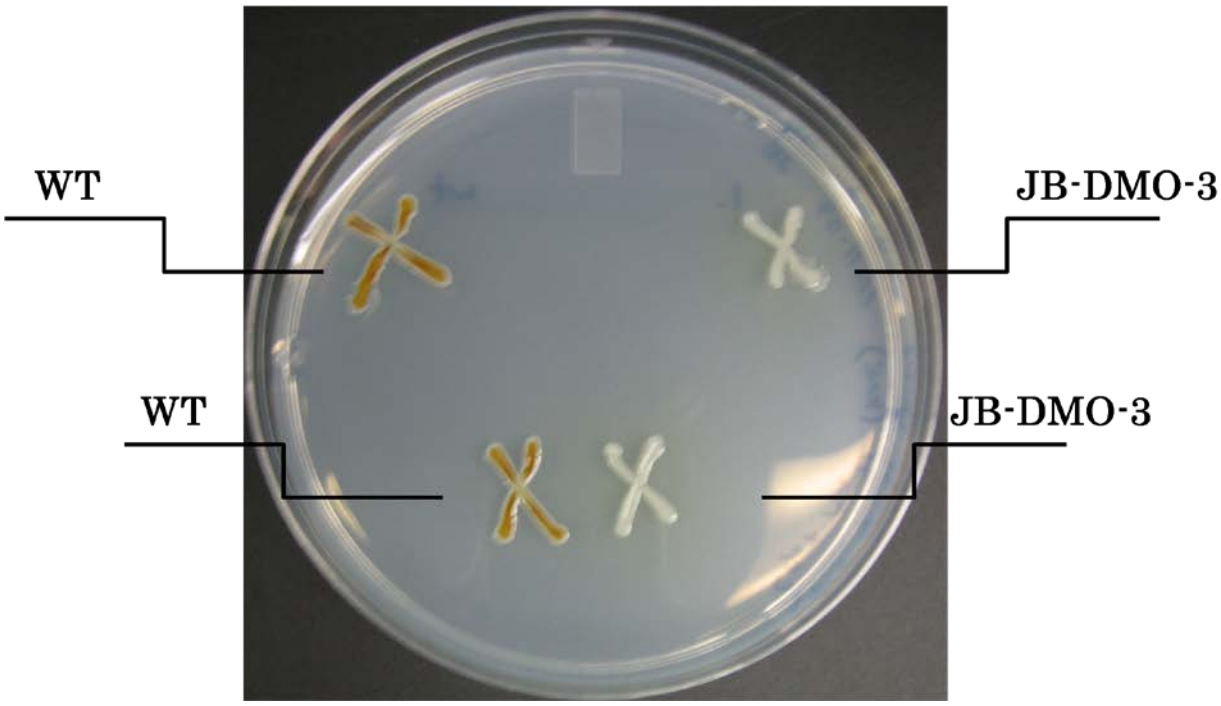

Figure B.3. Transposon mutagenesis of *P. putida* GB-1 screened on solid Lept medium supplemented with 100 μM $MnCl_2$ and grown at room temperature for three days.

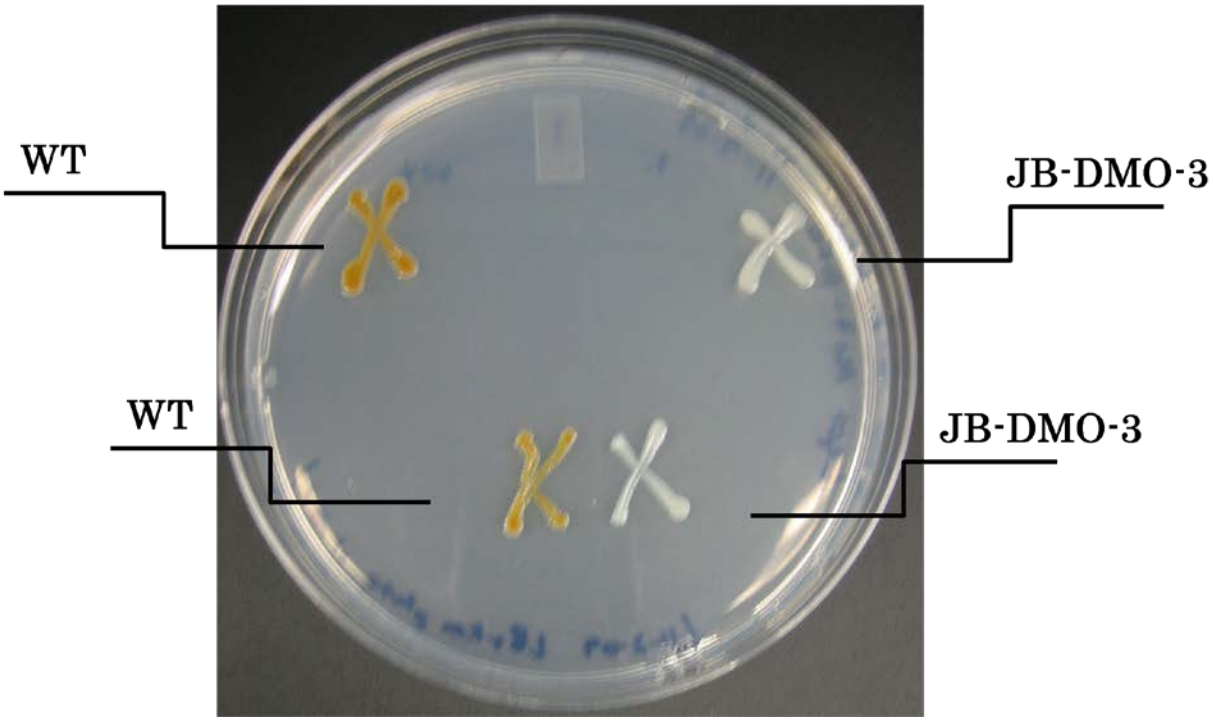

Figure B.4. Transposon mutagenesis of *P. putida* GB-1 screened on solid Lept medium supplemented with 100 μM Mn(III)-DFO and grown at room temperature for three days.

The mapped Tn5 insertion of JB-DMO-3 is in the PputGB1_3896, which encodes *ccmA* involved with cytochrome c maturation (JGI Integrated Microbial Genomes http://img.jgi.doe.gov/cgi-bin/pub/main.cgi, Markowitz and Kyrpides, 2007). JB-DMO-3 cannot oxidize either Mn(II) or Mn(III) even after three days (Figures B.3 and B.4). Previous transposon mutagenesis screens resulted in mutants with insertions in the *ccmF*, *ccmA*, and *ccmE* genes which resulted in the complete abolishment of Mn (II) oxidation (Caspi et al., 1998 and De Vrind et al., 1998). Thus these results are in agreement to what was previously known. This

study also indicates that the *ccm* operon is also essential for Mn(III) oxidation. A possible link of the *ccm* operon to Mn(II) oxidation is the integration of cytochrome c to the transfer of electrons from the putative Mn(II) oxidase(s) to terminal electron acceptor oxygen as an intermediate in an electron transport chain (De Vrind et al., 1998). Other possible explanations are that c-type cytochromes are directly associated with the Mn(II) oxidase in a protein complex (De Vrind et al., 1998 and Caspi et al., 1998).

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Appendix C. MS/MS Data

Introduction

LBB positive outer membrane protein, loosely bound protein, and concentrated medium Native PAGE gel bands were excised and analyzed by MS/MS analysis at the Shared Protein Resource facility at Oregon Health & Science University (OHSU) as described previously (Anderson et al. 2009). Twenty micrograms of active partially purified proteins post hydrophobic interaction and size exclusion chromatography FPLC (See Chapter 2) were also sent for MS/MS protein identification. The resulting protein identification data were pooled and closely looked at for Mn(II) oxidase and Mn(II) oxidation related proteins.

Results

Table C.1. MS/MS Protein Identification of concentrated medium. Active fractions were excised from Native PAGE gel in four independent preparations (Chapter 2).

Proteins identified from the concentrated medium	1	2	3	4
multicopper oxidase type 2 PputGB1_2665		X		X
hypothetical protein PputGB1_2552		X		
hypothetical protein PputGB1_2553		X		
hypothetical protein PputGB1_0186	X	X	X	X
integrase family protein	X			
glucose-methanol-choline oxidoreductase	X			
glutamine synthetase, type I	X			
succinyl-CoA synthetase subunit beta				X
NAD-glutamate dehydrogenase				X
DNA-directed RNA polymerase subunit beta'				X
catalase/peroxidase HPI PputGB1_2231				X
phosphate ABC transporter, periplasmic phosphate-binding protein				X
putative aminopeptidase 2			X	
GTP cyclohydrolase I				X
arginine deiminase				X

aldehyde dehydrogenase	X
inositol-5-monophosphate dehydrogenase	X
polynucleotide phosphorylase/polyadenylase	X
aspartate carbamoyltransferase catalytic subunit	X
F0F1 ATP synthase subunit alpha	X
hypothetical protein PputGB1_5111	X

Table C.2. MS/MS Protein Identification of loosely bound protein fraction. Active fractions were excised from Native PAGE gel (Chapter 2).

Proteins identified from loosely bound protein fraction
flagellin domain-containing protein
catalase/peroxidase HPI PputGB1_2231
phosphate ABC transporter, periplasmic phosphate-binding protein
cystine transporter subunit
outer membrane porin
extracellular solute-binding protein
N-acetyl-gamma-glutamyl-phosphate reductase
transketolase central region
hypothetical protein PputGB1_0806
hypothetical protein PputGB1_0807
outer membrane porin
extracellular solute-binding protein
carbohydrate-selective porin OprB
extracellular solute-binding protein
membrane protein involved in aromatic hydrocarbon degradation
extracellular solute-binding protein
aspartate-semialdehyde dehydrogenase
aminopeptidase N
OmpF family protein
outer membrane lipoprotein OprI
bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase
branched-chain amino acid aminotransferase
outer membrane autotransporter
alpha/beta hydrolase fold
dihydrolipoamide dehydrogenase
flagellin domain-containing protein
flagellar hook protein FlgE
cationic amino acid ABC transporter, periplasmic binding protein
hypothetical protein PputGB1_4051
outer membrane porin

extracellular ligand-binding receptor
extracellular solute-binding protein
extracellular ligand-binding receptor
azurin
hypothetical protein PputGB1_5030
extracellular solute-binding protein
hypothetical protein PputGB1_5366
phosphate binding protein
extracellular solute-binding protein
OmpF family protein

Table C.3. Pooled MS/MS Protein identification data of outer membrane fractions. Crude active fractions excised from Native PAGE gel in three independent preparations (Chapter 2).

Proteins identified from the outer membrane protein fractions	1	2	3
ornithine carbamoyltransferase	X	X	X
hypothetical protein PputGB1_0186	X	X	X
glutamine synthetase, type I	X	X	
flagellin domain-containing protein	X		
DNA-directed RNA polymerase subunit beta		X	
succinyl-CoA synthetase subunit beta		X	X
NAD-glutamate dehydrogenase		X	
DNA-directed RNA polymerase subunit beta'		X	
catalase/peroxidase HPI PputGB1_2231	X	X	
phosphate ABC transporter, periplasmic phosphate-binding protein	X		
putative aminopeptidase 2	X	X	
arginine deiminase	X		X
aldehyde dehydrogenase	X	X	
inositol-5-monophosphate dehydrogenase	X	X	
polynucleotide phosphorylase/polyadenylase	X	X	
aspartate carbamoyltransferase catalytic subunit	X	X	
F0F1 ATP synthase subunit alpha	X	X	
outer membrane porin		X	
N-acetyl-gamma-glutamyl-phosphate reductase	X		
transketolase central region	X		
carbohydrate-selective porin OprB		X	
extracellular solute-binding protein	X		
aspartate-semialdehyde dehydrogenase	X		
aminopeptidase N	X		
OmpF family protein	X	X	

outer membrane lipoprotein OprI		X	
bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	X		
branched-chain amino acid aminotransferase	X		
outer membrane autotransporter			
alpha/beta hydrolase fold	X		
dihydrolipoamide dehydrogenase	X		
flagellin domain-containing protein	X		
extracellular ligand-binding receptor	X		
azurin	X		
F0F1 ATP synthase subunit beta	X	X	
extracellular solute-binding protein	X	X	X
elongation factor G	X	X	
OmpF family protein	X	X	
pyruvate dehydrogenase subunit E1	X	X	
phosphopyruvate hydratase	X		
bacterioferritin		X	
phosphate uptake regulator, PhoU		X	
OmpA/MotB domain-containing protein		X	X
lactate/malate dehydrogenase	X		
LppC family lipoprotein		X	X
50S ribosomal protein L25/general stress protein Ctc		X	
elongation factor Ts	X		
alpha-ketoglutarate decarboxylase		X	
hypothetical protein PputGB1_3030	X	X	X
F0F1 ATP synthase subunit B		X	
peptidoglycan-associated lipoprotein		X	
phosphoserine aminotransferase	X		
pyruvate kinase		X	
arginine decarboxylase		X	
malate:quinone oxidoreductase		X	
glucose-6-phosphate 1-dehydrogenase	X	X	
lipoprotein		X	X
ornithine decarboxylase	X	X	
lipoprotein		X	X
hypothetical protein PputGB1_1040	X	X	
oligopeptidase A	X		
30S ribosomal protein S2		X	
trigger factor	X		
phosphoribosylaminoimidazole carboxylase ATPase subunit	X		
F0F1 ATP synthase subunit gamma	X	X	
signal peptidase I		X	X
methylmalonate-semialdehyde dehydrogenase	X	X	X
aromatic amino acid aminotransferase	X		

nucleoside-diphosphate kinase	X		
AsmA family protein		X	X
hypothetical protein PputGB1_3037		X	X
PQQ-dependent dehydrogenase glucose/quinate/shikimate family protein		X	X
4-aminobutyrate aminotransferase	X	X	
isocitrate dehydrogenase, NADP-dependent		X	
outer membrane protein assembly complex, YaeT protein		X	
succinate dehydrogenase flavoprotein subunit	X		
bacterioferritin		X	
thymidylate synthase			
DNA gyrase subunit A		X	
carbon starvation protein CstA		X	X
LytTR family two component transcriptional regulator		X	
PQQ-dependent dehydrogenase glucose/quinate/shikimate family protein			X
glutamate synthase subunit alpha	X	X	
sarcosine oxidase alpha subunit family protein		X	
3-oxoacyl-(acyl carrier protein) synthase III	X		
chaperonin GroEL	X	X	
dihydroorotase	X	X	
molecular chaperone DnaK	X		
30S ribosomal protein S4		X	
polyphosphate kinase		X	
beta-lactamase		X	X
saccharopine dehydrogenase	X		
peptidylprolyl isomerase FKBP-type		X	
acyl-CoA dehydrogenase domain-containing protein	X		
gamma-aminobutyraldehyde dehydrogenase	X		
lipoprotein			X
fructose-1,6-bisphosphate aldolase	X		
acetyl-CoA carboxylase biotin carboxylase subunit		X	
acetate permease			X
sulfatase		X	
aromatic amino acid aminotransferase	X		
carboxynorspermidine decarboxylase	X		
glycogen/starch/alpha-glucan phosphorylase		X	
TOBE domain-containing protein	X		
ThiJ/PfpI domain-containing protein	X		
NADH:flavin oxidoreductase/NADH oxidase	X		
redoxin domain-containing protein	X		
aspartyl-tRNA synthetase	X		
argininosuccinate synthase		X	
methyl-accepting chemotaxis sensory transducer with Cache sensor	X	X	X
hypothetical protein PputGB1_3484		X	

2-methylcitrate dehydratase		X	
oxidoreductase FAD/NAD(P)-binding subunit	X		
lipoprotein		X	
3-ketoacyl-(acyl-carrier-protein) reductase	X	X	
malate dehydrogenase	X		
deoxycytidine triphosphate deaminase	X		
agmatine deiminase	X		
serine hydroxymethyltransferase	X		
carbohydrate-selective porin OprB	X	X	
malate dehydrogenase	X		
30S ribosomal protein S11		X	
preprotein translocase subunit SecY			X
preprotein translocase subunit SecD		X	
thioredoxin			
type I phosphodiesterase/nucleotide pyrophosphatase		X	
3-isopropylmalate dehydrogenase	X		
bifunctional NADH:ubiquinone oxidoreductase subunit C/D	X	X	
Ferritin Dps family protein	X		
fumarylacetoacetase	X		
peptidase U62 modulator of DNA gyrase	X		
diaminopimelate decarboxylase	X		
peptidase S45 penicillin amidase		X	
S-adenosyl-L-homocysteine hydrolase	X		
trehalose synthase	X		
phosphate ABC transporter, ATPase subunit		X	
sarcosine oxidase gamma subunit family protein		X	
50S ribosomal protein L1		X	
acyl-CoA dehydrogenase domain-containing protein	X		
alpha-2-macroglobulin domain-containing protein	X	X	
carbamate kinase	X		
phosphoenolpyruvate carboxylase		X	
alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen		X	
cysteinyl-tRNA synthetase	X		
DoxX family protein			X
electron transfer flavoprotein alpha subunit	X		
electron transfer flavoprotein alpha/beta-subunit	X		
PhzF family phenazine biosynthesis protein	X		
chaperone protein DnaJ		X	
hypothetical protein PputGB1_4928			X
lipoprotein		X	
methyl-accepting chemotaxis sensory transducer		X	
transcription-repair coupling factor		X	
imidazole glycerol phosphate synthase subunit HisH	X		

zinc-dependent hydrolase		X
transcriptional regulator MvaT, P16 subunit	X	
aldehyde dehydrogenase	X	
NADH dehydrogenase subunit G	X	
aldehyde dehydrogenase	X	
alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	X	
aldehyde oxidase and xanthine dehydrogenase molybdopterin binding protein	X	
NADH dehydrogenase I subunit F	X	
dihydroxy-acid dehydratase	X	
alcohol dehydrogenase	X	
phosphogluconate dehydratase	X	
extracellular solute-binding protein	X	
succinate-semialdehyde dehydrogenase I	X	
glutamate--cysteine ligase	X	
glutamate--putrescine ligase	X	
dihydrolipoamide acetyltransferase	X	
aspartyl/glutamyl-tRNA amidotransferase subunit A	X	
phosphoenolpyruvate synthase	X	
dihydrolipoamide dehydrogenase	X	
membrane dipeptidase	X	
unknown	X	
betaine aldehyde dehydrogenase	X	
cystathionine gamma-lyase	X	
aspartyl/glutamyl-tRNA amidotransferase subunit B	X	
N-isopropylammelide isopropylaminohydrolase	X	
isochorismatase hydrolase	X	
acetyl-CoA acetyltransferase	X	
urocanate hydratase	X	
malate synthase G	X	
succinylarginine dihydrolase	X	
4-hydroxyphenylpyruvate dioxygenase	X	
pyruvate carboxylase subunit B	X	
amidohydrolase 3	X	
extracellular solute-binding protein	X	
peptidoglycan-binding LysM	X	
aconitate hydratase	X	
delta-aminolevulinic acid dehydratase	X	
dihydropyrimidinase	X	
catalase	X	
pyruvate dehydrogenase (acetyl-transferring)	X	
histidine ammonia-lyase	X	
methylmalonate-semialdehyde dehydrogenase	X	
OsmC family protein	X	

histidinol dehydrogenase	X
hypothetical protein PputGB1_2411	X
flagellar hook-associated 2 domain-containing protein	X
glycine dehydrogenase	X
formaldehyde dehydrogenase, glutathione-independent	X
hypothetical protein PputGB1_2241	X
malate:quinone oxidoreductase	X
glutathione synthetase	X
phosphonate ABC transporter, periplasmic phosphonate-binding protein	X
dihydrolipoamide acetyltransferase	X
bifunctional GMP synthase/glutamine amidotransferase protein	X
pyridoxal phosphate biosynthetic protein PdxJ	X
ATP phosphoribosyltransferase regulatory subunit	X
hypothetical protein PputGB1_1261	X
3-hydroxyisobutyrate dehydrogenase	X
peptidase M24	X
peptidase U62 modulator of DNA gyrase	X
dihydroorotase	X
ribonuclease PH	X
Hsp33-like chaperonin	X
phosphoribosylformylglycinamidine synthase	X
enoyl-CoA hydratase/isomerase	X
Dyp-type peroxidase family protein	X
amidase	X
oligopeptidase B	X
extracellular solute-binding protein	X
hypothetical protein PputGB1_4475	X
cytochrome c class I	X
histone deacetylase superfamily protein	X
acyl-CoA synthetase	X
extracellular solute-binding protein	X
propionyl-CoA carboxylase	X
hypothetical protein PputGB1_4999	X
soluble pyridine nucleotide transhydrogenase	X
short-chain dehydrogenase/reductase SDR	X
transketolase central region	X
peptidase M16 domain-containing protein	X
phosphoribosylamine--glycine ligase	X
hypothetical protein PputGB1_5084	X
carbamoyl-phosphate synthase L chain ATP-binding	X
isocitrate lyase	X
acyl-CoA dehydrogenase domain-containing protein	X
glucose-1-phosphate thymidylyltransferase	X

hypothetical protein PputGB1_4547	X
peptidase S16 lon domain-containing protein	X
putative aminotransferase	X
putative aminotransferase	X
keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase	X
flagellar hook-associated protein FlgK	X
glycogen debranching enzyme GlgX	X
carbamoyl phosphate synthase large subunit	X
hypothetical protein PputGB1_5422	X
indole-3-glycerol-phosphate synthase	X
xanthine dehydrogenase, small subunit	X
DNA topoisomerase I	X
ABC transporter, periplasmic binding protein	X
succinylglutamic semialdehyde dehydrogenase	X
homogentisate 1,2-dioxygenase	X
xanthine dehydrogenase, molybdopterin binding subunit	X
transaldolase B	X
flagellar hook-associated protein FlgL	X
gluconate 2-dehydrogenase acceptor subunit	X
acyl-CoA dehydrogenase domain-containing protein	X
ATP phosphoribosyltransferase catalytic subunit	X
cysteine synthase B	X
elongation factor G	X
aldehyde dehydrogenase	X
3-methyl-2-oxobutanoate dehydrogenase (2-methylpropanoyl-transferring)	X
3-hydroxybutyryl-CoA dehydrogenase	X
L-carnitine dehydratase/bile acid-inducible protein F	X
glutamate dehydrogenase	X
nicotinate-nucleotide pyrophosphorylase	X
O-acetylhomoserine aminocarboxypropyltransferase	X
2Fe-2S iron-sulfur cluster binding domain-containing protein	X
acetyl-CoA acetyltransferase	X
peptidase M16 domain-containing protein	X
hypothetical protein PputGB1_4098	X
glycerol kinase	X
ornithine carbamoyltransferase	X
hydroxydechloroatrazine ethylaminohydrolase	X
aminotransferase AlaT	X
heavy metal translocating P-type ATPase	X
Serine O-acetyltransferase	X
glyceraldehyde-3-phosphate dehydrogenase, type I	X
alkylhydroperoxidase	X
glutamate--putrescine ligase	X

dienelactone hydrolase	X
Beta-agarase	X
poly(hydroxyalkanoate) granule-associated protein	X
allantoate amidohydrolase	X
DNA polymerase III subunit beta	X
RND family efflux transporter MFP subunit	X
NADH:flavin oxidoreductase/NADH oxidase	X
fumarate hydratase	X
hypothetical protein PputGB1_1452	X
phospho-2-dehydro-3-heoxyheptonate aldolase	X
2-methylisocitrate lyase	X
acetolactate synthase	X
GMC oxidoreductase	X
dihydrodipicolinate synthetase	X
phosphoheptose isomerase	X
acyl-CoA thioesterase II	X
5,10-methylenetetrahydrofolate reductase	X
formyltetrahydrofolate deformylase	X
bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	X
pyrroloquinoline quinone biosynthesis protein PqqB	X
peptidyl-prolyl cis-trans isomerase cyclophilin type	X
succinylglutamate desuccinylase/aspartoacylase	X
L-carnitine dehydratase/bile acid-inducible protein F	X
carbamoyl phosphate synthase small subunit	X
XRE family transcriptional regulator	X
peptide synthase	X
dihydroorotase	X

Table C.4. MS/MS Protein Identification. Active outer membrane fractions were partially purified using hydrophobic interaction and size exclusion FPLC (Chapter 2).

Proteins identified post hydrophobic interaction and size exclusion FPLC
peptidase M24
extracellular solute-binding protein
histone family protein DNA-binding protein
PpiC-type peptidyl-prolyl cis-trans isomerase
2-methylisocitrate lyase
AcnD-accessory protein PrpF
bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase
hypothetical protein PputGB1_1973
B12-dependent methionine synthase

short-chain dehydrogenase/reductase SDR
hypothetical protein PputGB1_2156
catalase/peroxidase HPI PputGB1_2231
luciferase family protein
outer membrane porin
aldehyde oxidase and xanthine dehydrogenase molybdopterin binding protein
2Fe-2S iron-sulfur cluster binding domain-containing protein
5-dehydro-4-deoxyglucarate dehydratase
aromatic amino acid aminotransferase
hypothetical protein PputGB1_2396
branched-chain amino acid aminotransferase
hypothetical protein PputGB1_2475
hypothetical protein PputGB1_2552
hypothetical protein PputGB1_2553
gluconate 2-dehydrogenase acceptor subunit
gluconate 2-dehydrogenase acceptor subunit
beta-ketoadipyl CoA thiolase
N-isopropylammelide isopropylaminohydrolase
multicopper oxidase type 2
3-oxoacid CoA-transferase, B subunit
amidase
carboxynorspermidine decarboxylase
saccharopine dehydrogenase
delta-aminolevulinic acid dehydratase
glutaminyl-tRNA synthetase
hypothetical protein PputGB1_3029
hypothetical protein PputGB1_3030
hypothetical protein PputGB1_3037
phosphate ABC transporter, periplasmic phosphate-binding protein
prophage antirepressor
glutathione reductase
UTP-glucose-1-phosphate uridylyltransferase
alcohol dehydrogenase
isocitrate dehydrogenase, NADP-dependent
cupin 4 family protein
alpha/beta hydrolase fold
allantoate amidohydrolase
6-phosphogluconate dehydrogenase-like protein
glycogen debranching enzyme GlgX
acyl-CoA dehydrogenase domain-containing protein
elongation factor G
isocitrate lyase
NADH dehydrogenase I subunit F

NADH dehydrogenase subunit G
NADPH-dependent FMN reductase
succinyl-CoA synthetase subunit beta
dihydrolipoamide dehydrogenase
alpha-ketoglutarate decarboxylase
succinate dehydrogenase flavoprotein subunit
aldehyde oxidase and xanthine dehydrogenase molybdopterin binding protein
xanthine dehydrogenase, small subunit
xanthine dehydrogenase, molybdopterin binding subunit
urate catabolism protein
flagellin domain-containing protein
flagellar hook-associated protein FlgK
dihydrolipoamide dehydrogenase
dihydrodipicolinate synthetase
hypothetical protein PputGB1_3981
succinylarginine dihydrolase
bifunctional N-succinyldiaminopimelate-aminotransferase/acetylornithine transaminase protein
acetyl-CoA synthetase
3-oxoacyl-(acyl carrier protein) synthase III
parallel beta-helix repeat-containing protein
cysteine synthase A
hypothetical protein PputGB1_4098
cystathionine gamma-lyase
Ferritin Dps family protein
ribonucleotide-diphosphate reductase subunit alpha
3-hydroxyisobutyrate dehydrogenase
FAD dependent oxidoreductase
Beta-agarase
succinylglutamate desuccinylase/aspartoacylase
methionyl-tRNA synthetase
argininosuccinate synthase
dihydroorotase
alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen
ornithine carbamoyltransferase
glycerol kinase
pyridoxal phosphate biosynthetic protein PdxJ
glycoside hydrolase family 3 protein
tartrate/fumarate subfamily Fe-S type hydro-lyase alpha subunit
aldo/keto reductase
hypothetical protein PputGB1_4547
extracellular solute-binding protein
fumarylacetoacetase
homogentisate 1,2-dioxygenase

trans-2-enoyl-CoA reductase
3-hydroxyisobutyrate dehydrogenase
methylmalonate-semialdehyde dehydrogenase
polynucleotide phosphorylase/polyadenylase
translation initiation factor IF-2
carbamoyl phosphate synthase large subunit
carbamoyl phosphate synthase small subunit
molecular chaperone DnaK
glutamate-1-semialdehyde aminotransferase
phosphoribosylamine--glycine ligase
NAD synthetase
HflK protein
transketolase
S-adenosyl-L-homocysteine hydrolase
adenosylmethionine--8-amino-7-oxononanoate transaminase
glutathione synthetase
ubiquinone/menaquinone biosynthesis methyltransferase
extracellular solute-binding protein
proline iminopeptidase
histidine ammonia-lyase
urocanate hydratase
glycogen/starch/alpha-glucan phosphorylase
glutamine synthetase, type I
preprotein translocase subunit SecB
phosphoglyceromutase
glutamate synthase subunit alpha
malate dehydrogenase
arginyl-tRNA synthetase
peptidase M16 domain-containing protein
dihydroxy-acid dehydratase
putative aminotransferase
glycine dehydrogenase
peptidase M24
amidohydrolase 3
aldehyde dehydrogenase
aldehyde dehydrogenase
ribonuclease PH
phosphoribosylaminoimidazole carboxylase ATPase subunit
pyruvate carboxylase subunit B
acetyl-CoA carboxylase
cobalamin synthesis protein P47K
dihydrolipoamide dehydrogenase
UDP-N-acetylglucosamine pyrophosphorylase

F0F1 ATP synthase subunit beta
F0F1 ATP synthase subunit alpha

Table C.5. MS/MS Protein Identification, Deduced Amino Acid Sequence, and Actual Peptide Mass of notable proteins.

Protein Identified	Deduced Amino Acid Sequence	Actual Mass
Multi-copper oxidase, type 2 PputGB1_2665	(K)LYFVNLEEHR(T)	1,318.61
	(R)HPVGSPAEGRPPGK(G)	1,385.81
	(R)HPVGSPAEGRPPGK(G)	1,386.12
	(K)MLPLPIDR(N)	953.0292
	(K)GPSGSNVSYAR(V)	1,094.40
	(K)AQIEmFLNR(H)	1,137.10
	(K)AQIEmFLNR(H)	1,137.57
	(R)DYDVNLVIADK(A)	1,264.45
	(R)GNEALQDGVNLR(F)	1,285.29
	(R)SDGTDTTPWTIK(T)	1,320.37
	(R)IGPDADSSEEVEMAIR(F)	1,717.09
	(R)IGPDADSSEEVEMAIR(F)	1,718.40
	(K)LSVTTTGTGPATPATVTVK(S)	1,801.37
	(R)SDGTDTTPWTIKTDGGFGYSmDPRR(I)	2,778.23
Hypothetical protein PputGB1_2552	(K)EASLcMR(N)	866.3992
	(R)AQTPVTLER(A)	1,013.80
	(R)PAAETFAVTAATVTAR(S)	1,576.33
	(R)DSLGALSTPGTVTVNVSPR(P)	1,870.50
	(K)DGLNQSLELcQSR(A)	1,518.43
	(R)AGVYTITHPYGVETVNVTSPGR(R)	2,318.26
	(R)PAAETFAVTAATVTAR(S)	1,575.91
	(R)LAVSNSTTIIPTAAPTATITTSPGTTR(T)	2,644.42
	(R)SSDEVTIPDMLAQGYGR(L)	1,838.36
	(R)DSLGALSTPGTVTVNVSPR(P)	1,870.38
	(R)VTTTTGVQTLGTATVPVNGR(W)	1,973.20
	(R)VTTTTGVQTLGTATVPVNGR(W)	1,973.90
Hypothetical protein PputGB1_2553	(K)GDVGPFLR(S)	859.7982
	(R)TLDLcLTK(A)	963.0282
	(R)TELFAISGK(L)	964.8642
	(R)IEGPGGIDLR(T)	1,025.65
	(R)IEGPGGIDLR(T)	1,026.19
	(R)FIGDPNLEER(V)	1,189.36

	(R)SVNGPYTEGNER(F)	1,322.66
	(R)VTGSPFNTNFVR(I)	1,338.18
	(K)TLSmTVSPIPPAK(V)	1,359.48
	(K)LSTVALPTPLMPQR(S)	1,406.91
	(R)VDVPTAGTYVVTHPYGVEVFDVPAAGR(R)	2,816.40
Catalase-peroxidase HPI PputGB1_2231	(K)SPAGAHQWRPK(E)	1,234.33
	(R)VDLVFGSHAQLR(A)	1,341.88
	(R)VDLVFGSHAQLR(A)	1,341.93
	(K)HGEEQNRDLSAER(N)	1,540.35
	(R)FKDNPDQLADAFAR(A)	1,607.26
	(R)ALSEVYGSSDGADKFVR(D)	1,800.72
	(R)FDPIYEPIAR(R)	1,220.77
	(K)SPAGAHQWRPK(E)	1,232.93
	(K)LGVFTDKPGTLSNDFFR(N)	1,913.54
	(K)LGVFTDKPGTLSNDFFR(N)	1,913.99
	(K)AQAPGQGDLVAEPAK(H)	1,450.93
	(K)AQAPGQGDLVAEPAK(H)	1,451.22
	(K)AQAPGQGDLVAEPAK(H)	1,451.39
	(K)ADPSTIGEQDIAALK(A)	1,528.31
	(K)HGEEQNRDLSAER(N)	1,541.99
	(K)ISLADLIVLAGTAAVEK(A)	1,683.70
	(R)NLLDMSVEWKPTSADNETFEGR(D)	2,538.97
	(R)ALSEVYGSSDGADKFVR(D)	1,800.51
	(K)THGAGPADNVGPEPEAAGLELQGLGWANK(F)	2,856.11
	(K)THGAGPADNVGPEPEAAGLELQGLGWANK(F)	2,856.29
	(K)LGVFTDKPGTLSNDFFR(N)	1,913.82
	(R)ADVWEPDEDVYWGSEK(V)	1,925.12
	(K)GGDAITSGLEVIWTSTPTK(W)	1,933.33
	(R)VDASEAQTDVESFAVLEPLADGFR(N)	2,566.33
	(R)VDASEAQTDVESFAVLEPLADGFR(N)	2,567.61
Hypothetical protein PputGB1_0186	(R)TFENLELGGAAK(V)	1,249.25
	(R)TFENLELGGAAK(V)	1,250.81
	(K)HAALTFTLDDGK(T)	1,287.20
	(K)VSVTDTTDEVIAK(L)	1,376.91
	(K)VDVTDTTDEVVAK(L)	1,390.63
	(K)VDVTDTTDEVIAK(L)	1,405.32
	(K)YEHAAQGDDVYLDSGEISLGIK(S)	2,380.08
	(K)SIATVEGADVDKFEQ(L)	1,609.87
	(K)SIATVEGVDVDKFEK(L)	1,636.76
	(K)TTVTDEPGTPGNEGDLVK(V)	1,830.69
	(K)TVITVPANGTTGSVTVAAPDNVYVGANDPIVK(S)	3,141.71

	(K)TVITVPANGTTGSVTVIAPDNVYTGTNDPVVK(S)	3,201.45
	(K)TTVTDEPGTPGNPGGTNEGDLVK(V)	2,256.72
	(K)LTATPSVTEGGEITYTITLTNK(D)	2,310.20

Biographical Sketch

John Reino Buzzo was born in Ontonagon, Michigan on March 29th, 1986. He earned his Bachelor of Science Degree in Biological Sciences from Michigan Technological University, in 2008. While at MTU, he did undergraduate research under the direction of Dr. Heather Youngs studying peptides that mimic carbohydrate substrates of cellulase. He also worked as a laboratory assistant under the direction of Dr. Victor Busov and Dr. Jiqing Gou studying *Populus* genetics, particularly involving lateral root development.

John moved from Michigan to Portland, Oregon in the summer of 2008 to begin his graduate work in Dr. Bradley Tebo's laboratory at Oregon Health & Science University, which is presented in this thesis.