Genetic Analysis Reveals Complex Mn(II) Oxidation Regulation in *Pseudomonas putida* GB-1

Ai Yamaguchi
B.S., University of California, San Diego, 2006

Presented to the Division of Environmental and Biomolecular Systems within
The Department of Science & Engineering
and the Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirements for the degree of
Master of Science
in
Biochemistry and Molecular Biology

July 2009

Department of Science & Engineering
School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is certify that the Master's thesis of

Ai Yamaguchi

has been approved

Dr. Bradley Tebo
Thesis Research Advisor
Professor

Dr. Jorge Crosa
Professor
Department of Molecular Microbiology

Dr. Kati Geszvain
Senior Research Associate

Acknowledgment

It was my greatest honor to be working under my advisor Dr. Brad Tebo with his projects that provided me an opportunity to experience the excitement of the science. He gave me little restriction with experiments, and a huge support scientifically, psychologically, and financially. Also, this thesis would not have been possible without extensive help and encouragement from Dr. Kati Geszvain. She has taught me numerous molecular biology techniques, sometimes with her hand-made slides to make sure I understand the bases of the techniques. She has also spent her valuable time to read and correct my thesis multiple times.

I would also like to thank everyone in the Haygood-Tebo lab for their generous support and providing positive experiences inside and outside of the lab. Especially, Carolyn Sheehan for helping me get oriented to the lab and be supportive all through out my graduate study. Dr. Craig Anderson has introduced me to the basic experimental techniques used in the lab, and helped me design my first experiment.

So many faculties and staffs have been very important for my graduate study at OGI. Nancy Christie has helped me with administrative matters in numerous occasions that without her I wouldn't have been able to get though my master's study.

I would not be able to finish this section without acknowledging my family. My mother was there for me whenever I needed someone to talk to. My father gave me an opportunity to study in the United States, and supported me financially for so many years. My parents have given me so much freedom, but also gave me many valuable advices though out my school years. My grandfather has also helped me financially, and was always excited to hear my work. I also need to thank my sister and brother for been very patient with me. Lastly, but not least, I would like to thank Alvaro for keeping up with my frustration and giving me encouragements.

Table of Contents

List of Tables

List of Figures

Abstract

Genetic Analysis Reveals Complex Mn(II) Oxidation Regulation in *Pseudomonas putida* GB-1

Ai Yamaguchi, B.S.

Master of Science
Division of Environmental and Molecular Systems within
The Department of Science & Engineering
And the Oregon Health & Science University
School of Medicine
July 2009

Thesis Advisor: Bradley Tebo

Manganese is the second most abundant transition metal element in the Earth's crust. Many different bacteria produce enzymes that catalyze Mn(II)-oxidation, but the biochemical pathway of this process is still not fully understood. Therefore, to identify genes responsible for Mn(II) oxidation, random transposon mutagenesis was performed on one of the known Mn(II)-oxidizing bacteria, *Pseudomonas putida* GB-1. Several mutants exhibiting decreased Mn(II)-oxidation were identified and the gene(s) disrupted in these mutants was identified by sequencing out from the site of transposon insertion. Unexpectedly, however, there were some mutants that started oxidizing Mn(II) earlier than the wild-type, and all those mutants had the transposon insertion in motility-related genes. When the motility on low agar concentration plates was tested, some of them were completely non-motile, and the rest were very slow swimmers. Complementation with plasmid-borne copies of the motility genes further confirmed a relationship between

motility and Mn(II) oxidation. This is the first time that a relationship between Mn(II) oxidation and motility of the bacterium and/or flagella synthesis has been observed. In addition, those mutants that had faster oxidation rate now had a slower than wild-type oxidation rate on the motility agar. Further investigation of the effect of growth conditions on Mn(II) oxidation by different mutant strains revealed a requirement for different genes depending on the growth condition, such as growth substrate and temperature. These results demonstrate the complexity of the regulation of Mn(II) oxidation in *P. putida* GB-1.

Chapter 1

Introduction to Manganese Oxidation in the Environment

1.1 Significance of Manganese in the Environment

Manganese is the second most abundant transition metal in the Earth's crust, only after iron. Most of the Mn in the environment is found in the Mn(II) or Mn(IV) state because Mn(III) is thermodynamically unstable and only persists in certain soluble organic complexes (Kostka et al. 1995, Klewicki & Morgan 1998, 1999), or as insoluble oxides, oxyhydroxides, and hydroxides often together with Mn(IV) (Tebo et. al., 2004). While Mn(III, IV) oxides are solid, Mn(II) occurs as a cation (Mn^{2+}) in solution and can exist at up to millimolar concentrations in natural waters (Tebo et. at., 2004).

Manganese (III, IV) oxides are very reactive and play an important role in redox chemistry in the environment. They can oxidize different types of organic and inorganic compounds, and control the distribution and availability of many elements in nature. In addition, due to their characteristic structure, Mn(III, IV) oxides can adsorb and sequester heavy metals (e.g., Cu, Co, Cd, Zn, Ni, and Pb), or promote the degradation of a wide array of complex organic compounds (e.g., humic substances, PCBs, phenols and chlorinated phenols, and chlorinated anilines [Stone & Morgan 1984a,b; Stone 1987; Ulrich & Stone 1989]). Because of these characteristics, Mn(III,IV) oxides are useful in bioremediation (Tebo et. al., 2004). Mn oxide minerals have been used as a filtration medium to remove Fe(II), Mn(II), arsenic and hydrogen sulfide from drinking water

(Casale et. al. 2002). Mn(IV) oxides also can stabilize Pb in soil to protect plants from taking up Pb (Hettiarachchi et. al., 2000). Many toxic metals, like Pb, becomes associated or incorporated with Mn(IV) oxide minerals and become less mobile, but there are some toxic metals that are mobilized upon oxidation by Mn(IV) oxide, such as Cr and U (Tebo et. al., 2004), which makes it important to be able to monitor and predict Mn concentrations in the environment.

1.2 Microbial Manganese Oxidation

Mn(IV) oxides are abundant in both marine and terrestrial environments, and so are the bacteria that catalyze Mn(II) oxidation to form those Mn(IV) oxides. Many different species of bacteria that oxidize Mn(II) have been isolated from different environments, such as *Pseudomonas putida* GB-1 from fresh water in Green Bay off of Lake Michigan (JGI, 2009), *Aurantimonas* sp. strain SI85-9A1 from the oxic/anoxic interface of a stratified fjord (Dick et. al., 2008), and *Bacillus* sp. strain SG-1 (Nealson & Ford, 1980) and *Erythrobacter* sp. strain SD-21 from oceanic surface sediments (Francis et. al., 2001). This wide distribution of Mn(II)-oxidizing bacteria implies a physiological benefit of the Mn(II) oxidation reaction for these bacteria, although the benefit is still unclear. Some of the proposed biological functions are: protection from toxic heavy metals, reactive oxygen species, or UV light (Ghiorse 1984); storage of an electron acceptor for later use in anaerobic respiration (Tebo, 1983); or breakdown of refractory organic matter into utilizable substrates (Sunda & Kieber 1994). There has been some evidence suggesting that bacteria can harness and utilize the energy from the oxidation reaction of Mn(II) to Mn(III,IV) (Ehrlich 1983, Kepkay & Nealson 1987, Ehrlich & Salerno 1990) because it is thermodynamically favorable, but this still needs further investigation.

Biological Mn-oxidation can increase the rate of Mn biomineralization by orders of magnitude faster than either abiotic catalysis on mineral surfaces or homogeneous oxygenation in aqueous solution, where oxidation of Mn is thermodynamically favorable but kinetically slow (Tebo et. al., 2004). As previously mentioned, the Mn redox cycle plays an important role in chemical cycling; therefore, it is important to understand the

physiology and biochemistry of this process by microorganisms since they speed up the Mn redox cycle drastically. As a result of many studies, multiple genes have been found to be important in Mn oxidation, and the proteins encoded by those genes are: Cytochrome *c*, multi-copper oxidases (MCO), heme peroxidase, and general secretory proteins.

Cytochrome *c*

Random transposon mutagenesis of the aerobic, fresh-water bacteria *Pseudomonas putida* MnB1 and GB-1 created multiple mutants that are deficient in Mn(II) oxidation. One of the major transposon insertion sites that appeared in multiple mutants was in the cytochrome *c* maturation (*ccm*) operon. In *P. putida* MnB1, mutations in *ccmF, ccmA,* and *ccmE* that are crucial for the assembly of mature cytochrome *c* abolished cytochrome *c* production and Mn(II) oxidation (Caspi et. al., 1998). Mutation in the *ccmF* gene in *P. putida* GB-1 also showed the same phenotype (de Vrind et. al., 1998).

It is not clear how the *ccm* operon is involved in Mn(II) oxidation. One possibility is that cytochrome *c* is involved in electron transfer from Mn(II) to oxygen, possibly as an intermediate in an electron transport chain involving a terminal cytochrome *c* oxidase, so the complete cytochrome *c* structure is necessary (de Vrind et. al., 1998). Another possibility is that the cytochrome *c* is a part of Mn(II)-oxidizing complex (de Vrind et. al., 1998).

Multi-Copper Oxidase

The activation of dioxygen is an important reaction in biological systems. Multi-copper oxidases (MCO) are a family of enzymes that use multiple Cu atoms as cofactors in reducing O_2 in a four-electron reduction to H_2O while coupling one-electron oxidation of a reducing substrate (Solomon, 1996). MCOs have a wide range of cellular functionality, ranging from trace metal uptake and homeostasis to lignin degradation and antibiotic biosynthesis.

A relationship between MCO and Mn(II) oxidation was first speculated from mutant strains of a spore-forming marine bacterium *Bacillus* sp. SG-1 (van Waasbergen et. al., 1996). There are many *Bacillus* species known to oxidize Mn(II) as metabolically dormant spores using an Mn(II)-oxidizing enzyme located in the outermost layer of the spore coat (Rosson & Nealson, 1982). The Mnx region, which is assigned due to its involvement in Mn(II) oxidation, was first discovered by transposon mutagenesis (Van Waasbergen et. al., 1993). After further study, Van Waasbergen et. al.(1996) suggested one of the proteins, MnxG, is similar to an MCO according to the amino acid sequence, and that it may be causing Mn(II) oxidation because copper at low concentration enhanced Mn(II) oxidation. After further investigation with another Mn(II)-oxidizing *Bacillus* strain PL-12 using tandem mass spectrometric (MS/MS) analysis of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) purified Mn(II)-oxidase, it was confirmed that MnxG is the major component of the Mn(II)-oxidase (Dick et. al., 2008)

MCO homologues have been found in other Mn(II)-oxidizing bacteria and they may also play an important role in Mn(II) oxidation. *Leptothrix discophora* strain SS-1, a freshwater Proteobacterium, secretes an Mn(II)-oxidizing factor into media (Boogerd and de Vrind, 1987). A MCO homolog, *mofA*, from this strain seems to encode for a structural component of the oxidizing factor (Corstjens et. al., 1997). Results from transposon mutagenesis also suggested an MCO homolog, *cumA*, from *P. putida* GB-1 is involved in Mn(II) oxidation (Brouwers et. al., 1999). However, a Δ*cumA* mutant did not lose the Mn(II) oxidation phenotype, and also the transposon mutant isolated by Brouwers et. al. (1999) possessed another spontaneous mutation elsewhere in the genomic DNA, suggesting *cumA* may not be involved in Mn(II)-oxidation in *P. putida* GB-1 (K. Geszvain, personal communication).

It is also important to mention that none of the MCOs alone have been able to catalyze the direct Mn(II) oxidation. Therefore, additional factors may be necessary for the complete oxidation of the Mn(II). For example, studies with *Erythrobacter* sp. strain SD-21, a marine aerobic phototrophic bacterium, suggested involvement of a quinoprotein in Mn(II) oxidation (Johnson & Tebo, 2008).

Heme Peroxidases

Recently, Ca^{2+} binding heme peroxidase, MopA (for Mn(II)-oxidizing peroxidase), was identified as the enzyme that catalyzes Mn(II) oxidation in *Aurantimonas manganoxydans* strain SI85-9A1 and *Erythrobacter* sp. strain SD-21 (Anderson et. al., 2009). Mn(II) oxidase from *A. manganoxydans* strain SI85-9A1 was isolated using protein chromatography, and using native polyacrylamide gel electrophoresis in-gel activity assays from *Erythrobacter* sp. strain SD-21. In fungus, heme-containing manganese peroxidases (MnPs) have already been known to oxidize Mn(II) (Thurston, 1994, Camarero et. al., 2000, Palma et. al., 2000). During the catalytic cycle, H_2O_2 oxidizes the active center of MnPs. Then, two successive oxidations of Mn(II) to Mn(III) occur during the reduction of the active center (Glenn et. al., 1986, Wariishi et. al., 1992). In some cases, MnPs and MCOs cooperate; MCOs catalyze Mn(II) oxidation and produce H_2O_2, which is necessary for the MnPs to oxidize additional Mn(II) (Schlosser & Höfer, 2002). This raises a possibility that bacteria may also utilize the Mop-MCO system to oxidize Mn(II). An attempt to identify Mop as the Mn(II) oxidase in *P. putida* GB-1 has been made, but it seems that Mop is not the Mn(II) oxidase in this strain because the inframe deletion of *mop* did not affect the Mn(II) oxidation activity (K. Geszvain, personal communication).

General Secretory Proteins

In most of the Mn(II)-oxidizing bacteria, the oxidation reaction occurs extracellularly, e. g. the sheath of *Leptothrix discophora* SP-6 (Emerson and Ghiorse, 1992), the exosporium of *Bacillus* spores (Rosson & Nealson, 1982), or the outer membrane of *P. putida* (Okazaki et. al., 1997) and *Erythrobacter* sp. strain SD-21 (Francis et. al., 2001). Secretion mutants that cannot secrete Mn(II) oxidase and thus accumulate the oxidase intracellulary were discovered by transposon mutagenesis (de Vrind *et. al*., 1998). These mutants had transposon insertions in *xcpA* and *xcpX*, which are the homologues of *gsp* (general secretory pathway) genes, and further study indicated that secretion of the Mn(II)-oxidizing factor depends on an Xcp-related transport system with a different operon structure than the regular Xcp machinery (de Vrind et. al., 2003).

1.3 Purpose of the thesis

That bacteria facilitate Mn(II) oxidation has been known for many years, and many efforts have been made to elucidate the physiological benefits and biochemical pathways of bacterial Mn(II) oxidation. We now know some genes necessary for the Mn(II) oxidation or that are the major components for Mn(II) oxidase. However, there are still many things to be done to fully understand this phenomenon. To discover genes responsible for constructing Mn(II) oxidase and genes regulating Mn(II) oxidation, a genetic approach was used. In chapter two, random transposon mutagenesis was used to screen for loss of the Mn(II) oxidation phenotype. None of the mutants lost the Mn(II) oxidation function completely, but some mutants were severely defective in Mn(II) oxidation. All of the insertion sites were not previously identified, and were diverse. During the screening, some mutants were selected due to their increased Mn(II) oxidation ability. Interestingly, all of the increased Mn(II)-oxidizing mutants had insertions in motility related genes. Chapter three will discuss the possibility of complex regulation between motility and Mn(II) oxidation.

While characterizing the IMOs in effort to understand the relationships between motility and Mn(II) oxidation, I discovered IMOs are defective in Mn(II) oxidation on motility agar. This made me ask if DMOs also alter their Mn(II) oxidation phenotype in different growth conditions. In addition, I have observed stronger attachment of cells on hard agar with IMOs, so I decided to measure the biofilm formation ability of the different mutants. All of these basic characterizations of mutants are reported in Chapter 4.

Two additional experiments were conducted with *P. putida* GB-1, which are described in Appendix B and C. In Appendix B, IMO mutants were tested whether the decreased Mn(II) oxidation on motility agar was due to diffusion of a Mn(II)-oxidizing enzyme. In Appendix C, possibility of chemotactic reaction towards Mn(II) was tested with the wild-type.

I have also included an experiment that was intended to show the relationship between Mn(II) oxidation and extracellular polysaccharide production of *Erythrobacter* sp. strain SD-21 in Appendix A.

1.4 References

Anderson CR, Johnson HA, Caputo N, Davis RE, Torpey JW, and Tebo BM. 2009. Mn(II) oxidation is catalyzed by heme peroxidases in "*Aurantimonas manganoxydans*" strain SI85-9A1 and *Erythrobacter* sp. strain SD-21. *Appl. Environ. Microbiol.* 75(12):4130-38

Brouwers GJ, de Vrind JPM, Corstjens PLAM, Cornelis P, Baysse C, and de Vrind-deJong EW. 1999. *cumA*, a gene encoding a multicopper oxidase, is involved in Mn^{2+} oxidation in *Pseudomonas putida* GB-1. *Appl. Environ. Microbiol.* 65(4):1762-68

Booger FC, and de Vrind JPM. 1987. Manganese oxidation by *Leptothrix discophora. J. Bacteriol.* 169(2):489-94

Camarero S, Ruiz-Duenas FJ, Sarkar S, Martinez MJ, and Martinez AT. 2000. The cloning of a new peroxidase found in lignocelluloses cultures of *Pleurotus eryngii* and sequence comparison with other fungal peroxidases. *FEMS Microbiol*. *Lett*. 191:37-43

Caspi R, Tebo BM, and Haygood MG. 1998. *c*-type cytochromes and manganese oxidation in *Pseudomonas putida* MnB1. *Appl. Environ. Microbiol.* 64(10):3549-55

Corstjens PLAM, de Vrind JPM, Goosen T, and de Vrind-deJong EW. 1997. Identification and molecular analysis of the *Leptothrix discophora* SS-1 *mof*A gene, a gene putatively encoding a manganese oxidizing protein with copper domains. *Geomicrobiol. J.* 14:91–108.

de Vrind JPM, Brouwers GJ, Corstjens PLAM, Dulk J, and de Vrind-de Jong EW. 1998. The cytochrome *c* maturation operon is involved in manganese oxidation in *Pseudomonas putida* GB-1. *Appl. Environ. Microbiol.* 64(10):3556-62

de Vrind J, de Groot A, Brouwers GJ, Tommassen J, and de Vrind-de Jong E. 2003. Identification of a novel Gsp-related pathway required for secretion of the manganese-oxidizing factor of *Pseudomonas putida* strain GB-1. *Mol. Microbiol.* 47(4):993-1006

Dick GJ, Podell S, Johnson HA, Rivera YE, Bernier RL, McCarthy JK, Torpey JW, Clement BG, Gaasterland T, and Tebo BM. 2008. Genomic insights into Mn(II) oxidation by the marine alphaproteobacterium *Aurantimonas* sp. strain SI85-9A1. *Appl. Environ. Microbiol.* 74(9):2646-58

Dick GJ, Torpey JW, Beveridge TJ, and Tebo BM. 2008. Direct identification of a bacterial manganese(II) oxidase, the multicopper oxidase MnxG, from spores of several different marine *Bacillus* species. *Appl. Environ. Microbiol.* 74(5):1527-34

Ehrlich HL. 1983. Manganese oxidizing bacteria from a hydrothermally active area on the Galapagos Rift. *Ecol. Bull.* 35:357–66

Ehrlich HL, and Salerno JC. 1990. Energy coupling in Mn^{2+} oxidation by a marine bacterium. *Arch. Microbiol.* 154:12–17

Emerson D, and Ghiorse WC. 1992. Isolation, cultural maintenance, and taxonomy of a sheath-forming atrain of *Leptothrix discophora* and characterization of manganese-oxidizing activity associated with the sheath. *Appl. Environ. Microbiol.* 58(12):4001-4010

Francis CA, Edgie-Mark CO, and Tebo BM. 2001. Enzymatic manganese(II) Oxidation by a Marine α-Proteobacterium. *Appl. Environ. Microbiol.* 67(9):4024-29

Ghiorse WC. 1984. Biology of iron- and manganese-depositing bacteria. *Annu. Rev. Microbiol.* 38:515–50

Glenn JK, Akileswaran L, and Gold MH. 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.* 251:688-696

Hettiarachchi GM, Pierzynski GM, and Ransom MD. 2000. In situ stabilization of soil lead using phosphorus and manganese oxide. *Environ. Sci. Technol.* 34:4614–19

Johnson HA, and Tebo BM. 2008. In vitro studies indicate a quinine is involved in bacterial Mn(II) oxidation. *Arch. Microbiol.* 189:59-69

Joint Genome Institute, U. S. Department of Energy. *Pseudomonas putida GB-1finished genome*. < http://genome.jgi-psf.org/finished_microbes/psepg/psepg.home.html>

Kepkay P, and Nealson KH. 1987. Growth of a manganese oxidizing *Pseudomonas* sp. in continuous culture. *Arch. Microbiol.* 148:63–67

Klewicki JK, and Morgan JJ. 1998. Kinetic behavior of Mn(III) complexes of pyrophosphate, EDTA, and citrate. *Environ. Sci. Technol.* 32:2916–22

Klewicki JK, and Morgan JJ. 1999. Dissolution of β-MnOOH particles by ligands: pyrophosphate, ethylenediaminetetraacetate, and citrate. *Geochim. Cosmochim. Acta* 63:3017–24

Kostaka JE, Luther III GW, and Nealson KH. 1995. Chemical and biological reduction of Mn(III)-pyrophosphate complexes—potential importance of dissolved Mn(III) as an environmental oxidant. *Geochim. Cosmochim. Acta* 59:885-94

Nealson KH, and Ford J. 1980. Surface enhancement of bacterial manganese oxidation: implications for aquatic environments. *Geomicrobiol. J.* 2:21-37.

Okazaki M, Sugita T, Shimizu M, Ohode Y, Iwamoto K, de Vrind-de Jong EW, de Vrind JPM, and Corstjens PLAM. 1997. Partial purification and characterization of manganese-oxidizing factors of *Pseudomonas fluorescens* GB-1. *Appl. Environ. Microbiol.* 63**:** 4793–4799

Palma C, Martinez AT, Lema JM, and Martinez MJ. 2000. Different fungal manganese-oxidizing peroxidases: a comparison between *Bjerkandera* se. and *Phanerochaete chrysosporium*. *J. Biotechnol*. 77:235-245

Rosson RA, and Nealson KH. 1982. Manganese binding and oxidation by spores of a marine bacillus. *J. Bacteriol.*151(2):1027-34

Schlosser D, and Höfer C. 2002 Laccase-catalyzed oxidation of Mn^{2+} in the presence of natural Mn^{3+} chelators as a novel source of extracellular H_2O_2 production and its impact on manganese peroxidase. *Appl. Environ. Microbiol*. 68:3514-3521

Solomon EI, Sundaram UM, and Machonkin TE. 1996. Multicopper oxidases and oxygenases. *Chem. Rev.* 96:2563-2605

Stone AT. 1987. Reductive dissolution of manganese(III/IV) oxides by substituted phenols. *Environ. Sci. Technol.* 21:979–88

Stone AT, and Morgan JJ. 1984a. Reduction and dissolution of manganese(III) and manganese(IV) oxides by organics. 1. Reaction with hydroquinone. *Environ. Sci. Technol.* 18:450–56

Stone AT, and Morgan JJ. 1984b. Reduction and dissolution of manganese(III) and manganese(IV) oxides by organics. 2. Survey of the reactivity of organics. *Environ. Sci. Technol.* 18:617-24

Thurston CF. 1994. The structure and function of fungal laccases. *Microbiology*. 140:19-26

Tebo BM, Bargar JR, Clement BG, Dick GJ, Murray KJ, Parker D, Verity R, and Webb SM. 2004. Biogenic manganese oxides: properties and mechanisms of formation. *Annu. Rev. Earth Planetary Sci.* 32:287-328

Ulrich HJ, and Stone AT. 1989. The oxidation of chlorophenols adsorbed to manganese oxide surfaces. *Environ. Sci. Technol.* 23:421–28

Van Waasbergen LG, Hoch JA, and Tebo BM. 1993. Genetic analysis of the marine manganese-oxidizing *Bacillus* sp. strain SG-1: protoplast transformation, Tn*917* mutagenesis, and identification of chromosomal loci involved in manganese oxidation. *J. Bacteriol.* 175(23):7594-7603

Van Waasbergen LG, Hildebrand M, and Tebo BM. 1996. Identification and characterization of a gene cluster in manganese oxidation by spores of the marine *Bacillus* sp. strain SG-1. *J. Bacteriol.* 178(12):3517-3530

Wariishi H, Valli K, and Gold MH. 1992. Manganese(II) oxidation by manganese perocidase from the basidiomycete *Phanerochaete chrysosporium*. *J. Biol. Chem.* 267:23688-23695

Chapter 2

A Screen For Genes Involved in Mn(II) Oxidation Using Random Transposon Mutagenesis

2.1 Introduction

Random mutagenesis has been used on Mn(II)-oxidizing *Pseudomonas putida* strains multiple times to identify genes that are necessary for Mn(II) oxidation. Previous random mutagenesis experiments have revealed some important genes for Mn(II) oxidation: genes necessary for c-type cytochrome biogenesis (de Vrind et. al., 1998), tricarboxylic acid cycle enzyme biogenesis (Caspi et. al., 1998), tryptophan biogenesis (Caspi et. al., 1998), general secretory pathways (de Vrind et. al., 2003), multicopper oxidases (MCO) (Brouwers et. al., 1999), and heme peroxidases (Anderson et. al., 2009). However, direct identification of the Mn(II) oxidase has not been successful, and complete biochemical pathways and regulation for Mn(II) oxidation are not yet understood.

Here, I utilized transposon mutagenesis to identify genes necessary for Mn(II) oxidation in *Pseudomonas putida* GB-1 by screening mutants for defects in Mn(II) oxidation. The genes identified from sequencing the transposon insertion sites fall in the following categories: an MCO gene, genes required for several major metabolic pathways, membrane transporter related genes, transcription regulation genes, and environmental signaling genes. Interestingly, none of the genes were previously identified as important for Mn(II) oxidation. A novel result from my experiments is that some mutants had

increased oxidizing ability on agar plates, and all of these had transposon insertions in flagella synthesis related genes.

2.2 Materials and methods

Bacterial strains, media, and growth conditions

The strains and plasmids used in this study are shown in Table 2.1.

P. putida GB-1 was routinely grown at room temperature or 30˚C on Luria-Bertania (LB) medium or Lept medium, previously called LD, *L. discophora* medium used for *L. discophora* SS-1 (Boogerd & de Vrind, 1987) [0.5 g yeast extract per liter, 0.5 g of Casamino Acids per liter, 5 mM $CaCl_2$, 5 mM D-(+)-glucose, 0.83 mM $MgSO_4$, 10 mM HEPES, 0.037 μM $FeCl_3$, 200 μM $MnCl_2$, and 1 ml of trace elements (10 mg of $CuSO_4 \cdot 5H_2O$, 44 mg of $ZnSO_4 \cdot 7H_2O$, 20 mg of $CoCl_2 \cdot 6H_2O$, and 13 mg of $Na_2MoO_4 \cdot 2H_2O$ per liter of distilled water)]. *Escherichia coli* strains were grown in LB medium at 37˚C. Solid media were prepared by adding 1.5% (wt/vol) agar prior to autoclaving. The following concentrations of antibiotics were used: kanamycin (Km) 30 μg /ml and ampicillin (Amp) 100 μg /ml.

To store a strain, an overnight culture was mixed with glycerol to a final concentration of 10% at room temperature, and transferred into -80˚C immediately.

Conjugation

The plasmid carrying transposon Tn5, pRL27, was transferred into *P. putida* GB-1 by triparental mating using helper plasmid pRK2013. All strains were grown to mid-exponential phase by inoculating 100 μl overnight culture into 5 ml LB medium with appropriate antibiotics, and shaken at optimal temperature for 3 hours. Next, strains were mixed at following volumes: 600 μl *P. putida* GB-1, 300 μl *E. coli* carrying pRK2013, and 1 ml *E. coli* carrying pRL27 or 600 μl for pRR10. The bacteria were mixed in a tube, pelleted by centrifugation (10,000 rpm for 1 minute at room temperature), resuspended in approximately 50 μl of remaining supernatant, and spotted on a LB agar plate, which was incubated overnight at 30˚C.

Culture from the conjugation was scraped off the plate, suspended in 1.0 ml of LB medium, and vortexed until the suspension was homogenized. 50 µl and 100 µl of the culture was then spread on LB with Km and Amp, and incubated overnight at 30˚C. *P. putida* GB-1 can grow on Amp, while the *E. coli* donor and helper strains cannot, and only GB-1 with a transposon integrated into its chromosome can grow on Km.

Conjugation between GB-1, pRK2013, and pRR10 was used for a positive control. Since pRR10 is a broad host range plasmid, it can replicate in *P. putida* or *E. coli*. Therefore, the plasmid does not have to be integrated into the GB-1 chromosome for the GB-1 to become Km resistant. On the other hand, conjugation using pRL27 can only create Km resistant GB-1 when the transposon gets integrated into the chromosome, so it should produce far less colonies than the conjugation with pRR10. In addition, DH5α was used as a negative control because it lacks a Km^R plasmid, therefore will not be able to create any colony on Km agar plate.

Table 2.1 Bacterial strains and plasmids used in this work

Strain or plasmid	**Genetic characteristic**	**Antibiotic^R**	**Source or reference**
Strains			
Pseudomonas putida			
GB-1	Manganese oxidizer, wild-type	Amp	(Okazaki et. al., 1997)
Escherichia coli			
GT155	*F¯ mcr*A Δ(*mrr-hsdRMS-mcr*BC) F80*lacZ*ΔM15 Δl*acX*74 *rec*A1 *end*A1 Δ*dcm, uidA(ΔMluI)::pir-116, ΔsbcC-sbcD*		(InvivoGen, CA)
Plasmids			
pRR10	Broad host range plasmid	Kan, Amp	(Fang et. al., 1992)
pRL27	Tn5	Kan	(Larsen et. al., 2002)
pRK2013	ColE1 replicon, *mob* RK2, *tra* RK2	Kan	(Figurski & Helinski, 1979)

Screening

Transformants were screened for Mn(II) oxidation on Lept agar. Colonies on LB agar with Km and Amp were replica plated to Lept agar. Any colonies that did not start Mn(II) oxidation after 72 hours were transferred to another Lept plate to compare their relative oxidation speed against the wild-type GB-1. In addition, they were incubated on LB agar with Km and Amp to make sure the transposon was incorporated into the chromosome of GB-1.

During the screening of the decreased Mn(II) oxidation mutants, one colony was found to start oxidizing after just overnight incubation. It retained the fast oxidation phenotype when isolated. After this discovery, fast oxidation colonies were selected to be screened as well.

Identification of insertion sites

Genomic DNA was isolated from 5 ml LB overnight cultures using the Wizard Genomic DNA Purification Kit (Promega, USA). 5 µg of purified gDNA was digested in a 50 µl reaction overnight at 37˚C with the restriction enzyme *Pst*I (New England BioLabs, MA, USA), which does not cut within the transposon. Before the ligation step, to eliminate enzymes and buffers, ethanol precipitation was performed. 100 ng of digested gDNA was ligated using T4 DNA ligase (New England BioLabs) in a 20 µl reaction overnight at room temperature, and then the enzyme was heat inactivated at 65 ˚C for 10 minutes.

Competent cells were made using the protocol from "Experiments with Gene Fusions" by Silhavy and his colleagues (1984). Briefly, *E. coli* GT115 culture was grown to early exponential phase (OD_{600}=0.3), and 100 mM $CaCl_2$ was used to make the cell competent. Heat shock transformation method was used to transform 5 µl of the ligated DNA into 200 µl of competent cells. The Tn5 transposon carries an origin of replication as well as a Km^R gene, thus the *Pst*I fragment of the gene in which Tn5 inserted will produce a Km^R plasmid after ligation. Therefore, LB agar with Km was used to select for *E. coli* cells transformed with the plasmid containing Tn5 and the *Pst*I fragment of the genome. Plasmids were purified from multiple colonies that grew on the selective plate

using QIAprep Spin Miniprep Kit (Qiagen). Purified plasmids were digested with *Pst*I and *Bam*HI. Then, plasmids with different digestion patterns were sent for sequencing, using the following primers: 5'-AACAAGCCAGGGATGTAACG-3' and 5'-CAGCAACACCTTCTTCACGA-3'. The sequenced genes were identified using a BLAST search against the *P. putida* GB-1 genome database on the Integrated Microbial Genomes website (http://img.jgi.doe.gov/).

2.3 Results

Isolation of mutants

Approximately 1600 mutants from about 10 different matings were screened for the loss of function phenotype, which is a loss in Mn(II) oxidation. 13 mutants were originally isolated as having a severe defect in Mn(II) oxidation, but none of them had a complete loss of Mn(II)-oxidation as they exhibited at least slight oxidation after incubation for more than a week. These strains were annotated as DMO (Decreased Mn Oxidation). Also, 15 mutants were isolated as having a minor defect in oxidation, meaning they were able to start oxidizing Mn(II) within a week of incubation, but slower than the wild-type GB-1. They were annotated as MDMOs (Moderately Decreased Mn Oxidation). Unexpectedly, during the course of this screen, I identified strains that started oxidizing within 12 hours of incubation. This is faster than the wild-type, which typically starts oxidizing after at least 24 hours. Since I was not expecting this phenotype in the beginning, I was only able to isolate 4 mutants with the increased oxidation ability. They were annotated as IMO (Increased Mn Oxidation).

All of the mutants were plated on Lept agar multiple times to insure that they have the phenotype of my interest. As a result of these screenings, two of the DMOs regained wild-type level of Mn(II) oxidation, so they were eliminated from the strains. Unexpectedly, one DMO strain instead reproducibly oxidized faster than wild type, so it was renamed IMO. Therefore, the site of Tn5 integration was mapped for 10 DMO and five IMO strains, and results are organized in table 2.2.

The Mn(II) oxidation phenotypes were re-screened after a year of storage at -80˚C. As a result, some strains did not retain their original phenotype that was observed when

first isolated, as shown in Figure 2.1. (Note that pictures with DMOs were taken after 72 hours of incubation, but the one with IMOs were taken after 24 hours to best show the differences between the wild-type and the mutants the best.) DMO8 reverted to having the wild-type oxidation phenotype. DMO1, 3, 4, 7, 9, and 10 also started the oxidation faster than previously observed, but the brown color intensity of those colonies remained weaker than the wild-type even after 2 weeks of incubation (data not shown), which suggests, even though they start producing Mn(II) oxidase about the same time as the wild-type, they can't oxidize as much Mn(II) as the wild-type. This may be because they don't produce as much Mn(II) oxidase as the wild-type due to a defect in biochemical pathway or alteration in regulation, they utilize a different oxidase than the wild-type, or are affected in some other property. All of the IMOs retained their faster oxidation phenotype.

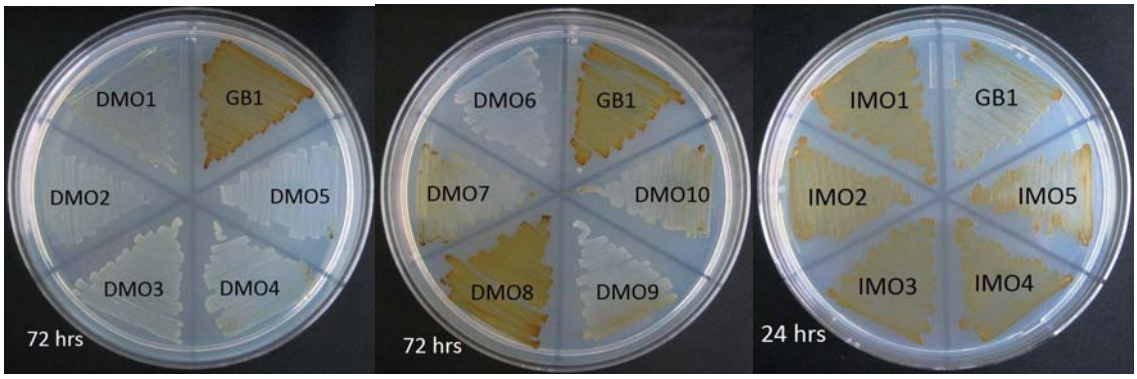

Figure 2.1 GB-1 and all of the sequenced mutants plated on Lept after storing in -80˚C for a year. First two plates from the left with DMOs were taken after 72 hours of incubation at 30˚C. The picture on the right with IMOs was taken after 24 hours of incubation at 30˚C.

Table 2.2 Transposon insertion sites of sequenced mutants

Mutant	Possible genes disrupted	Locus tag
DMO1	Uroporphyrin-III C-methyltransferase	PputGB1_3604
DMO2	1) Multicopper oxidase type 2	PputGB1_2665
	2) Nucleotidyl transferase*	PputGB1_0437
	Heat shock protein DnaJ domain protein	PputGB1_0438
DMO3	Beta-ketoacyl-acyl-carrier-protein synthase I	PputGB1_3940
DMO4	Hypothetical protein	PputGB1_4907
DMO5	1) Transmembrane regulator PrtR	PputGB1_2893
	2) Acyl-CoA synthetase*	PputGB1_2182
	Thiamine pyrophosphate protein TPP binding domain protein	PputGB1_2183
DMO6	1) Transmembrane regulator PrtR	PputGB1_2893
	2) Acyl-CoA synthetase*	PputGB1_2182
	Thiamine pyrophosphate protein TPP binding domain protein	PputGB1_2183
DMO7	1) Ammonium transporter	PputGB1_5294
	2) Major facilitator superfamily MFS_1	PputGB1_1293
DMO8	1) Transmembrane regulator PrtR	PputGB1_2893
	ECF sigma factor PrtI*	PputGB1_2894
	2) ABC-3 protein	PputGB1_0132
	ABC transporter related*	PputGB1_0133
DMO9	Transcriptional regulator, ArsR family	PputGB1_5015
DMO10	Mg(II) and Co(II) transporter protein, CorC*	PputGB1_4843
	Apolipoprotein N-acyltransferase	PputGB1_4844
IMO1	Flagellar cap protein FliD	PputGB1_3937
IMO2	Anti-sigma-28 factor, FlgM	PputGB1_3956
IMO3	Flagellin domain protein; flagellin hook IN repeat protein	PputGB1_3939
IMO4	1) Flagellin domain protein; flagellin hook IN repeat protein	PputGB1_3939
	2) Integral MB sensor signal transduction histidine kinase	PputGB1_0930
	Two component transcriptional regulator, Fis family	PputGB1_0931
IMO5	1) Na+/H+ antiporter	PputGB1_1141
	2) RNA polymerase, sigma 28 subunit, FliA/WhiG	PputGB1_3910
	Flagellar number regulator FleN*	PputGB1_3911

Genes were identified by BLAST search against *P. putida* GB-1 genome database on the Integrated Microbial Genomes website (http://img.jgi.doe.gov/).

* transposon insertion is in this gene, and the other gene is possibly co-transcribed.

Sequence analysis

Plasmids from five colonies from each strain that survived on LB agar with Km were digested to make sure all of the possible transposon insertion sites are sequenced. If all of the five plasmids gave the same digestion pattern, then most likely there is only one insert site, but if there is more than one pattern, then there may be as many inserts as the number of the different digestion patterns. Therefore, all of the plasmids with different digestion patterns were sent for sequencing. BLAST results are shown in Table 2.2. DMO2, 5, 6, 7, 8, IMO4 and 5 each had two different insertion sites. If the gene with the transposon insertion had a downstream gene within 20 bps, the downstream gene was also listed because there is a high possibility that its transcription is also disrupted.

BLAST results revealed there are several different categories of genes that may be important in Mn(II) oxidation. DMO2 had one of its MCO genes disrupted. In another study with *P. putida* GB-1, another MCO gene has been proposed to be important in Mn(II) oxidation (Brouwers et. al., 1999), but this is the first time that a type 2 MCO is reported to be important in Mn(II) oxidation. There were some genes important for major metabolic pathways to properly function, such as amino acid synthesis or fatty acid synthesis related genes. Also, some genes necessary for membrane transporters were identified. Multiple transcription regulators, including some that respond to environmental signals, were identified as well. All of the IMO mutants had a transposon insertion in flagella related genes, in addition to some second insertion sites.

The sequence analysis showed that DMO5 and 6 have the same insertion sites for both of the inserts. In addition, they have the same oxidation phenotype on Lept plates. Therefore, the two strains are probably derived from the same mother cell.

2.4 Discussion

Decreased manganese oxidation mutants

Ten DMO mutants were isolated from the transposon mutagenesis for having a severely defective Mn(II) oxidation phenotype, and the disrupted genes were sequenced. Here, I categorize most of the identified genes (Table 2.2) into 4 different categories according to their proposed cellular functions.

Multicopper oxidase:

A multicopper oxidase (MCO) encoding gene, *cumA*, has been reported to be essential for Mn(II) oxidation in GB-1 from a previous transposon mutagenesis experiment (Brouwers et. al., 1999). Another MCO encoding gene, *mnxG*, has also been shown to be important in Mn(II) oxidation. MnxG was originally isolated from *B. subtilis* PL-12 and MB-7 as the major component of the Mn(II) oxidase (Dick et. al., 2008). When an *mnxG* in-frame deletion strain was tested for Mn(II) oxidation with *P. putida* GB-1, it showed a significant delay in oxidation (J. McCarthy, personal communication). In this experiment, I identified an MCO gene that encodes another type of MCO, which was disrupted in DMO2. This raises the question if there is a specific type of MCO that is involved in Mn(II) oxidation, or if multiple MCOs are necessary for the normal function of Mn(II) oxidation.

However, in-frame deletions of both *cumA* and the MCO type 2 encoding gene resulted in strains that are, if at all, only mildly defective in Mn(II) oxidation (K. Geszvain, personal communication). DMO2 had another insertion site that disrupted transcription of a nucleotidyl transferase gene or a heat shock protein gene. The original *cumA* mutant strain GB-1-007 also had a spontaneous mutation in a sensor kinase encoding gene (K. Geszvain, personal communication). Therefore, the severe Mn(II) oxidation defects of DMO2 and GB-1-007 are most likely due to an additional mutation elsewhere on the chromosome, so it is still unclear if an MCO is functioning as a Mn(II) oxidase in GB-1, and if so, which MCOs are actually involved in Mn(II) oxidation.

Major metabolic pathways:

DMO5 and 6 had a transposon insertion in the gene encoding thiamine pyrophosphate (TPP) protein binding domain protein. However, further bioinformatic analysis suggested this gene may actually encode for acetolactate synthase (ALS) large subunit, which has the TPP binding domain. Acetolactate synthase is required for synthesis of branched amino acids (valine, leucine, and isoleucine) (Chipman et. al., 1998). A growth defect was observed with the ALS mutant of *E. coli* grown on acetate or oleate as the sole carbon source Dailey & Cronan, 1986). Since the Lept medium

contained Casamino Acids, all of the branched amino acids were provided for the cell growth in my case. Therefore, it is unlikely that the lack of ALS will cause a major effect on cell growth. Furthermore, DMO5 and 6 have an additional transposon mutation elsewhere on the genome. However, I cannot rule out the possibility this gene is required for Mn(II) oxidation, particularly because it is uncertain whether the gene disrupted in DMO5 and 6 actually functions as ALS.

Uroporphyrin-III C-methyltransferase, the gene for which was disrupted in DMO1, is a protein responsible for *S*-adenosyl-methionine-dependent methylation of a corrin ring of uroporphyrinogen III during vitamin B_{12} synthesis (Rehse et. al., 2005). The cofactor form of vitamin B_{12} is called 5'-deoxyadenosylcobalamin, and is a cofactor of methylmalonyl-CoA mutase. This mutase is necessary for the final round of oxidation of odd-chain fatty acid into succinyl-CoA, which can then enter the citric acid cycle. Conversion of uroporphyrinogen III to siroheme is also dependent on the uroporphyrin-III C-methyltransferase (Raux et. al., 2003). Siroheme is a prosthetic group of sulphite and nitrite reductases that catalyze reduction of sulfite and nitrite to sulfide and ammonia (Raux et. al., 1999). The deficiency of Mn(II) oxidation in DMO1 may be due to a limited ability to convert odd-chain fatty acids into a useful form, or to the defect in sulphite and/or nitrite reduction.

Vitamin B_{12} is only important in the last step of odd-chain fatty acid oxidation, but acyl-CoA synthetase is important for the initial activation of the fatty acid for oxidation (Voet & Voet, 2003). It catalyzes the reaction of fatty acid, CoA, and ATP into acyl-CoA, AMP, and PP_i. The gene encoding this enzyme is disrupted in DMO5 and 6, which also have a disruption of the gene encoding the transmembrane regulator, PrtR, discussed later. I do not think lack of one of the acyl-CoA synthetases is the major reason for the lack of Mn(II)-oxidation phenotype in these strains because usually there are multiple acyl-CoA synthetases, specific for different lengths of fatty acid chains. Therefore, it is unlikely that lacking the ability to activate just one kind of the fatty acid will cause loss of Mn(II)-oxidation.

The opposite reaction to the fatty acid oxidation is the biosynthesis of fatty acids. β-ketoacyl-ACP (acyl-carrier protein) synthase I is an enzyme that catalyzes the reaction

between acyl-ACP and malonyl-ACP, to produce acetoacetyl-ACP (Voet & Voet, 2003). The gene necessary to produce this enzyme is disrupted in DMO3. It can be assumed that by lacking one of the necessary enzymes for fatty acid biosynthesis, the bacterium will lose the major way to balance the fatty acid concentration in the cell and the energy requirement. Possibly this lack of balance prevents DMO3 cells from producing the Mn(II) oxidase, or affects the activity in some other manner.

DMO10 has an insertion in apolipoprotein N-acyltransferase, the enzyme catalyzing the conversion of an apolipoprotein to mature a lipoprotein. Apolipoproteins are the protein components of the lipoproteins that have to be N-acylated by the N-acyltransferase. It ensures lipoproteins are efficiently recognized by the Lol (lipoprotein localization) system, which transports them from the plasma membrane to the outer membrane (Fukuda et. al., 2002). Lipoproteins have multiple functions, such as: structural protein, binding proteins, transporter, adhesions, toxins, antigens, and enzymes (Babu & Sankaran, 2002). Since Mn(II) oxidation of GB-1 has been observed on the cell surface, a lipoprotein may be necessary to transport the Mn(II) oxidase out. Conversely it may be required to transport signaling chemicals in.

Membrane transporter:

One of the disrupted genes in DMO7 encodes an ammonium transporter. Not only does this protein mediate the transport of NH_3 across the membrane for nitrogen uptake, but it is proposed to function as an ammonium sensor and regulates cellular metabolism in response to changes in external ammonium concentrations in fungi and gram-negative bacteria (Tremblay & Hallenbeck, 2009). If one signal regulating Mn(II) oxidation is the concentration of NH_3, then lack of the ammonium transporter sensor could affect Mn(II) oxidation. As in other bacteria, GB-1 also has three different ammonium transporters. However, they share relatively little homology, with the transporter most similar to the one disrupted in DMO7 only 66% identical. It is highly possible that they regulate different cellular metabolisms, if they are acting as sensors.

DMO7 had another transposon insertion in a gene encoding a major facilitator superfamily (MFS) protein, which is also a transporter system. The MFS is also called the

uniporter-symporter-antiporter family, and transporters in this family can only transport small solutes in response to chemiosmotic ion gradients (Pao et. al., 1998). According to a BLAST search, multidrug resistant transporter was the closest MFS to the one disrupted in DMO7 that has been characterized, but most of the matches were uncharacterized transporters from the MFS. Therefore, it is unclear transport of what substance is actually affecting the Mn(II) oxidation of DMO7.

The gene for another type of membrane transporter, an ATP-binding cassette (ABC) transporter, was disrupted in DMO8. This is an important class of transporters that utilizes the energy of ATP hydrolysis to transport various substrates across the membranes. This specific ABC transporter had 71% identity with ABC-type Mn/Zn transporter systems from *Magnetospirillum magneticum* AMB-1 (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which means the disrupted gene may be also a transporter of manganese, and the disruption of the optimum concentration of Mn in the cell causes decrease in Mn(II) oxidation. However, it is important to remember that DMO8 reverted to the wild-type Mn(II) oxidation phenotype after prolonged storage. This might mean the disrupted gene encodes the primary ABC transporter for Mn(II), or another substrate that is necessary for Mn(II) oxidation, but the cell was able to adapt to losing this gene by using another set of very similar genes, since there are over 100 genes that encode ABC transporters in GB-1 (http://img.jgi.doe.gov/). In addition, DMO8 has a second insertion site, which means the Mn(II) oxidation defect may not have been due to disruption in the ABC transporter encoding gene.

The magnesium and cobalt ion transporter protein, CorC, studied in *Salmonella typhimurium*, was disrupted in DMO10. Since Mn(II) is also a divalent cation, there is a possibility Mn(II) gets transferred through the Mg(II) and Co(II) ion transporter. However, mutation at *corC* has only showed diminished capacity for Mg(II) efflux with no significant effect on influx activity, therefore how directly CorC is involved in metal transport is unclear (Gibson et. al., 1991). Further information is necessary to determine how CorC is affecting the Mn(II) oxidation, but as a metal transporter, disruption of *corC* could be altering the concentration of Mn(II) inside of the cell.

Environmental signaling:

In *Pseudomonas fluorescens*, *prtI* and *prtR* encode a sigma factor and a transmembrane activator respectively, and they are necessary for protease expression at slightly above the optimal growth temperature (Burger et. al., 2000). DMO5 and 6 have an insertion at *prtR*, and DMO8 has an insertion in the upstream gene *prtI*. Analysis with *P. fluorescens* revealed *prtI/R* are translationally coupled (Burger et. al., 2000), and this is probably true with *prtI/R* of GB-1 as well because the stop codon of *prtI* overlaps the start codon of *prtR* just like in *P. fluorescens*. Therefore, DMO5 and 6 are only lacking PrtR, but DMO8 is lacking both PrtR and PrtI. Burger and his colleagues also showed PrtR can have a minor interaction with other sigma factors, but PrtI specifically requires active PrtR to form a functional RNA polymerase. In addition, inactive PrtI can still bind with the promoter, resulting in down regulation of genes regulated by PrtI. In summary, DMO5 and 6 will have down regulation of genes that are regulated by PrtI due to lack of PrtR, while DMO8 will not have activation from the extracytoplasmic signals. Only preliminary experiments on a temperature effect on Mn(II) oxidation have been done, which will be discussed in Chapter 4, so it is difficult to say if *prtI/R* has any role in regulation. However, it is possible that Mn(II) oxidation genes are regulated by PrtI RNA polymerase, and PrtR is necessary to sense an environmental condition change for the optimal Mn(II) oxidation.

The PrtIR system of *Pseudomonas fluorescens* is a temperature sensing regulator, but DnaJ, which had its encoding gene disrupted in DMO2, is one of the heat shock proteins. Heat shock proteins function to facilitate the proper folding of newly synthesized polypeptides as they emerge from the ribosome, and to unfold proteins in preparation for their transport through membranes (Voet & Voet, 2003). Jürgen Gamer and his colleagues (1992) also discovered that some of the heat shock proteins can negatively regulate heat shock gene expression by association with the heat shock-specific σ subunit of RNA polymerase, σ^{32}. As a result, strains with defects in *dnaK, dnaJ,* or *grpE* genes have shown defects in heat shock responses (Straus et. al., 1990). It is possible that Mn(II) oxidation is partially regulated by heat shock proteins.

ArsR is also a transcriptional regulator that senses the environmental condition. The *ars* operon from most bacteria encodes arsenate reductases, as well as proteins required for metal ion extrusion (Xu et. al., 1998). Studies have shown metalloregulator ArsR functions in resistance of As(III) and Sb(III) by sensing the presence of those metals (Busenlehner et. al., 2003), so it may have a potential of sensing Mn(II). The decrease in Mn(II) oxidation ability of DMO9 can be explained if Mn(II) sensing by ArsR is initially necessary for the transcription of Mn(II) oxidation related genes.

Increased manganese oxidation mutants

All of the IMOs had at least one mutation in flagella related genes. Therefore, it is reasonable to assume there is a regulatory relationship between proper flagella synthesis and Mn(II) oxidation. Here, I will discuss what each identified gene's functions are, but further detailed characterization and discussion will be presented in the next chapter.

One of the genes potentially disrupted in IMO5 encodes for a RNA polymerase sigma factor, σ^{28} subunit, also called FliA. This gene is required for transcription of class III flagellar genes, which is the last class of the flagellar structural proteins that gets made after the hook-basal body (HBB) structure has been made (Karlinsey et. al., 2000). σ^{28} subunit has an anti-sigma factor called FlgM, the gene for which has been disrupted in IMO2. FlgM binds to the σ^{28} subunit until completion of the HBB structure to prevent premature transcription of class III genes in case there is a defect in earlier proteins that makes up the HBB (Chevance & Hughes, 2008). It is reasonable to think that lack of FliA will prevent construction of a complete flagellum because class III genes can not be transcribed, and lack of FlgM will disturb the normal regulation and balance of the proteins made for flagella synthesis.

The transposon insertion in IMO5 was in *fleN*, the gene upstream of *fliA* that encodes for a flagella number regulator. A mutation in *fleN* of *Pseudomonas aeruginosa* caused multi-flagellation compared to monoflagellated wild-type (Dasgupta et. al., 2000). In addition, over-production of FleN caused the cell to lose the ability to create flagella.

As well as regulatory genes, structural genes were also disrupted in the IMO strains. IMO1 had an insertion in *fliD*, which encodes the flagellar cap protein. The

flagellar cap is necessary for proper assembly of the flagellum filament, and it is one of the class III proteins, whose expression is regulated by FliA (Macnab, 1992). Flagellin domain proteins, the gene for which is disrupted in IMO3 and 4, are important in constructing the flagellar hook. The flagellar hook is an important component to complete the assembly of HBB, which is necessary for the class III genes to be transcribed by the free σ^{28} subunit.

2.5 Future directions

In this chapter, I introduced some of the genes that may have an important role in Mn(II) oxidation. All of the IMOs were studied further, and the results will be discussed in the next chapter. However, I did not have time to conduct further study on the DMO mutants other than basic characterization of their phenotype in different conditions, which will be discussed in Chapter 4. Therefore, further analysis of some of the genes disrupted in DMO strains is recommended.

Complementation or construction of site directed mutants may be useful to make sure that the genes found to be potentially important in Mn(II) oxidation by transposon mutagenesis are actually responsible for the loss of Mn(II) oxidation in mutant strains.

A simple experiment of temperature dependency of Mn(II) oxidation by comparing cultures incubated in room temperature and 30˚C was performed, and the results are presented in Chapter 4. However, since there are multiple strains defective in gene regulators that depend on temperature, growing the strains at a higher temperature than 30˚C may further reveal some temperature dependent regulatory mechanisms of Mn(II) oxidation.

Understanding how different major metabolic pathways are participating in Mn(II)-oxidation will be difficult because there are so many different possibilities from energy requirements, to catabolic product requirements. However, when more information about the biochemical pathways of Mn(II) oxidation becomes available, we may be able to understand why these genes are important in Mn(II) oxidation.

2.6 References

Anderson CR, Johnson HA, Caputo N, Davis RE, Torpey JW, and Tebo BM. 2009. Mn(II) oxidation is catalyzed by heme peroxidases in "*Aurantimonas manganoxydans*" strain SI85-9A1 and *Erythrobacter* sp. Strain SD-21. ***Appl. Environ. Microbiol.*** 75(12):4130-38

Babu MM, and Sankaran K. 2002. DOLOP—database of bacterial lipoproteins. *Bioinformatics*. 18:641-43

Boogerd FC, and de Vrind JPM. 1987. Manganese oxidation by *Leptothrix discophora*. ***J. Bacteriol***. 169:489-494

Brouwers GJ, de Vrind JPM, Corstjens PLAM, Cornelis P, Baysse C, and de Vrind-deJong EW. 1999. *cumA*, a gene encoding a multicopper oxidase, is involved in Mn^{2+} oxidation in *Pseudomonas putida* GB-1. ***Appl. Environ. Microbiol.*** 65(4):1762-68

Busenlehner LS, Pennella MA, and Giedroc DP. 2003. The SmtB/ArsR family of metalloregulatory transcriptional repressors: structural insights into prokaryotic metal resistance. *Microbiology Reviews*. 27:131-143

Caspi R, Tebo BM, and Haygood MG. 1998. *c*-type cytochromes and manganese oxidation in *Pseudomonas putida* MnB1. ***Appl. Environ. Microbiol.*** 64(10):3549-55

Chevance FFV, and Hughes KT. 2008. Coordinating assembly of a bacterial macromolecular machine. *Nature Reviews Microbiol.* 6:455-465

Chipman D, Barak Z, and Scholoss VJ. 1998. Biosynthesis of 2-aceto-2-hydroxy acids: acetolactate synthases and acetohydroxyacid synthases. *Biochemica et Biophysica Acta*. 1385:401-419

Dasgupta N, Arora SK, and Ramphal R. 2000. *fleN*, a gene that regulates flagellar number in *Pseudomonas aeruginosa*. ***J. Bacteriol.*** 182(2):357-364

Dailey FE, and Cronan JE Jr. 1986. Acetohydroxy acid synthase I, a required enzyme for isoleucine and valine biosynthesis in *Excherichia coli* K-12 during growth on acetate as the sole carbon source. ***J. Bacteriol.*** 165(2):453-460

de Vrind JPM, Brouwers GJ, Corstjens PLAM, Dulk J, and de Vrind-de Jong EW. 1998. The cytochrome *c* maturation operon is involved in manganese oxidation in *Pseudomonas putida* GB-1. ***Appl. Environ. Microbiol.*** 64(10):3556-62

de Vrind J, de Groot A, Brouwers GJ, Tommassen J, and de Vrind-de Jong E. 2003. Identification of a novel Gsp-related pathway required for secretion of the manganese-oxidizing factor of *Pseudomonas putida* strain GB-1. *Mol. Microbiol.* 47(4):993-1006

Dick GJ, Torpey JW, Beveridge TJ, and Tebo BM. 2008. Direct identification of a bacterial manganese(II) oxidase, the multicopper oxidase MnxG, from spores of several different marine *Bacillus* species. *Appl. Environ. Microbiol.* 74(5):1527-34

Fang FC, Libby SJ, Buchmeier NA, Loewen PC, Switala J, Harwood J. and Guiney D G. 1992. *Proc. Natl. Acad. Sci. USA.* 89:11978–1198

Figurski DH, and Helinski DR. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA* 76: 648–1652

Fukuda A, Matsuyama S, Hara T, Nakayama J, Nagasawa H, and Tokuda H. 2002. Aminoacylation of the N-terminal cysteine is essential for Lol-dependent release of lipoproteins from membranes but does not depend on lipoprotein sorting signals. *J. Biol. Chem.* 45:43512-18

Gamer J, Bujard H, and Bukau B. 1992. Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor σ^{32}. *Cell.* 69:833-842

Gibson MM, Bagga DA, Miller CG, and Maguire ME. 1991. Magnesium transport in *Salmonella typhimurium*: the influence of new mutations conferring Co^{2+} resistance on the CorA Mg^{2+} transport system. *Mol. Microbiol.* 5:2753-62

Karlinsey JE, Tanaka S, Bettenworth V, Yamaguchi S, Boos W, Aizawa SI, and Hughes KT. 2000. Completion of the hook-basal body complex of the *Salmonella typhimurium* flagellum is coupled to FlgM secretion and *fliC* transcription. *Mol. Microbiol.* 37:1220-31

Larsen RA, Wilson MM, Guss AM, and Metcalf WM. 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch. Microbiol*. 178:193-201

Macnab RM. 1992. Genetics and biogenesis of bacterial flagella. *Annu. Rev. Genet.* 26:131-58

Okazaki M, Sugita T, Shimizu M, Ohode Y, Iwamoto K, de Vrind-de Jong EW, de Vrind JPM, and Corstjens PLAM. 1997. Partial purification and characterization of manganese-oxidizing factors of *Pseudomonas fluorescens* GB-1. *Appl. Environ. Microbiol.* 63**:** 4793–4799.

Pao SS, Paulsen IT, and Saier MH Jr. 1998. Major facilitator superfamily. *Microbiol. Mol Biol. Reviews*. 62:1-34.

Raux E, Schubert HL, Roper JM, Wilson KS, and Warren MJ. 1999. Vitamin B_{12}; insights into biosynthesis's Mount improbable. *Bioorg. Chem.* 27:100-118

Raux E, Leech HK, Beck R, Schubert HL, Santander PJ, Roessner CA, Scott AI, Martens JH, Jahn D, Thermes C, Rambach A, and Warren MJ. 2003. Identification and functional analysis of enzymes required for precorrin-2 dehydrogenation and metal ion insertion in the biosynthesis of sirohaem and cobalamin in *Bacillus megaterium. Biochem J.* 370:505-516

Rehse PH, Kitao T, and Tahirov TH. 2005. Structure of a closed-form uroporphyrinogen-III *C*-methyltransferase from *Thermus thermophilus. Acta Cryst.* D61:913-19

Silhavy TJ, Berman ML, Enquist LW. 1984. Experiments with gene fusions. *Cold Spring Harbor Laboratory*, N.Y. Procedure 37, p. 169

Straus D, Walter W, and Gross CA. 1990. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of σ^{32}. *Genes & Development*. 4:2202-09

Tremblay PL, and Hallenbeck PC. 2009. Of blood, brains and bacteria, the Amt/Rh transporter family: emerging role of Amt as a unique microbial sensor. *Mol. Microbiol.* 71:12-22

Voet DJ, and Voet JG. 2003. Biochemistry, 3rd edition. *Wiley, John & Sons, Inc.*

Woodcock DM, Crowther PJ, Doherty J, Jefferson S, DeCruz E, Noyer-Weidner M, Smith SS, Michael MZ, and Graham MW. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acid Res.* 17:3469-78

Xu C, Kuroda T, and Rosen BP. 1998. Metaloid resistance mechanisms in prokaryotes. *J. Biochem.* 123:16-23

Chapter 3

A Relationship between Flagella Synthesis, Motility, and Mn(II) Oxidation

3.1 Introduction

Bacterial flagella are complex structures that allow bacteria to swim, and also attach to a surface. The synthesis of flagella requires over 40 genes (Choy et. al., 2004), which includes multiple structural and regulatory genes. Because synthesis of flagella requires a lot of protein, there is strict regulation at different stages of flagellar synthesis to prevent energy waste. From the transposon mutagenesis, five strains were identified as having increased Mn(II) oxidation on hard agar, and all of those mutants had transposon insertions in flagella-related genes. This result implied that there may be some important regulatory relationships between flagella synthesis and/or motility and Mn(II) oxidation.

In this chapter, I describe testing the motility of the IMO strains, and complementation of the genes in each IMO strain. In addition, site directed mutagenesis was performed to disrupt two other flagella-related genes that were not disrupted in any of the IMO strains in an effort to understand the relationship between the flagella and Mn(II) oxidation.

3.2 Materials and methods

Complementation:

Strains and media used

The bacterial strains and plasmids used in this study are listed in Table 3.1. Growth conditions are described in Chapter 2.

PCR

PCR to amplify the gene of interest from wild-type *P. putida* GB-1 genomic DNA was performed in iCycler Thermal Cycler (Bio-Rad, USA), using *PfuTurbo* DNA polymerase (Stratagene, La Jolla) or Phusion Hot Start high-fidelity DNA polymerase (Finnzymes, USA) in 20 µl reaction volumes. The reaction mixture consisted of 40 ng of genomic DNA from GB-1, 0.2 µM each primer (Table 4.1), 0.2 mM of deoxynucleoside triphosphates (dNTP), 1% dimethyl sulfoxide, 2 µl 10X cloned *Pfu* reaction buffer or 4 µl 5X Phusion GC buffer, and 1 U of the DNA polymerase. PCR was performed as follows: initial denaturation of 2 min at 98˚C, followed by 30 cycles of denaturation for 30 sec at 98˚C, annealing for 30 sec at various temperatures between 55˚C and 62˚C, and extension for 30 sec at 72˚C. This was followed by a final extension for 10 min at 72˚C. To amplify *fliA*, initial denaturation at 98˚C was extended to 5 min, and 30 cycles of extension at 72˚C was increased to 1 min to limit nonspecific amplification.

Aliquots of the amplified products were examined by gel electrophoresis on a 1% Agarose (Fisher Scientific, USA) gel, stained with GelRed (Biotium, CA, USA). Except for flagellin domain protein encoding region (PputGB1_3939) and *fleN*, each reaction had nonspecific products, so the Geneclean II kit (MP Biomedicals, OH, USA) was used to purify the product of interest.

Table 3.1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant information	Source or reference
Strains		
E. coli TAM1	Competent cell	Active Motif
P. putida		
GB-1	Wild-type; Amp^r	(Okazaki et. al., 1997)
IMO1	GB-1 with a *fliD*::Tn5 mutation; Amp^r Km^r	This study
IMO2	GB-1 with a *flgM*::Tn5 mutation; Amp^r Km^r	This study
IMO3	GB-1 with a flagellin domain protein encoding region (3939)::Tn5 mutation; Amp^r Km^r	This study
IMO4	GB-1 with flagellin domain protein & sensor histidine kinase protein encoding region::Tn5 mutation; Amp^r Km^r	This study
IMO5	GB-1 with *fleN* & Na+/H+ antiporter encoding region::Tn5 mutation; Amp^r Km^r	This study
IMO1.38	IMO1 complemented with pAY38	This study
IMO2.37	IMO2 complemented with pAY37	This study
IMO3.35	IMO3 complemented with pAY35	This study
IMO4.35	IMO4 complemented with pAY35	This study
IMO5.36	IMO5 complemented with pAY36	This study
IMO5.39	IMO5 complemented with pAY39	This study
IMO5.40	IMO5 complemented with pAY40	This study
Plasmids		
pJET1.2/blunt	Plasmid used for cloning PCR products; Amp^r	Fermentas, USA
pBBR1MCS-5	Low copy number cloning vector; Gm^r	(Kovach et. al., 1995)
pAY27	flagellin domain protein encoding region amplified with 3939-F/-R ligated into pJET1.2/blunt; Amp^r	This study
pAY28	*fleN* amplified with Coby-F/-R ligated into pJET1.2/blunt; Amp^r	This study
pAY31	*flgM* amplified with FlgM-F2/-R2 ligated into pJET1.2/blunt; Amp^r	This study
pAY33	f*liD* amplified with 3937-F2/-R2 ligated into pJET1.2/blunt; Amp^r	This study
pAY34	*fleN-fliA* amplified with Coby-F/FliA-R2 ligated into pJET1.2/blunt; Amp^r	This study
pAY40	*fliA* amplified with FliA-F2/-R2 ligated into pJET1.2/blunt; Amp^r	This study
pAY35	*Xba*I/*Eco*RI fragment of pAY27 in *Xba*I/*Eco*RI cut pBBR1MCS-5; Gm^r	This study
pAY36	*Xba*I/*Eco*RI fragment of pAY28 in *Xba*I/*Eco*RI cut pBBR1MCS-5; Gm^r	This study
pAY37	*Xba*I/*Eco*RI fragment of pAY31 in *Xba*I/*Eco*RI cut pBBR1MCS-5; Gm^r	This study
pAY38	*Xba*I/*Eco*RI fragment of pAY33 in *Xba*I/*Eco*RI cut pBBR1MCS-5; Gm^r	This study
pAY39	*Xba*I/*Eco*RI fragment of pAY34 in *Xba*I/*Eco*RI cut pBBR1MCS-5; Gm^r	This study
pAY41	*Xba*I/*Eco*RI fragment of pAY40 in *Xba*I/*Eco*RI cut pBBR1MCS-5; Gm^r	This study
Primers[a]		
3939-F	5' **gaattc**GCAGTACCCATCCAACGAGG 3', *Eco*RI site incorporated	
3939-R	5' **tctaga**AGGGCGAAAGGGCACGTAAAC 3', *Xba*I site incorporated	
Coby-F	5' **gaattc**CAGCGAGACAAGGTAAAGGACAGAC 3', *Eco*RI site incorporated	
Coby-R	5' **tcttga**AGCCGCTGGCGTTCATAGC 3', *Xba*I site incorporated	
FlgM-F2	5' **gaattc**AAGGCAAGCGTCCAAATACCC 3', *Eco*RI site incorporated	
FlgM-R2	5' **tctaga**GTCCGTGAAGTCAGCGC 3', *Xba*I site incorporated	
3937-F2	5' **gaattc**GTGGTTTTGTCCGCCAAGG 3', *Eco*RI site incorporated	
3937-R2	5' **tctaga**GGCCGATGACATGAAGCC 3', *Xba*I site incorporated	
FliA-F2	5' **gaattc**TTTGTCGAGCGTCTGGTGCA 3', *Eco*RI site incorporated	
FliA-R2	5' **tctaga**GCGGGCTCAACGTGC 3', *Xba*I site incorporated	

[a] Lowercase denotes nucleotides added or modified to generate restriction enzyme recognition site

Construction of plasmids

After amplifying the genes of interest, the resulting PCR products were ligated into pJET1.2/blunt (Fermentas, USA) according to the manufacturer's instructions using T4-ligase in 20 µl reaction volumes. After 5 minutes of ligation reaction, 2 µl of the ligated plasmid was transformed into competent *E. coli* TAM1, Rapid Trans cells (Active Motif, CA, USA), and 150 µl was plated on LB with Amp. After overnight incubation at 37˚C, a few colonies were chosen for plasmid purification using QIAprep Spin Miniprep Kit (Qiagen), then digested with *Eco*RI and *Xba*I in a 40 µl reaction volume and reaction time of 3 hours at 37˚C. The digested products were electrophoresed, and the gene of interest was purified from the gel using Geneclean II Kit (MP Biomedicals, OH, USA). This gene product was ligated with pBBR1MCS-5 fragment digested with *Xba*I and *EcoR*I using Quick Ligation Kit (New England BioLab, USA) in the following reaction mix; 1 µl pBBR1MCS-5 fragment, 2 µl gene of interest, 5 µl buffer, 0.5 µl ligase, and 1.5 µl dH_2O. Ligation product was used to transform TAM1, and transformants were selected on LB with Gm.

Transformation into *P. putida* strain

Each bacterial strain was grown overnight, and washed once with 0.1 M $MgCl_2$ and once with TG salt [75 mM $Cacl_2$, 6 mM $MgCl_2$, and 15% glycerol]. Cells were then incubated in TG salt on ice for 15 min. The cell, then, was transformed with the appropriate plasmid, which was constructed to complement the gene disrupted by the transposon in each mutant, by heat shocking the cell for 2 min at 37˚C, and incubating for 1 hour at 30˚C. Strains were selected using LB with Gm.

<u>Site directed mutagenesis:</u>

Strains and media used

Bacterial strains in addition to the ones used for complementation, and plasmids and primers specifically used for site directed mutagenesis are shown in Table 3.2.

motA and *fliF* primers were selected to amplify approximately 300bp of the gene starting from ~100bp downstream of the start codon. *fliA* and *fleN* primers(*fleN* primers are named Coby because the original annotation of the *fleN* found on the IMG website

was Cobyrinic acid a,c-diamide synthase) were selected to generate ~500bp fragments from the upstream and downstream flanking regions that were joined by PCR SOEing (splicing by overlap extension) (Horton, 1997).

Table 3.2 Strains, plasmids and primers used for site directed mutagenesis

Strain, plasmid, or primer	Relevant information	Source or reference
Strains		
P. putida		
AY44	Δ*motA*::pKG161 plasmid integration mutant of GB-1; Gm^r, Amp^r	This study
AY45	Δ*fliF* ::pKG161 plasmid integration mutant of GB-1; Gm^r, Amp^r	This study
Plasmid		
pKG161	Δ*sacB* derivative of pEX18Gm, Gm^r	Geszvain, personal communication
pAY42	internal fragment (0.3kb) of m*otA* amplified with MotA-F/-R2 ligated into pJET1.2/blunt; Amp^r	This study
pAY43	internal fragment (0.3kb) of *fliF* amplified with FliF-F/-R2 ligated into pJET1.2/blunt; Amp^r	This study
pAY44	*Xba*I/*Xho*I fragment from pAY42 cloned into *Xba*I/*Sal*I cut pKG161; Gm^r	This study
pAY45	*Xba*I/*Xho*I fragment from pAY43 cloned into *Xba*I/*Sal*I cut pKG161; Gm^r	This study
pEX18Gm	Gene replacement vector with MCS from pUC18; Gm^r, $oriT^+$, $sacB^+$	(Kelly-Winterberg, 1993)
pAY46	*fliA* 5' flanking region amplified w/ FliA-3-F (upstream)/FliA-3-R (junction), f*liA* 3' amplified with FliA-4-F (junction)/FliA-4-R2 (downstream), joined by PCR SOEing with FliA-3-F/FliA-4-R2 and cloned into pJET1.2/blunt; Amp^r	This study
pAY48	*fleN* 5' flanking region amplified w/ Coby-3-F (upstream)/Coby-3-R (junction), *fleN* 3' amplified with Coby-4-F (junction)/Coby-4-R (downstream), joined by PCR SOEing with Coby-3-F/Coby-4-R and cloned into pJET1.2/blunt; Amp^r	This study
pAY49	*Xba*I/*Xho*I fragment from pAY46 cloned into *Xba*I/*Sal*I cut pEX18Gm; Gm^r	This study
pAY51	*Xba*I/*Xho*I fragment from pAY48 cloned into *Xba*I/*Sal*I cut pEX18Gm; Gm^r	This study
Primer		
MotA-F	5’ TGCCCATGGCCATCGTCAAGG 3’	
MotA-F2	5’ TGGCTATCGGCACCTCCA 3’	
MotA-R2	5’ CACGCTTTCCAGGCCGAGC 3’	
FliF-F	5’ GCAGTCGTCGAAAACGCCCC 3’	
FliF-F2	5’ CGGCCGCCGCAAAGA 3’	
FliF-R2	5’ GCGGCTGCCAGTTTCAGGC 3’	
FliA-3-F	5’ CAGGCGTTCAGTGAAATCGGCG 3’	
FliA-3-R	5’ GCGGGCTCAACGTGC*C ATCTTGAAGCCGCTGGC* 3’	
FliA-4-F	5’ *GCCAGCGGCTTCAAGATG* GCACGTTGAGCCCGC 3’	
FliA-4-R2	5’ CCCTGACGTGACTCAGCCGTT 3’	
Coby-3-F	5’ CGCCGCTAAGAATTACCTGGTGC 3’	
Coby-3-R	5’ TGGCGTTCATAGCACGGGTCC *TACGGGATGCATGCTACCCAT* 3’	
Coby-4-F	5’ *ATGGGTAGCATGCATCCCGTA* GGACCCGTGCTATGAACGCCA 3’	
Coby-4-R	5’ TTCGACCTGGCCACTGGC 3’	
FlgM-3-F	5’ GGGCGAAGGTGACGTGGC 3’	
FlgM-3-R	5’ GCGCTGGGCTTCGAAATC*ACTGAAGTCGATGACCATGATCAG* 3’	
FlgM-4-F	5’ *CTGATCATGGTCATCGACTTCAGT*GATTTCGAAGCCCAGCGC 3’	
FlgM-4-2R	5’ ACTTGGCTGAGGGCGCG 3’	

PCR

The initial PCR was performed using DyNAzyme II DNA Polymerase (Finnzymes, USA) in 20 µl reaction volumes. The reaction mixture consisted of 40 ng of template DNA from GB-1, 0.8 µl each of 10 µM corresponding primers (MotA-F/R2, FliF-F/R2, FliA-3-F/3-R, FliA-4-F/4-R2, Coby-3-F/3-R, Coby-4-F/4-R) , 0.2 mM concentrations of dNTP, 1% dimethyl sulfoxide, 2 µl of 10X buffer, and 0.5 µl of DNA polymerase. PCR was performed as follows: initial denaturation of 3 min at 95˚C, followed by 30 cycles of denaturation for 15 sec at 95˚C, annealing for 15 sec at 59˚C, and extension for 30 sec at 72˚C. This was followed by final extension for 5 min at 72˚C.

With the PCR products from *fliA* and *fleN* primer sets, a second round of PCR was performed to construct ~1kb product without the internal region of the target gene. First, to make an ~1kb template for the next PCR, a short PCR cycle was performed as follows: initial denaturation (2 min at 95˚C), followed by 2 cycles of denaturation (15 sec at 95˚C), annealing (15 sec at 57˚C), extension (1 min at 69˚C). The reaction mixture contained 0.25 µl of 3' and 5' PCR products from the initial PCR, 2 µl 10 X buffer, 0.2 mM dNTP, and 0.5 µl DyNAzyme DNA polymerases. At last, the following PCR was performed to amplify the ≈1kb product from the previous PCR products after adding 0.8 µl of 10 µM 3-F and 4-R(2) primers: initial denaturation (2 min at 95˚C), followed by 30 cycles of denaturation (15 sec at 95˚C), annealing (15 sec at 62˚C), and extension (1 min at 68˚C). They were followed by a final extension (5min at 68˚C). A ≈1kb product was purified using Geneclean II kit (MP Biomedicals, OH, USA) for further cloning.

Plasmid construction

PCR products were cloned into pJET1.2/blunt (Fermentas, USA) as above. Purified plasmid was digested with *Xba*I and *Xho*I, and fragments from pAY42, 43 were ligated into pKG161, and fragments from pAY46, 48 were ligated into pEX18Gm (Table 3.2). pKG161 and pEX18Gm were digested with *Xba*I and *Sal*I, and phosphatase (Calbiochem, NJ, USA) was added to prevent self-ligation. Transformants were selected using LB with Gm.

Conjugation

Conjugation was performed as described in Chapter 2. Strains were mixed using the following volumes: 500 µl *P. putida* GB-1, 250 µl *E. coli* carrying pRK2013, and 1 ml *E. coli* carrying pAY44, 45, 49, or 51. *Pseudomonas* isolation agar (Fluka Analytical, USA) with Gm was used to select for colonies that underwent homologous recombination with the plasmid.

For plasmid integration, colonies that grew on selective agar plate were transferred on to LB with Amp, and then transferred again on *Pseudomonas* isolation agar with Gm to ensure chromosomal integration of the plasmid with Gm resistant gene.

Assays:

Motility assay

To test the motility, 0.25% agar Lept plates were used. This specific concentration of agar was selected after testing the motility with different concentrations of agar between 0.2% and 0.5%. This concentration was chosen because the 0.2% agar plate was too soft that *P. putida* GB-1 was able to spread over entire plate within 24 hours, and the 0.3% agar plate was too viscous that it took 48 hours for GB-1 to colonize the entire plate. Strains were incubated in LB medium with appropriate antibiotic, and 2.5 µl of the overnight culture was spotted in the middle of the plate.

Electron microscopy

Sample preparations were done and electron microscopy pictures were taken by Wendy Smythe. The slide to be observed was prepared by incubating an autoclaved poly-L-Lysine-coated slide in 30 ml Lept medium with the bacterial strain, and shaken for about a week at room temperature. Then the slide was fixed using a method adapted from the book "A Manual of Applied Techniques for Biological Electron Microscopy" by Michael J. Dykstra (1993), and stained with 0.15% Safranin O or 0.15% Alcian Blue dissolved in 0.1 M Cacodylate buffer using method adapted from Erlandsen and his colleagues (2004). Scanning electron micrographs of Au-Pd coated slides were taken using FEI Siron HR-SEM.

3.3 Results and Discussion

Complementation

FliD:

The transposon was inserted in *fliD* in IMO1. FliD is a protein that makes up the filament cap of the flagella and enables proper filament polymerization (Homma & Iino, 1985). In *Salmonella typhimurium,* without this protein, flagella failed to assemble and motility was lost (Homma et. al., 1985). This is most likely the same case in IMO1, because the complementation with *fliD* (pAY38) restored the motility (Figure 3.1). In addition, IMO1 complemented with pAY38 and the wild-type transformed with pAY38 showed a similar oxidation rate on both hard and soft agar (Figure 3.1 and 3.2). This means, FliD is required for complete assembly of flagella in *P. putida* GB-1 as well, and it is required for oxidation of Mn(II) at wild-type level. This also proved that it was not a coincidence that all IMO strains had a mutation in a flagella-related gene, but there actually is some relationship between having an incomplete flagella and fast Mn(II) oxidation on hard agar.

Surprisingly, IMO1 no longer exhibited increased oxidation on soft agar. In fact, it had decrease oxidation compared to the wild-type, because some part of the colony failed to oxidize at all. This effect of the different growth surface on Mn(II) oxidation is investigated further in Chapter 4.

The slight decrease in Mn(II) oxidation on both soft and hard agar by GB-1 transformed with pAY38 is probably due to the plasmid backbone of pAY38, because there was a slight oxidation decrease with GB-1 transformed just with pBBR1MCS-5 (Figure 3.2, picture not shown for soft agar).

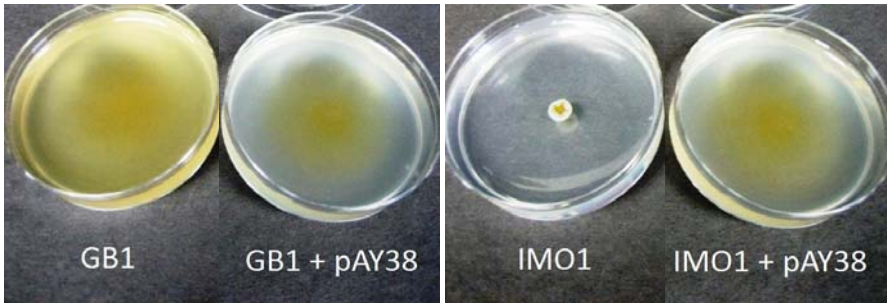

Figure 3.1 Complementation of motility-deficient *P. putida* IMO1 mutant by a wild-type *fliD* allele. Over night culture was spotted on 0.25% agar Lept plate, and incubated at room temperature for 48 hours. *P. putida* GB-1 and *fliD*::Tn5 mutant IMO1 were transformed with plasmid expressing *fliD* (pAY38).

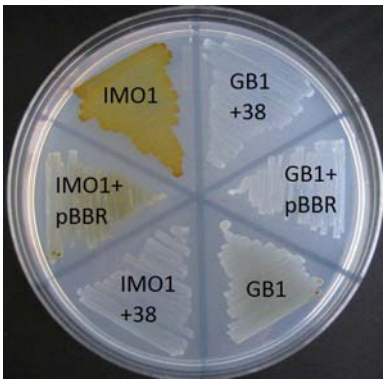

Figure 3.2 Complementation of increased Mn(II)-oxidizing *P. putida* IMO1 mutant by a wild-type *fliD* allele. Strains were inoculated on Lept hard agar, and incubated at room temperature for 72 hours.

Flagellin domain protein:

Flagellin is the major subunit of the flagellum and it makes up the filament. IMO3 only had a transposon insertion in this gene, while IMO4 had another insertion site in addition to this gene, and as a result, IMO3 was non-motile, but IMO4 was partially motile (Figure 3.3). Complementation of motility and wild-type level of Mn(II) oxidation on hard agar failed with both IMO3 and 4 (Figure 3.3 and 3.4), which suggests loss of motility with IMO3 and partial loss of motility with IMO4, and faster oxidation on hard agar was not due to disruption of flagellin domain protein encoding gene. One feasible explanation for the failure of the complementation is the disruption of transcription of downstream genes actually being responsible for the phenotype of IMO3 and 4. The downstream genes of the flagellin domain protein (PputGB1_3939) is a flagellar protein encoding gene (PputGB1_3938), which is only 77bp away from the stop codon of 3939, and *fliD* which is 81bp away from the stop codon of 3938. There is a possibility that 3938

and *fliD* are co-transcribed with 3939, and the phenotype of IMO3 and 4 is caused by the disruption of 3938, *fliD*, or any of them together.

Unexpectedly, IMO4 transformed with pAY35 lost its motility (Figure 3.3). It is hard to understand this phenomenon because the wild-type transformed with pAY35 did not lose its motility, which means over production of flagellin domain protein is not the cause of the loss of motility with IMO4.35.

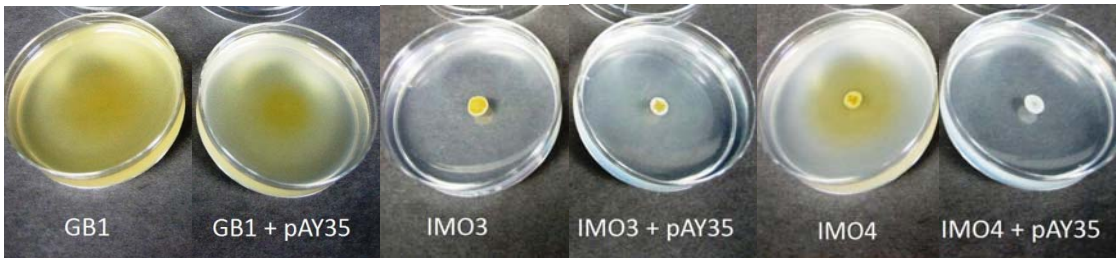

Figure 3.3 Complementation of motility-deficient *P. putida* IMO3 mutant and partially deficient mutant IMO4 by a wild-type flagella domain protein encoding region. Over night culture was spotted on 0.25% agar Lept plate, and incubated at room temperature for 48 hours.

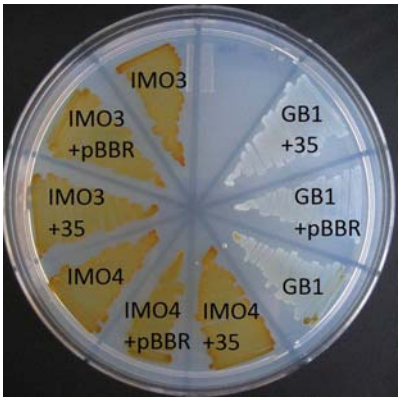

Figure 3.4 Complementation of increased Mn(II)-oxidizing *P. putida* IMO3 and 4 mutants by a wild-type flagella domain protein encoding region. Strains were inoculated on Lept hard agar, and incubated at room temperature for 72 hours.

FleN:

This gene was originally annotated as encoding Cobyrinic acid a,c-diamide synthase, but as a result of a BLAST search this gene was also annotated as *fleN*, which is a negative regulator of the number of flagella. There have been some studies done with *Pseudomonas aeruginosa* on *fleN*, which showed a loss of motility and an increased number of shorter flagella on a Δ*fleN* strain (Dasgupta et. al., 2000). The same phenotype

was observed with IMO5; loss of motility (Figure 3.5) and electron microscopy revealed an increase in number of shorter flagella (Figure 3.7).

Complementation of IMO5 with a plasmid containing *fleN* (pAY39) has failed; the motility was not restored and Mn(II) oxidation on hard agar remained increased. However, this result correlates with the complementation of the Δ*fleN* strain of *P. aeruginosa*, in which restoration of the motility failed with a multi-copy plasmid because the over-production of FleN repressed the synthesis of flagella (Dasgupta et. al., 2000). This is most likely the case with the failure of complementation with IMO5 as well, because GB-1 transformed pAY39 also became non-motile (Figure 3.5). GB-1 transformed with pAY39 also acquired the increased Mn(II) oxidation phenotype on hard agar (Figure 3.6).

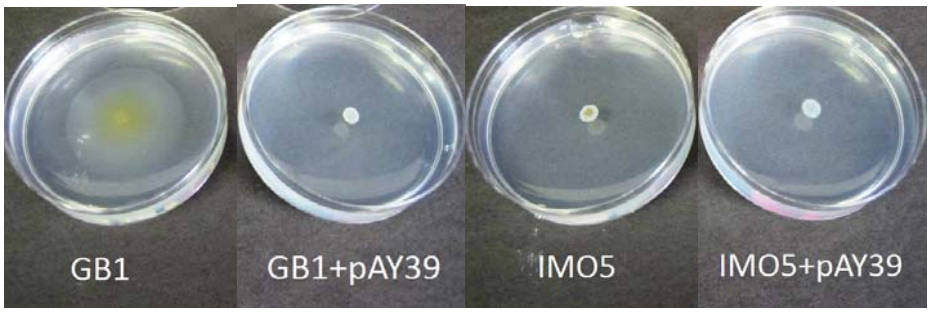

Figure 3.5 Complementation of motility-deficient *P. putida* IMO5 mutant by a wild-type *fleN* allele. Over night culture was spotted on 0.25% agar Lept plate, and incubated at room temperature for 24 hours. *P. putida* GB-1 and *fleN*::Tn5 mutant IMO5 were transformed with plasmid expressing *fleN* (pAY39).

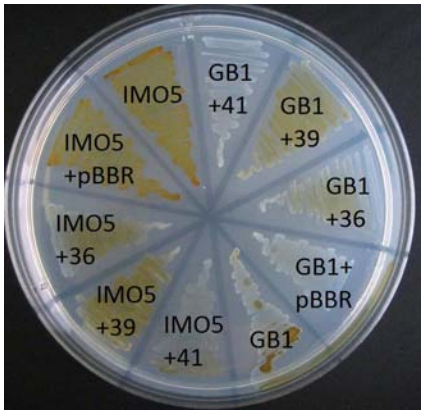

Figure 3.6 Complementation of increased Mn(II)-oxidizing *P. putida* IMO5 by wild-type genes. Strains were inoculated on Lept hard agar, and incubated at room temperature for 48 hours. *P. putida* GB-1 and IMO5 were transformed with pAY36 (*fleN, fliA*), pAY39 (*fleN*), or pAY41 (*fliA*).

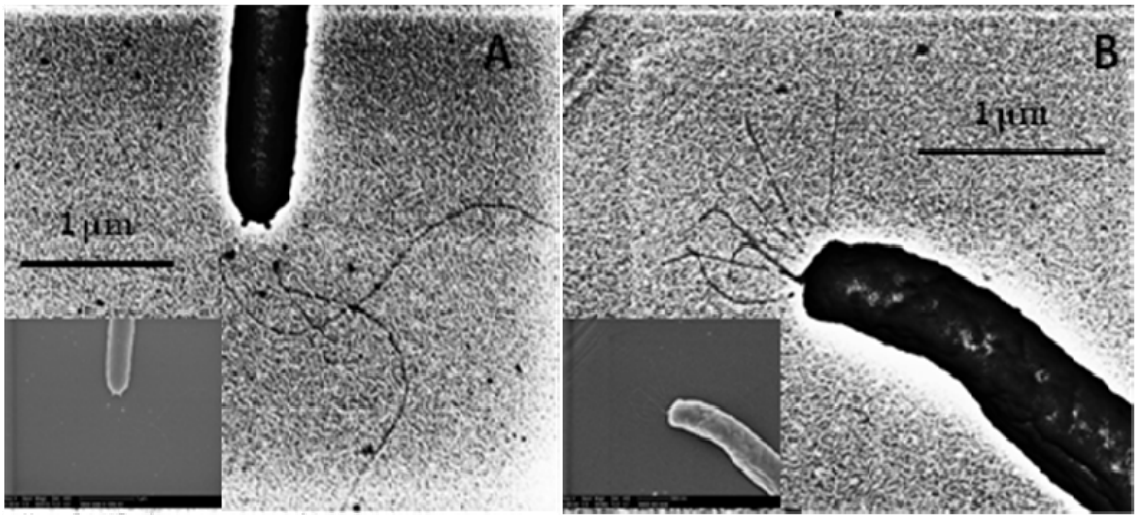

Figure 3.7 Scanning electron micrographs of *P. putida* GB-1 (A) and mutant IMO5 (B). Smaller picture on the left corner is the original picture, and the main picture was modified by inverting and equalizing it using Adobe Photoshop CS3 Extended to enhance the flagella structures.

FliA:

FliA is a sigma factor (σ^{28}) that is responsible for the transcription of late flagellar structural components, such as the flagellins and motor structures, after the hook-basal body structure is complete (Chevance & Hughes, 2008). Therefore, without *fliA*, there will be no flagella structure made beyond the hook-basal body.

fliA was not directly disrupted by transposon insertion, but it is downstream of *fleN* and possibly co-transcribed with *fleN*. Thus, *fliA* may be disrupted by the transposon insertion at *fleN*. As a result of complementation with *fliA*, IMO5 became motile, though slower than the wild-type (Figure 3.8), and Mn(II) oxidation on hard agar became slower, though still faster than the wild-type (Figure 3.6). This result indicates that *fliA* is at least partially responsible for the loss of motility and increased Mn(II) oxidation on hard agar exhibited by IMO5. However, *fliA* may not be the only reason for the phenotype of IMO5 because GB-1 transformed with *fliA* (pAY41) had better motility and less oxidation on hard agar than IMO5 complemented with *fliA*, which means over production of *fliA* is not the reason for the failure of the complete complementation of IMO5 with *fliA*.

Alternatively, *fliA* might not be the reason at all. Dasgupta et. al. (2000) showed that mutation in *fleN* does not affect transcription of *fliA* in *P. aeruginosa*. If this is also true in *P. putida*, partial complementation of IMO5 with pAY41 may be due to over

production of *fliA* that can now support the increased number of flagella due to disruption of *fleN*.

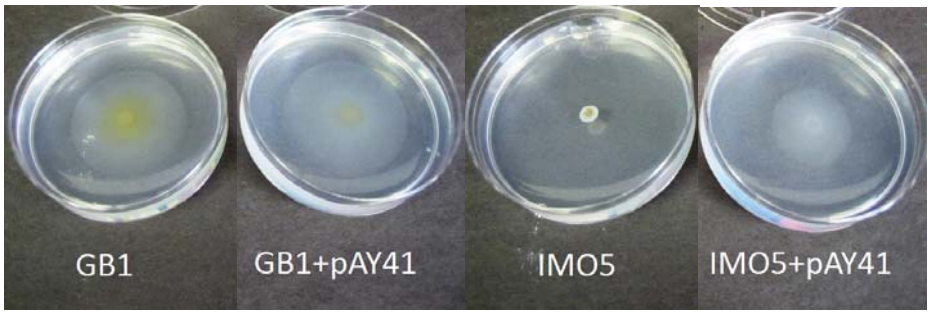

Figure 3.8 Complementation of motility-deficient *P. putida* IMO5 mutant by a wild-type *fliA* allele. Over night culture was spotted on 0.25% agar Lept plate, and incubated at room temperature for 24 hours. *P. putida* GB-1 and *fleN*::Tn5 mutant IMO5 were transformed with plasmid expressing *fliA* (pAY41).

FleN & FliA:

Since *fleN* and *fliA* are possibly co-transcribed, complementation of IMO5 with both *fleN* and *fliA* on the same plasmid was done. The results were the same as when IMO5 was complemented with *fleN* (pAY39) alone: loss of motility on soft agar, and increased Mn(II) oxidation on hard agar (compare Figure 3.5 and 3.9, and pAY36 and pAY39 of Figure 3.6). This is likely due to the characteristics of FleN that excess amount of it will interfere with synthesis of flagella, which results in loss of motility and increased Mn(II) oxidation.

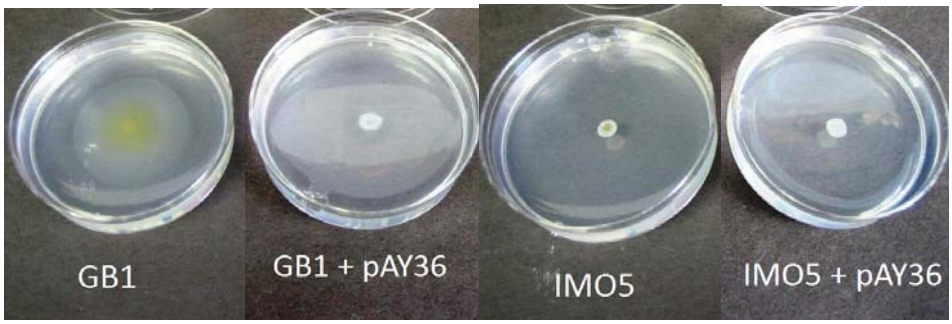

Figure 3.9 Complementation of motility-deficient *P. putida* IMO5 mutant by a wild-type *fleN* and *fliA* allele. Over night culture was spotted on 0.25% agar Lept plate, and incubated at room temperature for 24 hours. *P. putida* GB-1 and *fleN*::Tn5 mutant IMO5 were transformed with plasmid expressing *fleN* and *fliA* (pAY36).

FlgM:

IMO2 has a transposon insertion in *flgM*. FlgM is the anti-sigma factor of FliA. It binds to σ^{28} and prevents the association of σ^{28} with RNA polymerase, resulting in inhibition of late flagella gene transcription (Chevance & Hughes, 2008). Once the hook-basal body structure is complete, FlgM dissociates from FliA and is secreted out through the hook-basal body structure. Without FlgM, it is assumed that late flagella genes will be transcribed before the hook-basal body structure is made, but there has been no publication that describes the consequence without FlgM, so it is unclear what happens to the cell without FlgM.

From my results, IMO2 had a mixed population of motile and non-motile cells, which means some cells lost the ability to form complete flagella. Complementation with *flgM* (pAY37) exhibited more uniform motility than the original mutant, which was very similar to GB-1 transformed with pAY37 (Figure 3.10). Both GB-1 and IMO2 pAY37 transformants had slightly slower motility and had some non-motile cells. This is most likely due to over production of FlgM, because if there is too much FlgM, the cell could have difficulty exporting the FlgM so that it can start transcribing the later flagella genes.

Mn(II) oxidation on hard agar was also not fully complemented to the wild-type level, but it became slower and less intense than the IMO2 (Figure 3.11).

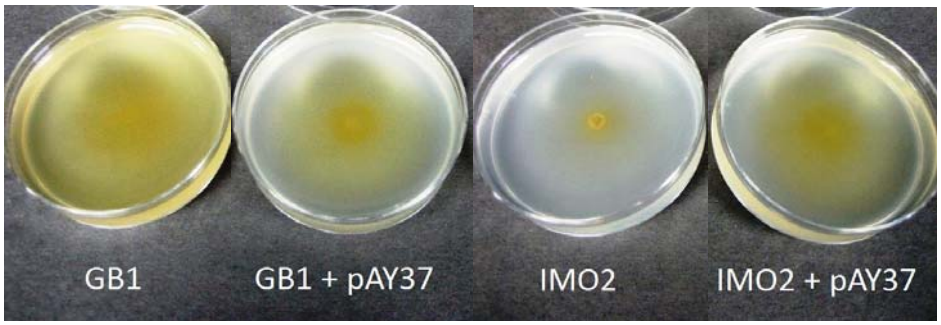

Figure 3.10 Complementation of partially motility-deficient *P. putida* IMO2 mutant by a wild-type *flgM* allele. Over night culture was spotted on 0.25% agar Lept plate, and incubated at room temperature for 48 hours. *P. putida* GB-1 and *flgM*::Tn5 mutant IMO2 were transformed with plasmid expressing *flgM* (pAY37).

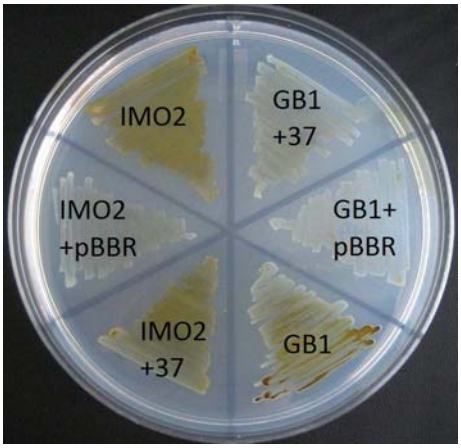

Figure 3.11 Complementation of increased Mn(II)-oxidizing *P. putida* IMO2 mutant by a wild-type *flgM* allele. Strains were inoculated on Lept hard agar, and incubated at room temperature for 72 hours

Site-directed mutations

MotA:

mot genes encode for proteins that are part of a sodium-driven flagellar motor, which is part of the late transcribed genes. In *Vibrio cholerae*, a *motA* mutation resulted in flagellated but non-motile cells (Lauriano et. al., 2004). I generated a plasmid integration *motA* mutant of *P. putida* GB-1 to investigate whether disruption of flagella synthesis, or loss of motility is responsible for increased Mn(II)-oxidation. Figure 3.12 and 3.13 show the motility and oxidation phenotype of four potential *motA* mutants. Unfortunately, the attempt to verify plasmid integration at the correct site by PCR failed. This may be due to loss of the integrated plasmid while growing the culture, or because there was no insert at the correct place. Therefore, I cannot say that the MotA strains truly had a defective motor system. However, MotA-1 and 2 had a severe defect in motility, and had more increased Mn(II) oxidation than MotA-3 and 4 on hard agar. If MotA-1 and 2 really have a mutation in the *motA* gene, it means it is not the energy conservation from not making the flagella structure that is allowing motility-deficient mutants to have increased Mn(II) oxidation on hard agar.

Unfortunately, after storing the strains in -80˚C, all of the isolates lost their defects in motility and the increased Mn(II) oxidation phenotype. When the isolates stored in -80˚C were inoculated on LB agar plates, only a few colonies appeared after 24 hours. When isolates were inoculated on LB agar plate with Gm, no growth was observed. Colonies grew on LB agar plates were tested for the oxidation rate on Lept agar plates

and for the motility on soft Lept agar plates, but both assay results showed all of the colonies had the wild-type phenotype. The reversion may be because loss of MotA is an unfavorable phenotype. In support of this, there was a severe growth defect on Lept plates with all of the MotA strains. This also explains why there was no *mot* mutation with transposon mutagenesis. It can also be because the plasmid backbone used for plasmid integration mutagenesis was not favorable in *P. putida* GB-1. This growth defect might also be the reason for *motA*::pKG161 mutants not exhibiting as increased Mn(II) oxidation as IMO5.

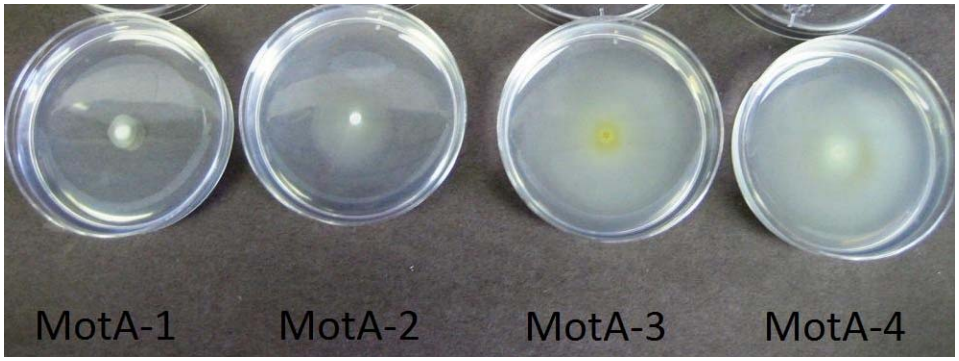

Figure 3.12 Motility test on different *motA*::pKG161 isolates. While creating plasmid integration mutant of *motA*, mutants with different degree of motility defects were isolated. Overnight culture was spotted and incubated at room temperature for 48 hours.

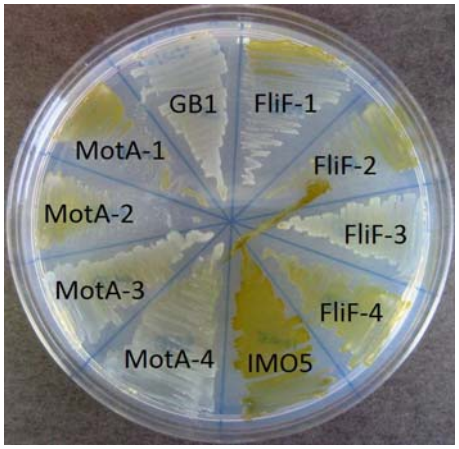

Figure 3.13 Mn(II)-oxidation on hard agar by *motA*::pKG161 and *fliF*::pKG161 strains. Strains were inoculated on Lept hard agar, and incubated at room temperature for 48 hours.

FliF:

FliF is the basal-body membrane and supramembrane ring and collar. The initiating event of flagellar biogenesis is the insertion of FliF into the membrane and its self-assembly into the ring structure (Ueno et. al., 1992). Without FliF, bacteria will not be able to assemble any flagella structure. Therefore, I chose to disrupt *fliF* in *P. putida* to determine the effect of loss of flagella on Mn(II) oxidation. As shown in Figure 3.14, FliF-4 lost its motility completely as expected, and was confirmed by PCR to have the plasmid inserted into *fliF*. FliF-1 and 2 also had a deficiency in motility, but the plasmid integration into the correct site was not confirmed by PCR, probably due to the same reasons as PCR failure of MotA mutants. FliF-3 did not show any phenotypic difference compared to the wild-type, which suggests its plasmid was lost at very early stage or was not inserted correctly. When the Mn(II) oxidation on hard agar was tested, FliF-4 showed the most increased oxidation and FliF-1 and 2 showed a slight increase in oxidation. This means the more motility-deficient the strain is, the more increased Mn(II) oxidation it exhibits.

FliF-4 was confirmed to have the plasmid integrated at the *fliF* with PCR reaction, so the result shown with FliF-4 is the result due to the loss of flagella. However, FliF mutants also reverted to the wild-type after storing in -80˚C, possibly for the same reason as MotA mutants.

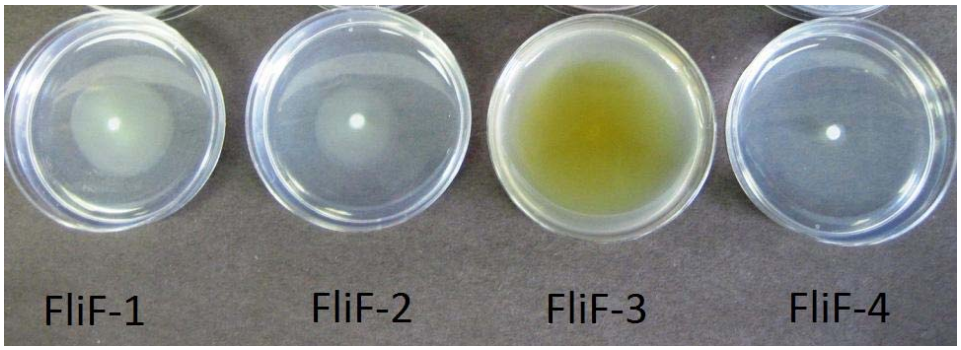

Figure 3.14 Motility test on different *fliF*::pKG161 isolates. While creating plasmid integration mutant of *fliF*, mutants with different degree of motility defects were isolated. Overnight culture was spotted and incubated at room temperature for 48 hours.

Correlation between Motility or/and Flagella synthesis and Mn(II) oxidation

From the transposon mutagenesis result, it seemed that there is a correlation between disruption of flagella synthesis and increased Mn(II) oxidation on hard agar. This correlation was confirmed by the complementation of IMO1with *fliD* and partial complementation of IMO5 with *fliA*. However, it seems that the real correlation is between motility and Mn(II) oxidation because if the reason for the increased oxidation on hard agar is due to energy used synthesizing or regulating the flagella assembly being redirected to Mn(II) oxidation, then the mutants should keep the increased Mn(II) oxidation phenotype even on the soft agar. Another reason energy use is an unlikely explanation is that IMO5 had an increased number of flagella confirmed by the electron micrograph (Figure 3.7) but exhibited increased Mn(II) oxidation on hard agar. In addition, if my MotA mutant had plasmid integrated at *motA*, we predict they would have the complete flagella without the motor, but they still exhibited increased Mn(II) oxidation.

All of the IMO strains had defects in motility, but IMO2 and 4 exhibited some motility. Therefore, complete loss of motility is not the requirement for fast Mn(II) oxidation on hard agar, rather, any degree in motility defect seems to increase the Mn(II) oxidation on hard agar. Strains constructed by plasmid integration mutagenesis of *motA* and *fliF* showed that the less motile the colony on soft agar, the faster the oxidation on hard agar. However, of the two IMO mutants with motility, only IMO2 exhibited slightly slower oxidation than the other IMO strains. Thus, the motility defect does not perfectly correlate with the rate of Mn(II) oxidation. Therefore, there has to be some other factor that has more direct effect on Mn(II) oxidation.

3.4 Future directions

Here, I have shown that there is some link between Mn(II) oxidation and motility of *P. putida* GB-1. However, I have not been able to understand the mechanism of this correlation. It seems that any kind of defect in motility causes the strain to increase the Mn(II) oxidation on hard agar. The question is why they would have any regulation depending on motility while they are on a solid surface where they cannot swim. There have been some studies done to show a correlation between motility and virulence

systems through the FliA-FlgM regulatory system (Eichelberg & Galan, 2000; Wang et. al., 2005). Eichelberg and Galan's result indicated FliA is necessary for expression of the virulence proteins. Also, Wang et. al. (2005) found that when bacteria are on a dry surface, flagellin assembly fails and FlgM is not excreted, resulting in down regulation of all of the late flagella genes due to excess FlgM associating with FliA. From these results, it is possible that FliA is regulating the Mn(II) oxidation. However, in the case of the virulence factors, they are down-regulated in absence of FliA, but in case of Mn(II) oxidation, disruption of late flagella genes up-regulates the oxidation. Therefore, FliA may be transcribing a protein that down-regulates Mn(II) oxidation. This can explain why IMO5 was able to partially restore the wild-type phenotype with *fliA*. However, it cannot explain why IMO2 had the increased Mn(II) oxidation on hard agar because without FlgM, IMO2 should have decreased Mn(II) oxidation on hard agar. This has to be further investigated by constructing inframe deletions of *fliA* and *flgM*. I have attempted to construct inframe deletions of *fliA*, *fleN* and *flgM*, but was not successful due to some complications with the plasmid used.

To further understand the correlation between motility and Mn(II) oxidation, isolating more transposon mutants with increased oxidation on hard agar will be useful. Since I isolated increased Mn(II) oxidation mutants from only a few conjugations, it is too early to conclude that *P. putida* GB-1 only increases Mn(II) oxidation when it has disruption in flagella related genes. In addition, reconstructing *motA* and *fliF* plasmid integration mutants may be useful to obtain more definite results. Furthermore, making chemotaxis mutants may give further important information about the relationship between motility and Mn(II)-oxidation, because some of the non-motile mutants may be moving, but not through a nutrient gradient, therefore not showing any movement on the soft agar. It will be also helpful to look at more strains with electron microscopy to check the existence of flagella, and if they possess flagella, it will be useful to look under the light microscope to see if they are actually not moving at all or they just can't move on soft agar.

This was the first time that the possibility of the relationship between motility and Mn(II) oxidation has been discovered. There are still many questions as to how they are

correlated, but understanding this relationship can enrich our understanding of Mn(II) oxidation.

3.5 References

Chevance FFV, and Hughes KT. 2008. Coordinating assembly of a bacterial macromolecular machine. *Nature Reviews*. 6:455-465

Choy WK, Zhou L, Syn CKC, Zhang LH, and Swarup S. 2004. MorA defines a new class of regulators affecting flagellar development and biofilm formation in diverse *Pseudomonas* species. *J. Bacteriol.* 186(21):7221-7228

Dasgupta N, Arora SK, and Ramphal R. 2000. *fleN*, a gene that regulates flagellar number in *Pseudomonas aeruginosa*. *J. Bacteriol.* 182(2):357-64

Eichelberg K, and Galan JE. 2000. The flagellar sigma factor FliA (sigma 28) regulates the expression of *Salmonella* genes associated with the centisome 63 type III secretion system. *Infect. Immun*. 68:2735–2743

Erlandsen SL, Kristich CJ, Dunny GM, and Wells CL. 2004. High-resolution visualization of the microbial glycocalyx with low-voltage scanning electron microscopy: dependence on cationic dyes. *Journal of Histochemistry & Cytochemistry.* 52(11):1427-35

Homma M, and Iino T. 1985 Locations of hook-associated proteins in flagellar structures of *Salmonella typhimurium*. *J. Bacteriol*. 172:183-189

Homma M, Kutsukake K, and Iino T. 1985. Structural genes for flagellar hook-associated proteins in *Salmonella typhimurium*. *J. Bacteriol.* 163:464-71

Horton R M. 1997. In vitro recombination and mutagenesis of DNA. SOEing together tailor-made genes. *Methods Mol. Biol*. 67:141–149

Kelly-Wintenberg, K, South SL, and Montie TC. 1993. Tyrosine phosphate in a- and b-type flagellins of *Pseudomonas aeruginosa*. *J. Bacteriol*. 175:2458–2461

Lauriano DM, Ghosh C, Correa NE, and Klose KE. 2004. The sodium-driven flagellar motor controls exopolysaccharide expression in *Vibrio cholerae*. *J. Bacteriol.* 186(15):4864-4874

Ueno T, Oosawa K, and Aizawa SI. 1992. M ring, S ring and proximal rod of the flagellar basal body of *Salmonella typhimurium* are composed of subunits of a single protein, FliF. *J. Mol. Biol.* 227(3):672-7

Verma A, Schirm M, Arora SK, Thibault P, Logan SM, and Ramphal R. 2006. Glycosylation of b-type flagellin of *Pseudomonas aeruginosa*: Structural and genetic basis. *J. Bacteriol.* 188(12):4395-4403

Wang Q, Suzuki A, Mariconda S, Porwollik S, and Harshey RM. 2005. Sensing wetness: a new role for the bacterial flagellum. *European molecular Biology Organization Journal*. 24:2034-42

Chapter 4

Characterization of Mutant Strains

4.1 Introduction

In Chapter 2, I described the 10 DMO strains and 5 IMO strains that I isolated and the genes disrupted in these strains. Then, in Chapter 3, all five IMOs were further analyzed to understand the correlation between Mn(II) oxidation and motility and/or flagella regulation. During the analysis of IMOs, the mutants were tested for their motility on soft agar. Surprisingly, all of the non-motile mutants became very slow oxidizers, opposite to the fast oxidizing phenotype that they exhibited on hard agar. Therefore, I wanted to screen the effect of growth on soft agar, as well as growth in liquid media, on the ability of all the DMO and IMO mutants to oxidize Mn(II).

While testing the Mn(II) oxidation in liquid, there were differences in amount of biofilm attached to the glass tube among different strains. Also, as mentioned in the introduction, biofilm formation and Mn(II) oxidation has been thought to have some kind of correlation because most of the Mn(II) oxidases identified are secreted and/or loosely bound to the outer membrane (Emerson and Ghiorse, 1992; Okazaki et. al., 1997; Johnson & Tebo, 2008; Francis &Tebo, 2002). From this information, I thought it would be interesting to compare the oxidation phenotypes with biofilm formation phenotypes to confirm any correlations between those two.

In addition, while growing *P. putida* GB-1 in the 30˚C incubator, I noticed a more intense Mn(II) oxidation compared to when it was grown in room temperature.

Furthermore, there were some mutants with transposon insertions in heat-shock related protein encoding genes, so, the effect of temperature was tested by incubating the strains at different temperatures.

4.2 Materials and methods

Strains and media used

All of the strains and media used are the same as in Chapter 2. The motility assay using soft agar is explained in Chapter 3, section 2. The oxidation rate in liquid medium was tested by inoculating 1.5 µl of an overnight culture that had been grown in LB medium with appropriate antibiotic, into 5 ml of Lept medium, and placing on a shaker at room temperature. Mn(II) oxide formation was assessed by visual comparison to wild-type.

To test differences in Mn(II) oxidation rate at different temperature, strains were inoculated onto 1.5% agar Lept plates and incubated at room temperature (22 ~ 25˚C) and 30˚C.

Biofilm and cell growth measurements

The biofilm assay was modified from the method described in a paper by Stepanovic and his colleagues (2007). Bacteria were grown in Lept medium overnight before the OD_{600} was adjusted to 0.132 by diluting with Lept medium. Then it was further diluted 100 fold, vortexed, and 200 µl of the diluted culture was added to each well in a 96-well microtiter plate. All of the cultures were incubated in triplicate including the control, which was uninoculated Lept medium. Immediately after inoculation, OD_{600} was measured using a plate reader to record the initial cell density. After 24 hours of static incubation at room temperature, the plate was shaken with the plate shaking function on the plate reader, and then OD_{600} was measured.

Additionally, protein concentration was used to determine the growth difference between different strains. 150 µl of the culture was carefully transferred to another 96-well plate with 150 µl of Coomassie Plus Reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) in each well, and mixed by pipetting up and down about 10 times.

After 30 minutes of incubation at room temperature, the absorbance at 595 nm was measured.

The culture in the original 96-well plate was discarded, and the wells were washed three times with 300 µl PBS buffer. After the plate was air dried, 150 µl Crystal Violet (CV) was added to each well, incubated for 15 minutes, washed under running water, air dried again, and 150 µl of 95 % ethanol was added to elute the CV bound to biofilm. After 30 minutes of incubation, the absorbance at 570 nm was measured.

4.3 Results

Mn(II) oxidation at different temperatures

I wished to screen for a temperature effect on Mn(II) oxidation. Strains were inoculated on Lept hard agar, and incubated at two different temperatures, room temperature (22 ~ 25˚C) and 30˚C. The results are presented in Table 4.1. The wild-type colonies started oxidizing Mn(II) faster (Figure 4.1 E) and much more intensely (compare Figure 4.1 A & B) at 30˚C than at room temperature. Even after one week, the intensity of the Mn(IV) oxide color in wild-type cells grown at room temperature did not approach that of cells grown at 30˚C. DMO8 behaved in the same way as the wild-type, probably due to its reversion. IMO2 also exhibited notably slower oxidation in room temperature than at 30˚C, but the colony was fully oxidized after 48 hours, and the intensity of the oxide color was as intense as the colony incubated at 30˚C. DMO7, 9, and 10 showed almost no Mn(II)-oxidation in room temperature, even after 10 days, but some oxidation in 30˚C. DMO1,2, IMO1, 3, 4, and 5 only showed slightly slower Mn(II)-oxidation at room temperature compared to 30˚C, and did not demonstrate much difference in intensity of Mn(IV) oxide color after it reached maximum oxidation. Contrary to those results, DMO3 and 4 produced more Mn(IV) oxides at room temperature than at 30˚C. DMO5 and 6 both did not show any oxidation in either condition even after 10 days of incubation.

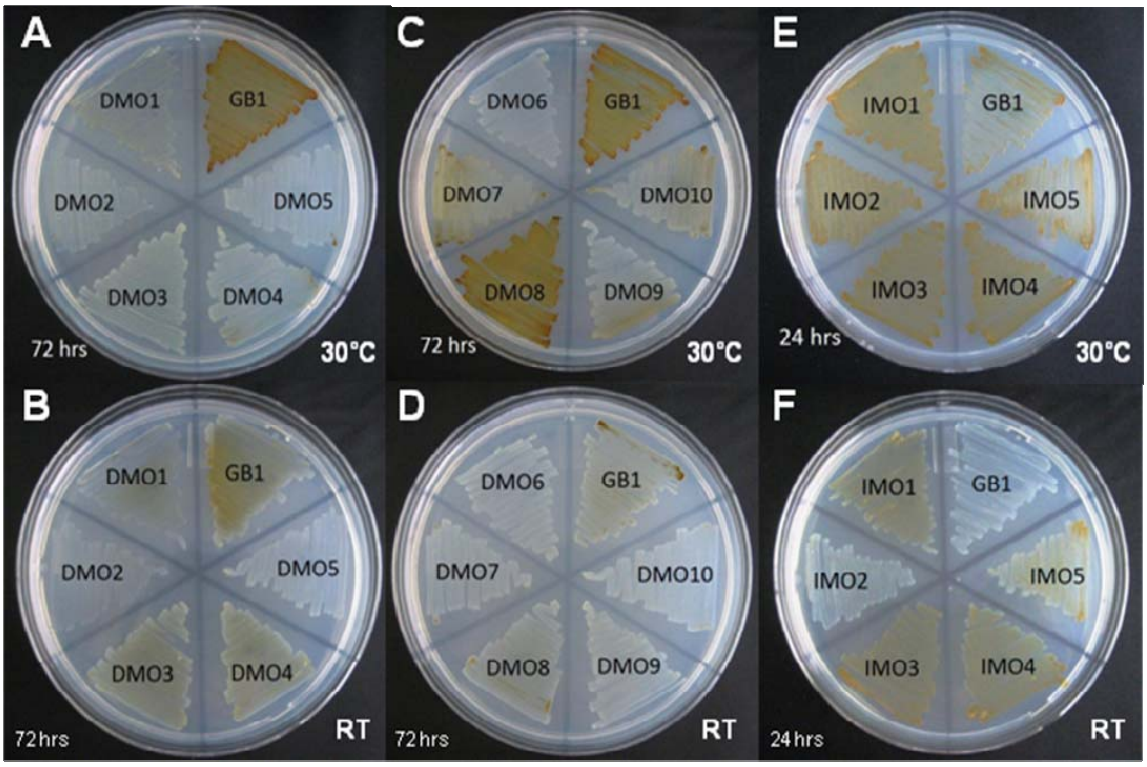

Figure 4.1 Mn(II) oxidation differences at different temperatures. Strains were inoculated on Lept plate, and incubated at room temperature (22 ~ 25˚C) or 30˚C. Strain names are indicated on top of each strain. GB-1 annotates for *Pseudomonas putida* GB-1. Pictures A-D were take after 72 hours of inoculation, E and F were taken after 24 hours.
A. DMO1-5 incubated at 30˚C, B. DMO1-5 incubated at room temperature, C. DMO6-10 incubated at 30˚C, D. DMO6-10 incubated at room temperature, E. IMO1-5 incubated at 30˚C, F. IMO1-5 incubated at room temperature.

Mn(II) oxidation on soft agar

To test the effect of soft agar on Mn(II) oxidation, each of the mutant strains were spotted on 0.25% agar Lept plate. As expected, none of the DMO strains exhibited a motility defect under these conditions. For IMOs, swimming ability and oxidation ability were correlated, meaning the less motile the strain, the less oxidizing ability it had on soft agar. Results from DMOs did not have the same correlation pattern; therefore it is necessary to compare the results from soft agar with disrupted genes in each strain, and this will be discussed in the next section. Results of both motility and oxidation on soft agar after 49 hours of incubation at room temperature are summarized in Table 4.1, and representative photos are shown in Figure 4.2. It was also interesting that Mn(II) oxidation occurred faster on the soft agar compared to the hard agar with GB-1 (Figure 4.3) and some other DMO strains.

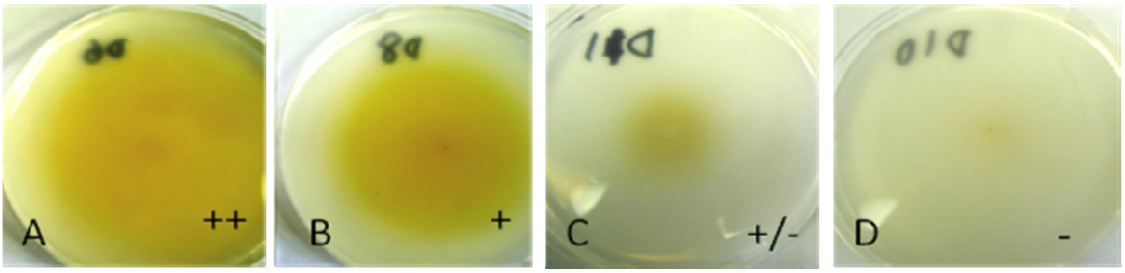

Figure 4.2 Motility and Mn(II) oxidation on soft agar. 2.5 µl of overnight culture was spotted in the middle of 0.25% agar Lept plate, and incubated for 48 hours at room temperature. Each strains are; A: DMO6, B: DMO8, C: DMO1, and D: DMO10. DMO6 exhibited same Mn(II) oxidation phenotype as the wild-type on soft agar, and DMO6 picture was chosen over the wild-type one due to its superior picture quality. Signs on the lower right hand corner of each picture are the oxidation intensity index used in the Table 4.1.

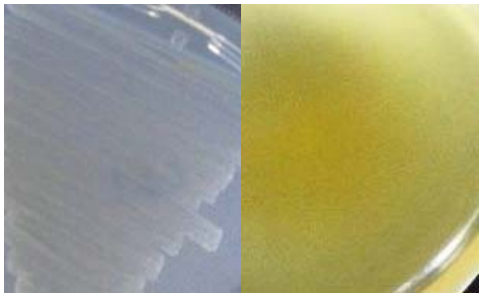

Figure 4.3 Difference in Mn(II) oxidation at 48 hours between hard agar (left) and soft agar (right). *P. putida* GB-1 was inoculated on hard agar and soft agar and incubated at room temperature for 48 hours.

Table 4.1 Oxidation ability of strains in different conditions

	[a] Hard agar			[c] Soft agar		[d] Liquid
Strains	49hrs	10days	[b] Temperature	motility	oxidation	oxidation
GB1	-/+	++	++	++	++	++
DMO1	-	-/+	-/+	++	-/+	+
DMO2	-	-/+	-/+	++	+	++
DMO3	-	-/+	-	++	+	+
DMO4	-	-/+	-	++	+	+
DMO5	-	-	-/+	++	+	++
DMO6	-	-	-/+	++	++	++
DMO7	-	-/+	+	++	-/+	-/+
DMO8	-/+	++	++	++	+	+
DMO9	-	-/+	+	++	++	-/+
DMO10	-	+	+	++	-	++
IMO1	++	++	-/+	-	-	++
IMO2	+	++	+	++	+	++
IMO3	++	++	-/+	-	-	++
IMO4	++	++	-/+	-/+	+	++
IMO5	++	++	-/+	-	-	-/+

[a] Strains were inoculated on 1.5% agar Lept plate for specified time length at room temperature. ++ indicates the entire colony oxidized Mn(II), + indicates more than half of the colony oxidized Mn(II), -/+ indicates some part of the colony started to oxidize Mn(II), - means the entire colony is still white.

[b] Strains were incubated at room temperature (RT) or 30˚C. ++ means both the intensity and the speed of the oxidation increased significantly at 30˚C compared to RT. + means there was some increase intensity and speed of oxidation at 30˚C but not as significant. -/+ means there was no significant difference between RT and 30˚C. – means oxidation at RT was more intense and faster than at 30˚C. Pictures are shown in Figure 4.1.

[c] 2.5 µl of overnight culture was spotted in the middle of 0.25% agar Lept plate, and incubated for 48 hours at room temperature. For motility; ++ means the strain covered the entire plate evenly, -/+ means it formed a colony where the culture was spotted, but still swim across the plate, and – means the strain did not move from the originally spotted area and formed a thick colony. For oxidation; ++ means the entire plate was oxidized, + means more than 40% but not the entire plate was oxidized, -/+ means less than 40% of the plate was oxidized, and - means less than 5% of the colony was oxidized. Pictures are shown in Figure 4.2.

[d] 1.5 µl of overnight culture was inoculated in Lept medium, and incubated for 24 hours in room temperature. There was no culture that showed more intensive color of Mn(IV) oxide than wild-type, so the oxidation amount of wild-type was set as ++. + means less intense than wild-type, and -/+ means there was very little Mn(IV) oxide observed in the tube. Pictures are shown in Figure 4.4 as a reference.

Mn(II) oxidation in liquid

Mn(II) oxidation in the liquid environment was tested by inoculating strains in test tubes with Lept medium. The intensity of oxidation was determined visually by comparison to wild-type. Pictures of the cultures were taken after 24 hours of shaking at room temperature (Figure 4.4), and results are summarized in Table 4.1. In this condition DMO7, 9 and IMO5 produced significantly less Mn(IV) oxides than wild-type. DMO2 produced a large amount of Mn(IV) oxides, but most of the oxides were in the liquid as particles unlike with the other strains that had most of the oxides attached to the glass surface of the tube (Figure 4.4). DMO5 and 6 also had more oxides in the liquid than other strains, but also had large amount of oxides on the wall of the tube. There was no apparent correlation between how the strains oxidized Mn(II) on hard agar and in liquid.

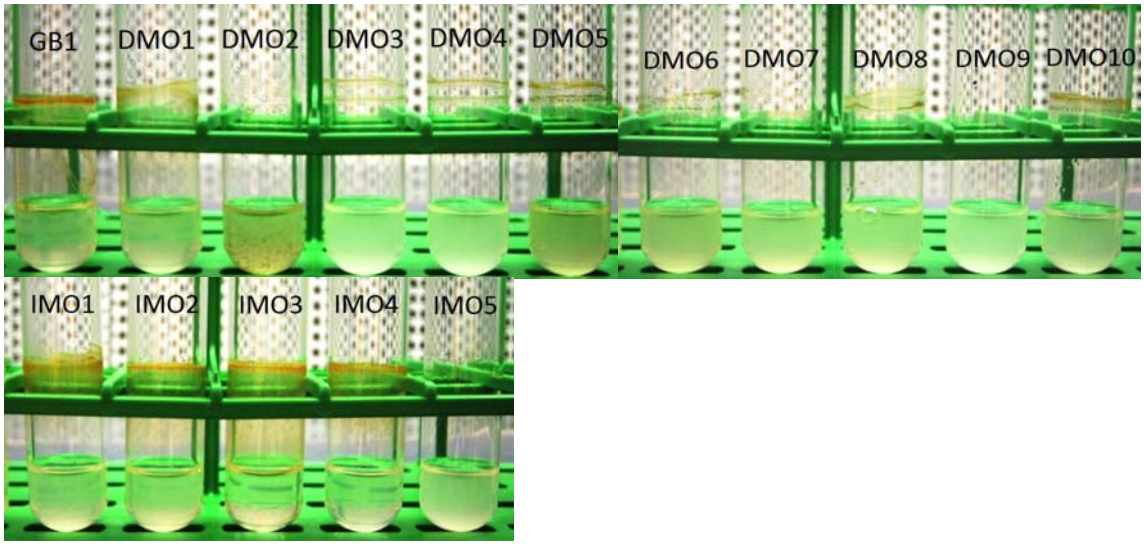

Figure 4.4 *P. putida* GB-1 and its mutant strains in liquid medium. 1.5 µl of overnight culture was inoculated into 5 ml of Lept medium in a test tube. Pictures were taken after shaking the culture for 24 hours at room temperature. The names of the strains are indicated on the top of the tube.

Biofilm formation

The amount of biofilm formed was measured using Crystal Violet. Strains were incubated in a 96-well plate for 24 hours at room temperature without shaking. In addition to the biofilm formation, cell growth was measured using OD_{600} and protein concentration. All of the results are shown in graphs (Figure 4.5).

For the cell growth, most of the DMOs did not have a significant difference in growth compared to the wild-type (Figure 4.5 B&C), except for DMO1 that consistently showed lower cell concentration with both measurements. In contrast to DMOs, all of the IMOs, except IMO2, had some growth defect.

The results showed that DMO1 secreted significantly more biofilm compared to wild type, and all other mutant strains produce the same or less biofilm compared to wild type (Figure 4.5 A). DMO1 had a significant growth defect, so the biofilm result suggests each DMO1 cell produces more than twice as much biofilm compared to all other strains tested. DMO5, 6, IMO2 and 5 produced less than half of the amount of biofilm compared to wild type. DMO5, 6, and IMO2 all had no significant growth defect, so the defect in biofilm formation is likely not due to a growth defect. IMO5 had a significant growth defect, but it had a more severe defect in biofilm formation, so it is unlikely that the decrease in biofilm formation is due to the growth defect. All of the IMO mutants exhibited relatively lower biofilm concentration compared to the wild-type, but IMO5 had the most severe defect in biofilm production.

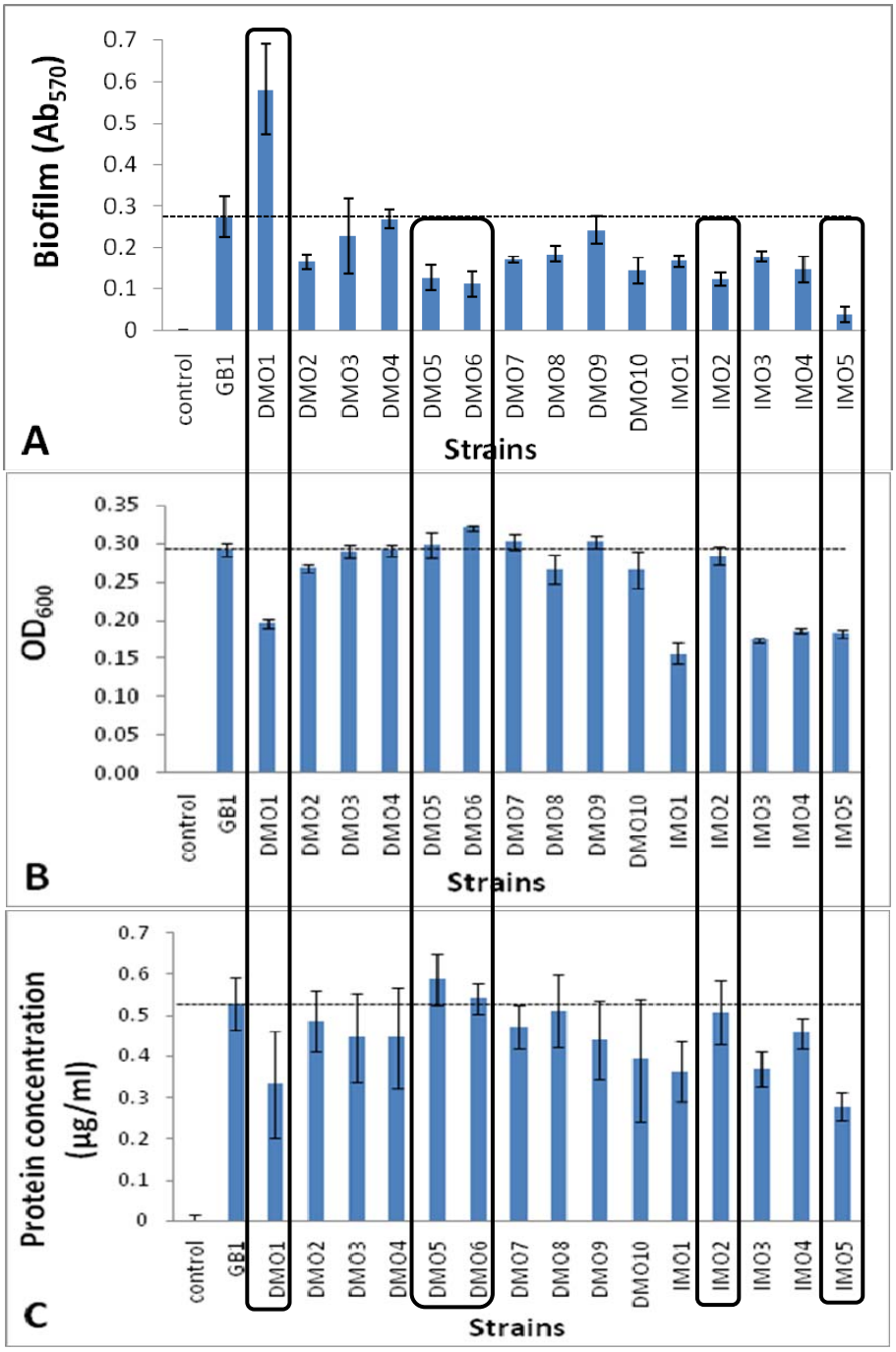

Figure 4.5 Biofilm concentration and cell growth.
Cells were incubated statically in 96-well plate overnight at room temperature for 24hours. Cultures were grown in triplicate, and the error bar indicates the standard deviation. The dotted horizontal line indicates the value of the wild-type
A. Biofilm concentration was measured using crystal violet.
B. OD_{600} was used to measure a cell concentration.
C. Protein concentration was measured using Coomassie plus reagent.

4.4 Discussion

Mn(II) oxidation in different environments

Mn(II) oxidation rate and intensity was tested in different conditions: solid surface vs. semi-solid surface vs. liquid, and room temperature vs. 30˚C. Results were compared to the Mn(II) oxidation of the wild-type, and will be discussed according to the disrupted gene(s) in each strain. It is important to note that the Mn(II) oxidation rate was faster at 30˚C in wild-type, possibly due to faster growth rate, and intensity was higher at 30˚C as well, but for some unknown reasons.

DMO1

This strain had a disruption in the uroporphyrin-III C-methyltransferase encoding gene, which is necessary for vitamin B_{12} and siroheme synthesis. It exhibited low Mn(II) oxidation ability on hard agar, but it did oxidize a little bit on soft agar and in liquid. Therefore, it is assumed that uroporphyrin-III C-methyltransferase itself or a product synthesized by this enzyme is necessary for optimal Mn(II) oxidation. In addition, there was no difference in intensity of oxidation between different temperatures, which may suggest uroporphyrin-III C-methyltransferase is involved in part of the oxidation mechanism that is affected by the temperature.

DMO2

This strain exhibited better Mn(II) oxidation on soft agar and in liquid than on hard agar. From the disrupted genes, MCO type 2, nucleotidyl transferase, and heat shock protein domain protein encoding genes, it is hard to understand this, but possibly one or more of those genes are required for Mn(II) oxidation on solid media.

One of the genes disrupted was a heat shock protein domain protein encoding gene, and there was no significant difference in Mn(II) oxidation between the two different temperatures. If the heat shock protein is one of the causes of the increased Mn(II)-oxidation in 30˚C, loss of temperature effect due to disruption of the gene related to the heat shock protein can be understandable.

DMO3&4

DMO3 and 4 had transposon inserts in different genes, β-ketoacyl-ACP (acyl-carrier protein) synthase I for DMO3 and a hypothetical protein for DMO4, but they showed the same phenotype in every condition. It is especially remarkable that they had slightly increased Mn(II) oxidation at room temperature, while all other strains showed same or decreased oxidation at room temperature compared to at 30˚C. Therefore, it is possible that the hypothetical protein disrupted in DMO4 is involved in fatty acid biosynthesis as well. With this assumption, we can say fatty acid biosynthesis is important in fully functional Mn(II) oxidation in every condition, and when strains are incubated at different temperatures, fatty acid biosynthesis is more critical at higher temperature. The decrease in Mn(II) oxidation at higher temperature may be due to a higher fluidity of a cell membrane, because fatty acid becomes more fluid at higher temperature and it is one of the major components of the membrane, and a defect in biosynthesis of fatty acid results in a more severe defect in Mn(II) oxidation.

DMO5&6

These two strains had the same transposon insertion sites, and showed similar phenotypes in different conditions, so they are discussed together. Both had mutations at the transmembrane regulator PrtR, and Acyl-CoA synthetase encoding genes, and possibly at acetolactate synthase large subunit encoding genes due to polar effect, and they both exhibited almost complete loss of Mn(II) oxidation on hard agar, at both temperatures. However, their oxidation ability was close to the wild-type level in semi-solid and liquid conditions. These results suggest the gene(s) disrupted are necessary in Mn(II) oxidation on hard surface where bacteria cannot swim, but is not necessary in semi-solid and liquid conditions. One possibility is that PrtR is not just sensing the temperature, but also sensing the surface environment that is necessary for turning on some biochemical pathway leading to Mn(II) oxidation on a solid surface.

DMO7

This strain had transposon insertions in ammonium transporter and major facilitator superfamily encoding genes, and exhibited very low Mn(II) oxidation in every condition, except when it was incubated in 30˚C. Even at 30˚C, the oxidation intensity was not high, but it was significantly more than at room temperature. This suggests that the disrupted gene(s) is necessary for Mn(II) oxidation at room temperature, but its loss can be partially over-come at higher temperature.

DMO8

This strain had a mutation in transmembrane regulator PrtR/I, and ABC transporter related protein encoding genes. It originally exhibited decreased Mn(II) oxidation phenotype on hard agar, but after a year of storage in -80˚C its oxidation phenotype reverted to the wild-type. DMO8 showed decreased oxidation ability under different conditions, but those experiments were done on different days, so possibly the strain still possessed the phenotype that it was originally isolated with. Therefore, it is hard to make any conclusions about the involvement of the disrupted genes, except that this phenotype was not favorable to retain.

DMO9

This strain had a mutation in a transcriptional regulator, which was annotated as ArsR but did not match with any ArsR from different genera. It showed very low Mn(II)-oxidizing ability on hard agar and in liquid, but did not show any defect on soft agar. This suggests this strain is not regulating Mn(II) oxidation depending on whether it can swim or not, but is sensing something else.

DMO10

This strain had mutations in Mg(II) and Co(II) transporter protein, CorC, and apolipoprotein N-acyltransferase encoding genes. It exhibited no oxidation on soft agar, and very limited oxidation on hard agar, but was able to oxidize in liquid. It is possible

that either or both of these genes are important in hard or viscous environment, but not too important in liquid environment for Mn(II) oxidation.

IMOs

All of the IMO strains had mutations in flagella related genes. They all exhibited fast oxidation on hard agar, and except for IMO5, only a small decrease compared to wild-type in liquid. On soft agar, each strain exhibited decreased oxidation relative to wild-type, with non-motile strains failing to oxidize completely. This was discussed further in Chapter 3.

IMO2 had increased Mn(II) oxidation at 30˚C compare to the room temperature. Other IMO strains may also have increased oxidation at 30˚C as well, but by the time I checked the culture after 24 hours incubation, the difference was indistinguishable even if there was any.

It was a surprise when IMO strains were originally screened for motility on soft agar, and they showed slower oxidation. Then, further investigations have shown not just the IMO strains, but also DMO strains change their oxidation phenotype depending on the growth media condition. These growth condition effects were not readily apparent with wild-type bacteria, except for the fact the oxidation occurs faster on the soft agar and in the liquid compared to on the hard agar. Therefore, my mutants reveal underlying complex regulation of Mn(II) oxidation.

Biofilm formation and Mn(II) oxidation

When the strains were incubated in liquid, some cultures produced defined Mn(IV) oxide rings around the test tube and some didn't. This made me wonder if there is any difference in biofilm formation among different strains. However, the biofilm result did not seem to correlate with how much oxide ring formed. For example, DMO1 seemed to produce about the same amount of oxide ring as the wild-type, but it produced almost twice as much biofilm compared to the wild-type. In contrast, DMO5 and 6 seemed to be only slightly defective in oxide ring formation, but they produced less than

half of the biofilm compared to the wild-type. Conversely, DMO7 and 9 produced significantly less oxide ring, but they were not seriously defective in biofilm formation compared to the wild-type. Therefore, IMO5 was the only strain that seemed to exhibit an obvious correlation between both oxide ring formation and biofilm formation.

There have been some reports that showed differences in attachment of cells to different materials (Toutain et. al., 2004), so one of the reasons for the non-correlation can be due to the material of the equipment that the culture was grown in. The first experiment, as seen in Figure 4.4, was in a glass test tube, which is composed of hydrophilic materials, and the biofilm experiment was in a plastic 96-well plate, which is a hydrophobic, nonpolar surface. Therefore, I do not have data to support the correlation between Mn(II) oxidation and biofilm formation, but also have no data to disprove the correlation possibility.

4.5 Future directions

I have found that Mn(II) oxidation is affected by growth conditions, especially among different hydration levels. However, since all of the experiments were only done once, it is important to make sure the results are repeatable. More importantly, it is necessary to measure the concentration of Mn(IV) oxide precisely, so that we can make a better comparison between Mn(II) oxidation and biofilm formation in liquid. Perhaps, Mn(IV) oxide formation measurement and biofilm formation measurement can be both done with a 96-well plate, or a 48-well plate, so that uncertainty caused due to differences in material of culturing equipment can be eliminated.

While growing the strains, IMO strains seemed to be more tightly attached to the agar surface compared to the wild-type and DMO strains. With the help of Wendy Smythe, I have tried to get electron micrographs of GB-1 and IMO5 colonies on hard agar to observe if there are any differences in cell morphology on this surface. However, due to a thick biofilm coating, I was not able to obtain a good micrograph of the IMO strain. At the same time, this result has suggested that there is a very high possibility that IMO strains are producing more biofilm on hard agar compared to the wild-type and DMO strains. This will be an opposite result from the biofilm assay done in liquid, where

IMO strains all exhibited lower biofilm production. If all IMO strains have increased biofilm production on plates, it may suggest some correlation between Mn(II) oxidation and biofilm formation. Thus, it is necessary to be able to quantify biofilm formation on hard agar for each strain.

I have also tried to test the Mn(II) oxidation in different temperatures, but this experiment has to be repeated with a wider range of temperatures. Since it is known that *Pseudomonas putida* GB-1 cannot grow at 37˚C, some temperature between 31˚C and 36˚C should be tested to ensure that heat-shock proteins are being expressed. In addition, it is important to check the plates with IMO strains after 12 hours of incubation because they can complete their growth and oxidation by 24 hours.

In this chapter I have shown that Mn(II)-oxidation in *P.putida* GB-1 is regulated in a more complex manner than we originally assumed. Now that we know different growth conditions can affect the Mn(II) oxidation phenotype in GB-1, characterization of mutants that have been already isolated in other studies may be interesting, because some of the Mn(II) oxidation deficient mutants may be able to oxidize Mn(II) on soft agar or in liquid.

4.6 References

Emerson D, and Ghiorse WC. 1992. Isolation, cultural maintenance, and taxonomy of a sheath-forming strain of *Leptothrix discophora* and characterization of manganese-oxidizing activity associated with the sheath. *Appl. Environ. Microbiol.* 58(12):4001-4010

Francis CA, and Tebo BM. 2002. Localization of Mn(II)-oxidizing activity and the putative Multicopper oxidase, MnxG, to the exosporium of the marine *Bacillus* sp. SG-1. *Arch. Microbiol.* 178:450-456

Johnson HA, and Tebo BM. 2008. In vitro studies indicate a quinine is involved in bacterial Mn(II) oxidation. *Arch. Microbiol.* 189:59-69

Okazaki M, Sugita T, Shimizu M, Ohode Y, Iwamoto K, de Vrind-de Jong EW, de Vrind JPM, and Corstjens PLAM. 1997. Partial purification and characterization of manganese-oxidizing factors of *Pseudomonas fluorescens* GB-1. *Appl. Environ. Microbiol.* 63**:** 4793–4799

Stepanovic S, Vukovic D, Hola V, Bonaventura GD, Djukic S, Cirkovic I, and Ruzicka F. 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS.* 115:891-9

Toutain CM, Caiazza NC, and O'Toole GA. 2004. Microbial Biofilms: Chapter 4, Molecular basis of biofilm development by *Pseudomonads*. *ASM Press*. Wachington, D.C.

Conclusion

Unfortunately, from all of the results, it is clear that I haven't found the one gene that is responsible for Mn(II) oxidation or what makes up the Mn(II) oxidase because all of my mutants oxidized Mn(II) in some conditions. It can be assumed that Mn(II) oxidase is made out of multiple proteins, because there are so many factors that can regulate the Mn(II) oxidation, such as growth surface and temperature, and also there are many genes that are involved in Mn(II) oxidation.

It has been assumed that Mn(II) oxidation is beneficial for the bacteria because there are so many bacteria that are capable of oxidizing Mn(II). However, from the fact that this phenotype can be affected by disruption of so many different genes that do not affect the growth of the cells, it seems that synthesis of Mn(IV) oxide is not crucial for the cells to grow in the case of *P. putida* GB-1. However, the observation that Mn(II) oxidation phenotype is regulated in so many different ways suggests this is a very important phenotype because each regulatory step is making sure that GB-1 is expressing this phenotype in the optimum way.

Transposon mutagenesis has been performed on *P. putida* GB-1 multiple times by different scientists and students, and each time, more genes have been identified to be important in Mn(II)-oxidation. However, we have not been able to fully understand the biochemical mechanism, nor have we been able to identify the Mn(II) oxidase using this genetic approach. Therefore, it may be more important to identify the Mn(II) oxidase biochemically first, so that we can analyze the transposon mutants according to the proteins that make up the Mn(II) oxidase. This will be also useful to separate genes that are required for synthesis of Mn(II) oxidase and regulation of Mn(II) oxidation.

This is the first evidence that showed Mn(II) oxidation is regulated differently depending on the growth condition, such as growth medium and temperature. There are still many questions to be answered, such as what is the actual factor that is affecting the regulation; is it the humidity, viscosity, or the cell attachment to the surface? Understanding of this complex regulation of Mn(II)-oxidation may lead us to understand one of the ultimate questions: why *P. putida* GB-1 oxidizes manganese.

Appendix A

Relationship between Mn(II) Oxidation and Extracellular Polysaccharide Formation of *Erythrobacter* sp. SD-21

A.1 Introduction

In all of the well-studied Mn(II)-oxidizing bacteria, Mn-oxide accumulation has been observed by transmission electron micrographs to be on the cell surface. Therefore, it was been hypothesized that the Mn(II)-oxidizing protein is on the outermost region of the cell and that is where oxidizing activity occurs. It has been confirmed that sheath-producing *Leptothrix discophora* SP-6 secrets the Mn(II) oxidase (Emerson and Ghiorse, 1992), and with *Pseudomonas putida* GB-1 (Okazaki et. al., 1997), *Erythrobacter* sp. SD-21 (Johnson & Tebo, 2008), and *Aurantimonas manganoxydans* strain SI85-9A1 (Anderson et. al., 2009) the Mn(II) oxidase has been identified as a soluble protein residing in the periplasm or loosely associated with the cell surface. In spore-forming *Bacillus* sp. SG-1, Mn(II) oxidase was found to be located in the exosporium, which is the outer-most layer of the spore coat (Francis &Tebo, 2002).

The attempt to identify the specific Mn(II)-oxidase was unsuccessful with SG-1, but it was successfully identified in two other Mn(II)-oxidizing *Bacillus* species, PL-12 and MB-7, and it was confirmed to be localized in the exosporium (Dick, et. al., 2008). From the observation that the Mn(II) oxidase from SG-1 had a much bigger molecular

mass, it seemed possible that the difference between SG-1 and the other two species in the ability to purify the Mn(II) oxidase activity was protein-protein cross-linking, glycosylation, or the composition of the exosporium that formed a complex with the Mn(II) oxidase (Dick, et. al., 2008). When Mn(II) oxidase purification from *Erythrobacter* sp. SD-21 was attempted, extracellular polysaccharide (EPS) prevented complete purification as well (Johnson & Tebo, 2008).

There has not been a successful method to overcome the difficulty of purifying Mn(II) oxidase from the EPS complex, but one of the possibilities is to try to purify secreted Mn(II) oxidase before an excessive amount of EPS starts to accumulate on the cell surface. However, it was not clear if Mn(II)-oxidase is secreted before the accumulation of the EPS, so first it was necessary to reveal the relationships between Mn(II) oxidase and EPS secretion. In addition to the investigation of the relationships between Mn(II) oxidation and EPS formation, cell-growth rate and Mn(III) formation was measured. Furthermore, two different conditions, with and without addition of Mn(II) to the growth medium, were tested to investigate any effect that Mn(II) has on EPS formation or growth of *Erythrobacter* sp. SD-21.

Most of the experiments were not successful due to lack of practice and experience. All of the experiments presented here were only done once; therefore, it is hard to draw a clear conclusion. Still, there are some interesting results implying relationships between EPS formation and Mn(II) oxidation. The EPS concentration was much higher in the culture with added Mn(II) compared to without, which suggest EPS secretion is stimulated by some conditions created by the presence of Mn(II). However, the increase in EPS occurred when Mn-oxidation reaction plateaued, which implies EPS is probably not directly catalyzing or stimulating the Mn(II) oxidation, but rather the presence of Mn(IV) oxide or excess amount of Mn(II) oxidase are stimulating the production of EPS.

A.2 Materials and methods

Bacterial media and growth conditions

One milliliter of *Erythrobacter* sp. SD-21 (Francis et. al., 2001), grown in a test tube for 6 days, was inoculated into 2.8 L flasks containing 1 L of K medium (van Waasbergen et. al., 1993) on an orbital shaker at room temperature. K medium contained: 0.5 g yeast extract, 2.0 g peptone, and 1 L of 75% natural seawater or modified artificial seawater (ASW; 0.2M $MgSO_4$, 1.2 M NaCl, 0.04 M KCl, and 0.04 M $CaCl_2 \cdot 2H_2O$). After autoclaving, 20 mM HEPES (*N*-2-hydrocyethylpiperazine-*N'*-2-ethanesulfonic acid, [pH 7.8]) was added. 100 μM $MnCl_2$ was added to some cultures as noted in the text.

Sample collection

10 ml samples of the 1 L culture were taken from the flask every 12 hours and placed in the collection tube on ice. 6 ml was stored in -20˚C to be used for subsequent cell count, protein quantification, and Alcian blue assays. 1 mM of pyrophosphate, $P_2O_7^{4-}$ (PP_i) was added to the remaining 4 ml to stabilize Mn(III) as a Mn(III)-PP_i complex for the further measurement (Webb et. al., 2005), and the presence of pyrophosphate-Mn(III) complex was monitored by absorbance at 258 nm (Schlosser & Hofer, 2002).

Growth measurement

As *Erythrobacter* sp. SD-21 grow, it secrets EPS that results in aggregation of the cells. Because of this cell aggregation problem, especially when the cell starts to accumulate Mn-oxides around its cell surface, OD_{600} was not used to measure the cell growth. Instead, protein concentration measurements and cell count using DAPI staining were used as an indication of the cell growth. To reduce the labor intensity, only samples every 24 hours were used to generate the data.

750 μl of collected sample was mixed with 933 μl of buffer solution (50 mM Tris-HCl [pH 8.0] and 20 mM EDTA [pH 8.0]) in a bead beating tube containing 0.5 mm and 2.5 mm zirconia and silica beads. The tube was placed in the bead beating machine (BIO101/Savant FastPrep FP120) for 20 seconds at a speed of 4.5, and then it was centrifuged (13,000Xg for 2 minutes at room temperature) (Gautsch et. al., 2004,

modified by Rick Davis, personal communication). Protein in the supernatant was measured using Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacture's instruction. Briefly, 150 µl of the supernatant and 150 µl of the Coomassie Reagent was mixed, incubated for 10 minutes, and the A_{595} was determined. A standard curve was constructed using Albumin Standard Ampules, which contained bovine serum albumin (BSA) at 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide (Thermo Fisher Scientific Inc., Rockford, IL, USA).

DAPI (4'-6-Diamidino-2-phenylindole) stained cells were counted under fluorescence microscope (AX10 Imager.M1, Carl Zeiss Microimaging, Thornwood, NY). The method was adapted from Pernthaler et. al. (2001), and was modified by Andrew Han (personal communication). Briefly, cells from 10 µl to 50 µl of samples were fixed using 4% paraformaldehyde (Pfa), and placed on 0.2 µm black polycarbonate filter supported with GFB filter underneath it. Fixed cells were incubated with DAPI stains for 5 minutes before it was vacuum filtered, and the cells placed on the filter were counted using the florescent microscope.

Manganese concentration measurement

Mn(II) concentration was measured colorimetrically using the formaldoxime assay and $MnSO_4 \cdot H_2O$ as a standard (Brewer & Spencer, 1971). To make formaldoxime stock solution, 10% hydroxylamine-HCl and 37% formaldehyde was mixed at a 20 to 1 ratio. Right before using, 10% NH_4OH was added to the formaldoxime stock at an approximately 1 to 2 ratio, but adjusted so when the mixture was added to the sample, the pH is between 8.8 and 8.9. The collected sample was filtered to eliminate any Mn-oxides and cells, and then 260 µl of filtered sample was mixed with 40 µl of the formaldoxime solution.

The concentration of both Mn-oxides, Mn(III) and Mn(IV), was measured using leukoberbelin blue (LBB) (Okazaki et. al., 1997). LBB forms a blue product when it is oxidized by Mn(III, IV). 50 µl of sample was added to 250 µl 0.04% LBB in 1% acetic acid, then the mixture was incubated for a minimum of 15 minutes for complete reduction of oxidized Mn, and the A_{595} was determined. The Mn(III) concentration was measured

exclusively in a similar manner except the sample was filtered to eliminate Mn(IV) oxide particles, and the sample was left overnight at room temperature after it was mixed with LBB to ensure the reaction because the Mn(III)-PP_i complex is stable (log $k_{apparent}$ = 31.35 at pH ≈ 8) (Parker et. al., 2004).

EPS measurement

Alcian blue is a dye that can stain acidic long unbranched polysaccharides, and can be used to measure the concentration of the EPS produced by the cell. When Alcian Blue forms a complex with polysaccharides, the dye becomes insoluble. Therefore, it is important to note that when calculating the EPS concentration, colorimetric data obtained has to be inverted, because the more polysaccharides there is, the less color will remain in the solution. Alcian blue 8GX (Sigma-Aldrich Chemical Co., Missouri, USA) was prepared in 7% (v/v) acetic acid to 0.015%, stirring with gentle heating for 5 minutes, then without heat for 2 hours. The solution was then centrifuged for 30 minutes at 5000rpm to eliminate any undissolved dye (McKellar RC, et. al., 2003).

Collected cell samples were assayed according to the method by Ryu & Berchet (2003): 1.0 ml of the sample was vortexed at maximum speed for 10 second, centrifuged (10,000Xg for 20 minutes at 4˚C), 0.3 ml of the supernatant was well mixed with 0.6 ml of 7% acetic acid, then mixed with 0.6 ml of 0.05% Alcian blue. After at least 30 minutes of incubation, it was centrifuged (10,000Xg for 5 minutes at room temperature) to eliminate the precipitated dye before A_{595} reading.

Xanthan gum (Sigma-Aldrich Chemical Co., Missouri, USA) was used as a standard. 1 mM of the *Xanthan* gum was resuspended in K medium with a small amount of 95% ethanol, and stirred under mild heat until the suspension was uniform.

A.3 Results and Discussion

Cell growth and biofilm accumulation

The concentration of EPS was measured by the colorimetric reaction with Alcian Blue. In both conditions, with $MnCl_2$ or without, the highest concentration of EPS recorded was at time 0 with approximately 1.1 mg/ml EPS as can be seen in Figure A.1. Then, one day later, the concentration of EPS dropped to less than 0.5 mg/ml. This is unexpected because 1 ml of mature starter culture was added to 1 L of medium, which is a 1000 fold dillution. Roughly calculating, this means the starter culture had 100 mg/ml concentration of EPS, which should be extremely viscous, but the starter culture wasn't. Therefore, at least the first two data points seem to not represent the actual concentration of the EPS.

Growth curves constructed using two different methods both showed linear growth, (Figure A.2). This is inconsistent with what Francis et. al., (2001) reported using OD_{600} measurement, which was close to the characteristic S shape growth observed with most bacterial growth curves. The growth curve constructed with protein concentrations from lysed cells was not optimal for measuring the cell growth, because is showed very little growth over the observed time period, and also had some points with significant error (Figure A.2 (a)). The growth curve based on cell counts did show significant increase in cell numbers (Figure A.2 (b)), so this data was used for further analysis.

Even though the growth curve data might not be accurate, it is important to point out that there is no difference in growth rate between the two different conditions, with Mn(II) or without. This shows that *Erythrobacter* sp. SD-21 probably does not utilize Mn(II) oxidation to harvest evergy, nor does oxidation benefit the growth of the culture.

Figure A.3 shows EPS concentration data divided by the number of the cells from the cell count data. Because there was a high concentration of EPS at time 0, and it was the point with the least amount of cells, the graph showed that individual cells have the most amount of EPS at time 0. After 50 hours, there was no change in amount of EPS per cell. As I mentioned earlier, the EPS concentration data up to 24 hours are not reliable, so I will only discuss observations after 24 hours. It seems like the concentration of EPS produced by an individual cell does not increase over time (Figure A.3 (b)).

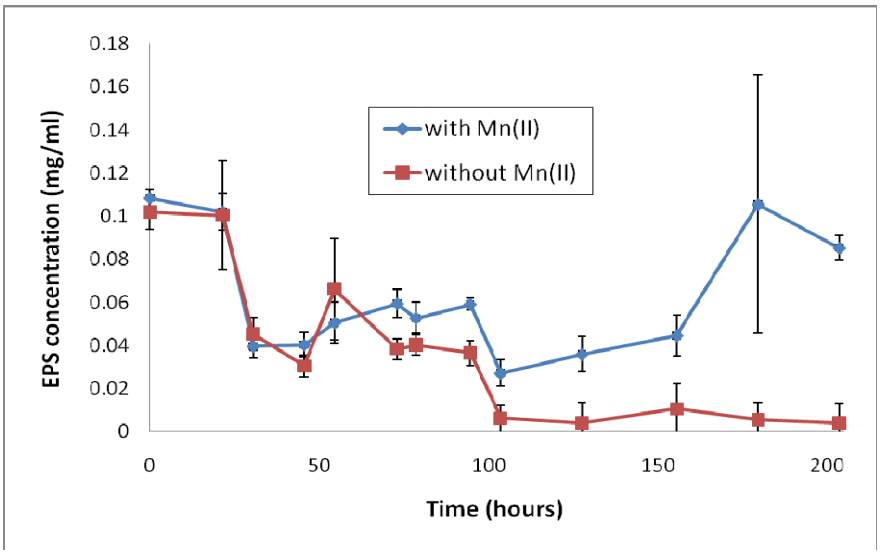

Figure A.1 EPS concentration. Duplicate cultures of *Erythrobacter* sp. SD-21 were incubated in the presence or absence of 100 μM $MnCl_2$. Three separate measurements of EPS concentration were taken from each of the two cultures.

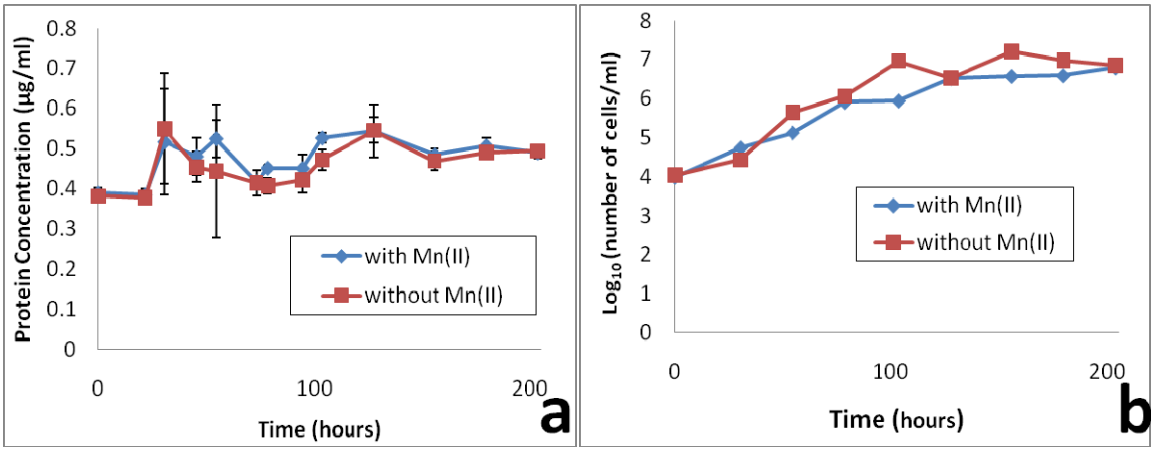

Figure A.2 Measurement of the cell growth of *Erythrobacter* sp. SD-21 using protein concentration (a) and cell count (b).

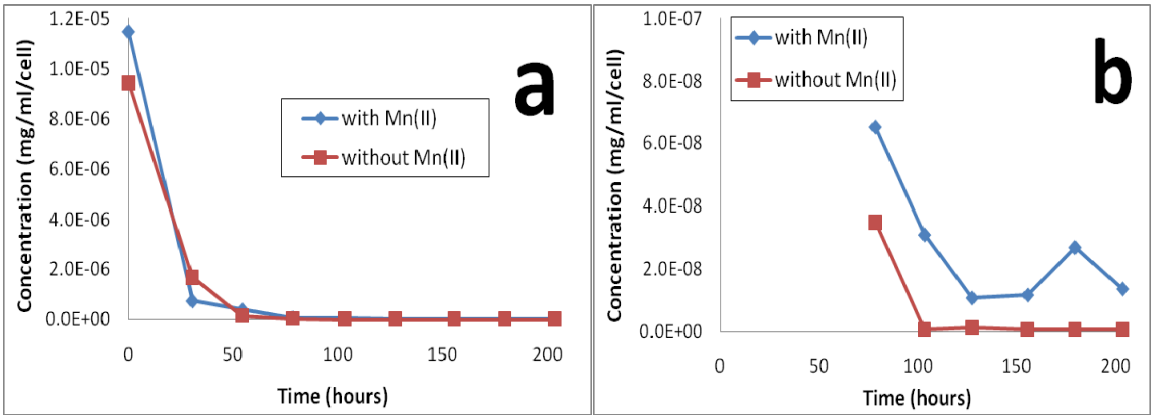

Figure A.3 EPS concentration normalized against cell number. First three time points were eliminated in (b) to observe a close up of (a).

Mn(II) oxidation and biofilm accumulation

Biofilm concentration was also compared with change in Mn concentrations. Mn(II) was not detected until approximately 70 hours, which has to be an error, since there was addition of 100 μM of $MnCl_2$ at time 0. Therefore, none of the data before 70 hours should be considered (Figure A.4). At 100 hours, almost all of the Mn(II) in the solution disappeared, mostly due to oxidation into Mn(IV). This is confirmed by the fact that the Mn(IV) concentration reaches its maximum at 150 hours, but rate of increase in Mn(IV) concentration drastically slows down at 100 hours (Figure A.5).

The concentration of Mn(III) increased slightly right before the increase of Mn(IV) takes off, but there is never a drastic change in its concentration (Figure A.5). However, it is interesting to note that Mn(IV) concentration showed a slight decrease when Mn(III) concentration increased slightly at 36 hours.

When EPS concentration and Mn(II) and Mn(IV)-oxide concentrations are compared, EPS production seems to start increasing when Mn(II) concentration is close to its lowest, and Mn(IV) concentration is close to the highest (Figure A.6). This result can mean that neither the Mn(II) oxidation reaction itself, nor the Mn(II) oxidase produced by the cell is stimulating the EPS secretion, but rather Mn(IV) oxide accumulation might be doing so. Also, there is a possibility that an excess amount of Mn(II) oxidase possibly residing around the cell, due to lack of Mn(II) to be oxidized, is stimulating the EPS production. It is also important to mention the culture without added Mn(II) decreased the production of EPS as time went by, but the culture with added Mn(II) eventually increased the EPS production after six days. This suggests that either absence of Mn(II) oxidase or Mn(IV) oxide may be suppressing the EPS accumulation. It is possible that without Mn(II) in the culture, genes responsible for Mn(II)-oxidase production are down regulated, and there is not much Mn(II) oxidase produced, and this prevents the stimulation of production of EPS by the Mn(II) oxidase.

It is hard to compare Mn(III) concentration with EPS production because there is only a slight increase of Mn(III) around 24 hours, and first two time points (0 hour and 12 hours) of EPS concentration seem to be not following the tendency from 24 hours, which suggests that those two data points are not appropriate to incorporate into the analysis.

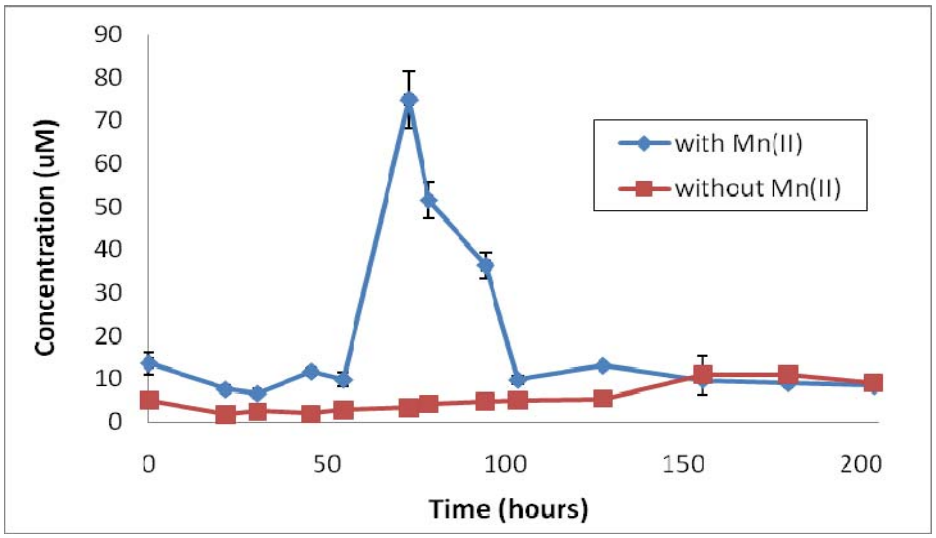

Figure A.4 Change in concentration of Mn(II).

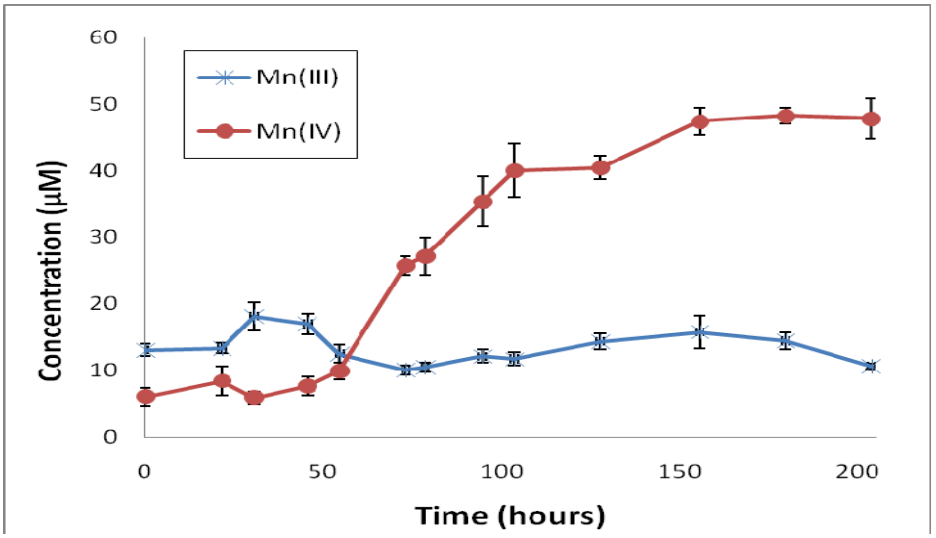

Figure A.5 Change in concentration of Mn(III) and Mn(IV).

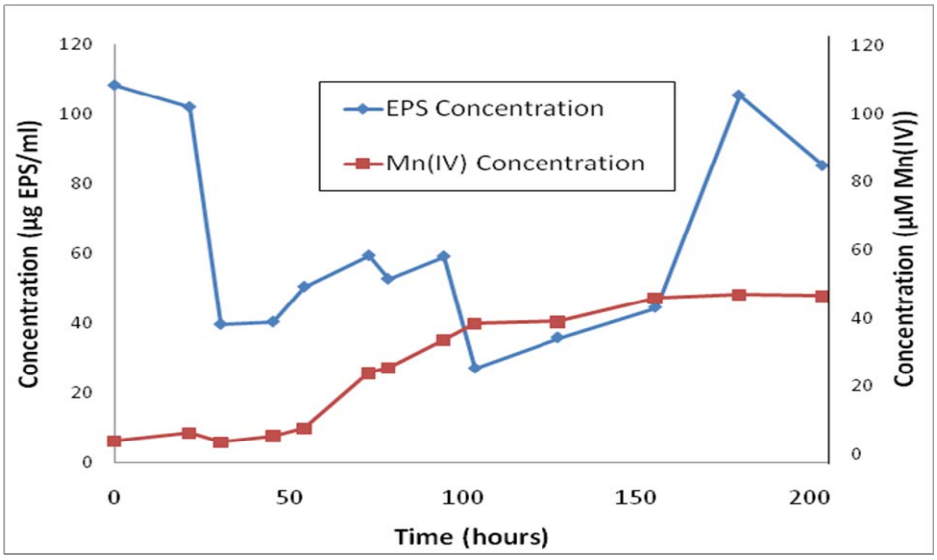

Figure A.6 Comparison of EPS concentration change and Mn(IV) concentration change.

A.4 Future direction

There were multiple problems with this project, some due to lack of experience with the assays used, some possible human errors, and some unknown issues. Since none of the assays were repeated, it is hard to identify whether the problems were due to human error or experimental error, but I will try my best to identify the problems.

The problem with the Mn(II) formaldoxime assay was most likely due to human error, because this assay has been used frequently to measure the concentration of the Mn(II). Furthermore, since the first five data points were the ones that seemed to have much lower readings than what they were suppose to be, it is most likely that I made some mistakes in the beginning due to lack of experience with the assay. The possible problem was not adjusting the pH to the reaction's optimal of pH8.8 to 8.9. Interruption of formaldoxime reaction by K medium was tested, but there was no notable difference compared to using water.

The data obtained for Mn(III) and Mn(IV) seemed to be reasonable, but it is too early to conclude that since the experiments were not repeated. Especially for the Mn(III) data, since these results do not correlate with the previous report that the intermediate Mn(III) concentration does not decrease after it reaches the maximum concentration (Johnson & Tebo, 2008).

The cell growth curves constructed using two different methods both seemed to have some inconsistency with previously reported growth curves of *Erythrobacter* sp. SD-21 as mentioned in the results and discussion. Hence, I should have adopted OD_{600} measurement as well, as the third method, so that I can compare which method gives the most reliable result. From the fact that protein concentration data showed almost no change over time, the high salt concentration of K medium might have interfered with the reagents used to measure the concentration. To overcome this problem, collected sample should have been pelleted, and resuspended in buffer. The only problem with cell count was its labor intensity. Since I froze down the entire sample, and did the cell count after all the samples were collected at the same time, my eyes got very tired. This can cause many human errors, although counting 20 different sections of each filter from the sample should even out the errors.

Alcian Blue has been used for quantifying EPS on several occasions. However, it was never used with *Erythrobacter* sp. SD-21, or in the K medium. After the data was collected, I realized K medium was interfering with the reaction between Alcian Blue and EPS. This made the assay sensitive to the incubation time, which was not seen when the assay was used with water or buffer (data not shown). Since I was not aware of this difference, I was not very careful with the incubation time. Therefore, differences between the first two data points, the next six data points, and the last five data points can be explained by the differences in the incubation time. In addition to the incubation time, the best method of separating the EPS from the cell should be further investigated. Different bacteria have different structures of EPS, therefore, how strongly it is associated with the cell surface could be different as well. I did check if there was any remaining EPS after 10 seconds of vortexing by resuspending the pelleted cell in MilliQ water, vortexing for an additional 15 seconds and testing with Alcian Blue. This result showed there was little remaining EPS after the 10 seconds vortexing, but there might be more EPS strongly associated with the cell. I have laid the groundwork for future students to pursue investigations of Mn(II)-oxidation in *Erythrobacter* sp. SD-21 by identifying optimal assay conditions and possible pitfalls.

A.5 Reference

Anderson CR. Johnson HA, Capto N, Davice RE, Torpey J, and Tebo BM. 2009. Mn(II) oxidation is catalyzed by heme peroxidase in *Aurantimonas manganoxydans* strain S185-9A1 and *Erythrobacter* sp. Strain SD-21. ***Appl. Environ. Microbiol.*** Accepted

Brewer PG, and Spencer DW. 1971. Colorimetric determination of manganese in anoxic waters. ***Limnol. Oceanogr.*** 16:107-112

Dick GJ, Torpey JW, Beveridge TJ, and Tebo BM. 2008. Direct identification of a bacterial manganese(II) oxidase, the multicopper oxidase MnxG, from spores of several different marine *Bacillus* species. ***Appl. Environ. Microbiol.*** 74(5):1527-34

Emerson D, and Ghiorse WC. 1992. Isolation, cultural maintenance, and taxonomy of a sheath-forming strain of *Leptothrix discophora* and characterization of manganese-oxidizing activity associated with the sheath. ***Appl. Environ. Microbiol.*** 58(12):4001-4010

Francis CA, Edgie-Mark CO, and Tebo BM. 2001. Enzymatic manganese(II) oxidation by a marine α-proteobacterium. *Appl. Environ. Microbiol.* 67(9):4024-29

Francis CA, and Tebo BM. 2002. Localization of Mn(II)-oxidizing activity and the putative Multicopper oxidase, MnxG, to the exosporium of the marine *Bacillus* sp. SG-1. *Arch. Microbiol.* 178:450-456

Gautsch JW, Saghbini MG, Lippman DA, and Dana RC. 2004. Method for isolating DNA. Bio 101, Inc., Vista, CA. United States Patent 6706498 B2.

Johnson HA, and Tebo BM. 2008. In vitro studies indicate a quinine is involved in bacterial Mn(II) oxidation. *Arch. Microbiol.* 189:59-69

McKellar RC, van Geest J, and Cui W. 2003. Influence of culture and environmental conditions on the composition of exopolysaccharide produced by *Agrobacterium radiobacter*. *Food Hydrocolloids*. 17(4):429-237

Okazaki M, Sugita T, Shimizu M, Ohode Y, Iwamoto K, de Vrind-de Jong EW, de Vrind JPM, and Corstjens PLAM. 1997. Partial purification and characterization of manganese-oxidizing factors of *Pseudomonas fluorescens* GB-1. *Appl. Environ. Microbiol.* 63**:** 4793–4799

Parker DL, Sposito G, and Tebo BM. 2004. Manganese(III) binding to a pyoverdine siderophore produced by a manganese(II)-oxidizing bacterium. *Geochimica et Cosmochimica Acta*. 68:4809-20

Pernthaler J, Glockner FO, Schonhuber W, and Amann R. 2001. Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes, in *Methods in Microbiology* (Paul J. H., ed.), Marine Microbiology. Academic Press Ltd, London, vol 30:207–226

Ryu JH, and Berchat LR. 2003. Development of method to quantify extracellular carbohydrate complexes produced by *Escherichia coli* O157:H7. *J. Appl. Microbiol.* 95:1304-1314

Schlosser, D. and Hofer, C. 2002. Laccase-catalyzed oxidation of Mn^{2+} in the presence of natural Mn^{3+} chelators as a novel source of extracellular H_2O_2 production and its impact on manganese peroxidase. *Appl. Environ. Microbiol.* 68:3514-3521

van Wassbergen LG, Hoch JA, and Tebo BM. 1993. Genetic analysis of the marine manganese-oxidizing *Bacillus* sp. Strain SG-1: protoplast transformation, Tn917 mutagenesis, and identification of chromosomal loci involved in manganese oxidation. *J. Bacteriol.* 175:7594-7603

Webb, S. M., G. J. Dick, J. R. Bargar, and B. M. Tebo. 2005. Evidence for the presence of Mn(III) intermediates in the bacterial oxidation of Mn(II). *Proc. Natl. Acad. Sci. USA* 102:5558–5563

Appendix B

Decreased Mn(II) Oxidation of IMO Strains of *Pseudomonas putida* GB-1 on Motility Agar is not due to Diffusion of Enzymes

B.1 Introduction

In Chapter 2, five Increased Mn(II) Oxidation (IMO) strains were isolated. They all exhibited more intense and faster Mn(II) oxidation compared to the wild-type on hard Lept agar plates. However, when they were tested for motility on soft agar in Chapter 3, not only were they non-motile or less motile than wild-type, they also had decreased Mn(II) oxidation. One hypothesis for the decreased Mn(II) oxidation on the soft agar was that the enzyme(s) required for the Mn(II) oxidation is secreted, and on soft agar it diffuses across the plate and IMO strains that lack motility cannot acquire enough enzyme to start the oxidation. This hypothesis was derived from the observation by Kati Geszvain (personal communication) that a non-oxidizing mutant started to oxidize Mn(II) when it was inoculated next to the wild-type on hard agar due to diffusion of an unknown activating factor that is necessary for Mn(II)-oxidation. The hypothesis for IMO strains was tested by spotting two separate colonies on one plate and observing if there is any difference in oxidation compared to the plate with just one colony.

B.2 Materials and methods

Pseudomonas putida GB-1 and all of the IMO strains isolated in Chapter 2 were used. Media used are the same as the ones used for the motility assay in Chapter 3. For the plate with just one colony, the overnight culture was spotted in the middle of the plate. For the plate with two colonies, two different spotting patterns were tested; one that has two spots very close to the edge of the plate on opposite sides, and another with a shorter distance between the colonies (Figure B.1). The plates were incubated at room temperature, and all of the pictures were taken after 12 hours, but observed for 48 hours.

B.3 Results

There was no difference in swimming speed, oxidation speed, or growth of the colony between the two different spotting patterns (Figure B.1, right two pictures). There was also no difference in swimming speed, oxidation speed, or growth of the colony between single spotted plate and two spotted plate (Figure B.1, B.2, B.3). (Pictures from IMO2 and IMO5 were selected because IMO2 has some motility, and IMO5 does not have any motility on soft agar. Also, they were selected over other IMO strains because they had the best pictures.) IMO strains still exhibited slower and less intense Mn(II) oxidation compared to the wild-type even after 48 hours of incubation in either one-spot or two-spot plate.

B.4 Discussion

The results showed that putting two culture spots on soft agar does not affect motility, Mn(II) oxidation, or growth. This result suggests that loss or decreased in Mn(II) oxidation of IMO strains on soft agar is not due to lack of sufficient concentration of an enzyme(s) that is necessary for Mn(II) oxidation. Having more cells on one plate also did not affect the Mn(II) oxidation speed of the wild-type. It may be because the activating factor found on hard agar is not the limiting factor for Mn(II) oxidation in the wild-type, that only small amount is sufficient for normal oxidation and excess of it does not affect the oxidation. The results from this experiment strongly suggests loss of fast Mn(II) oxidation phenotype of IMO strain on soft agar is a regulatory effect, and not from being unable to acquire enough enzyme for the reaction.

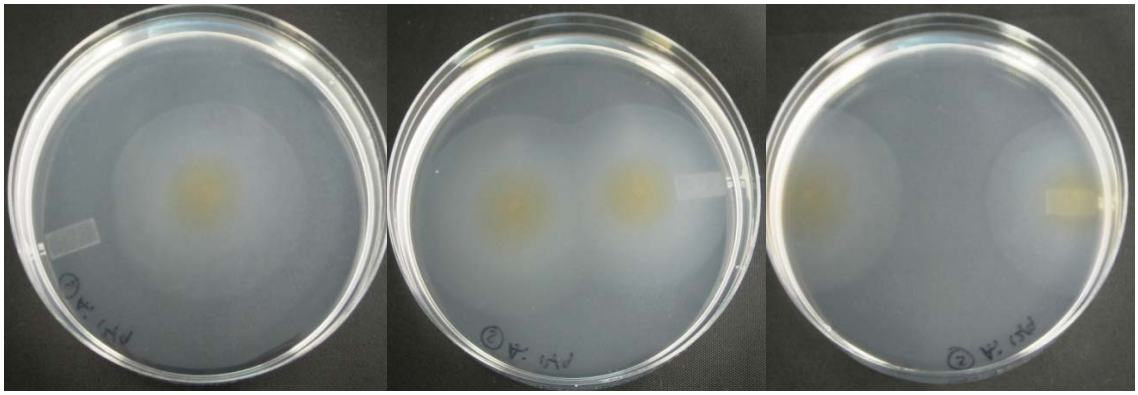

Figure B.1 Motility and Mn(II) oxidation of *P. putida* GB-1 on soft agar. 2.5 µl each of overnight culture was spotted either in the middle or on two different locations of the 0.25% agar Lept plate. The plates were incubated at room temperature for 12 hours.

Figure B.2 Motility and Mn(II) oxidation of IMO2 on soft agar. 2.5 µl each of overnight culture was spotted either in the middle or on two different locations of the 0.25% agar Lept plate. The plates were incubated at room temperature for 12 hours.

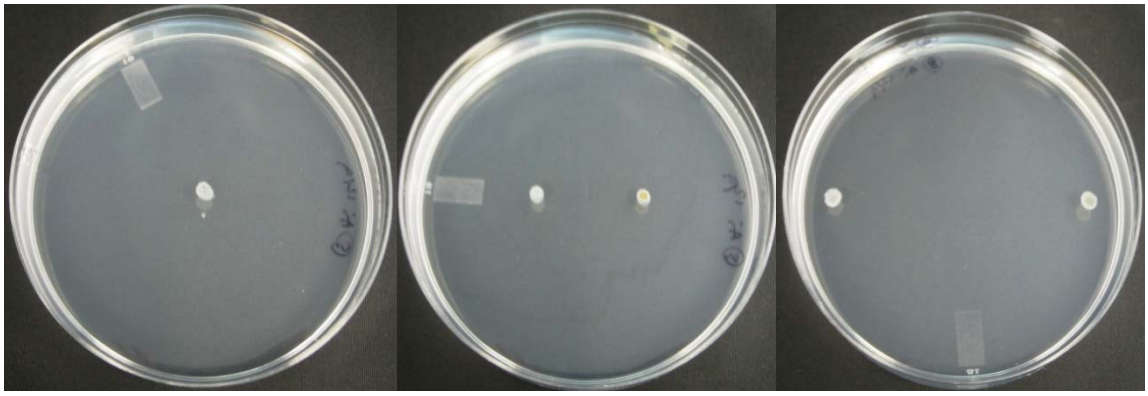

Figure B.3 Motility and Mn(II) oxidation of IMO5 on soft agar. 2.5 µl each of overnight culture was spotted either in the middle or on two different locations of the 0.25% agar Lept plate. The plates were incubated at room temperature for 12 hours.

Appendix C

Pseudomonas putida GB-1
Does not have Chemotaxis for Manganese

C.1 Introduction

It is suspected that Mn(II) oxidation is beneficial for the bacteria because different bacteria capable of Mn(II)-oxidation are wide-spread in the environment. Also, there are many proposed benefits to the bacterial cells from Mn(II) oxidation, which is introduced in Chapter 1, section 2. For those reasons, I assumed there may be chemotactic movement towards Mn(II). Furthermore, there has been a study that showed Mn(II) can substitute for iron metalizing mononuclear enzymes in case of iron deficiency (Anjem et. al., 2009). Therefore, I decided to test if *Pseudomonas putida* GB-1 has a chemotaxis towards Mn(II) under iron deficiency.

C.2 Materials and methods

Pseudomonas putida GB-1 was grown in LB medium, then 2.5 μl of the overnight culture was spotted on 0.25% agar Lept plate. Some of the Lept plate did not contain either Mn(II), Fe(III), or both. Also, 2 μl of 0.5 mM or 1 mM Mn(II) was spotted at the edge of the plate in some plates without Mn(II). The plates were incubated at room temperature for 24 hours.

C.3 Results and discussion

There was no chemotactic movement towards Mn(II), whether the Lept plate was supplemented with Fe(III) or not. Figure C.1.C had a spot of Mn(II) close to the edge of the plate (indicated by red arrow), but GB-1 exhibited even diffusion of the colony. Pictures are not shown, but even when Fe(III) was supplied in the medium, there was no change in the even swimming pattern of GB-1. This suggest GB-1 probably has no apparatus for chemotaxis towards Mn(II) because even on the relatively minimum medium with iron limited condition, GB-1 does not move towards Mn(II).

In addition, Mn(II) added by spotting on the plate that contained Fe(III) produced Mn(IV) oxides, but the plate without Fe(III) did not oxidize Mn(II) even after 48 hours. This is consistent with observation by Parker et. al. (2007), where Mn(IV) oxide formation was retarded under iron limiting condition by *P. putida* GB-1 due to complexation of Mn(III) with siderophore. It seems that the requirement of Fe(III) for Mn(II) oxidation is consistent even in different medium conditions, probably due to same reason.

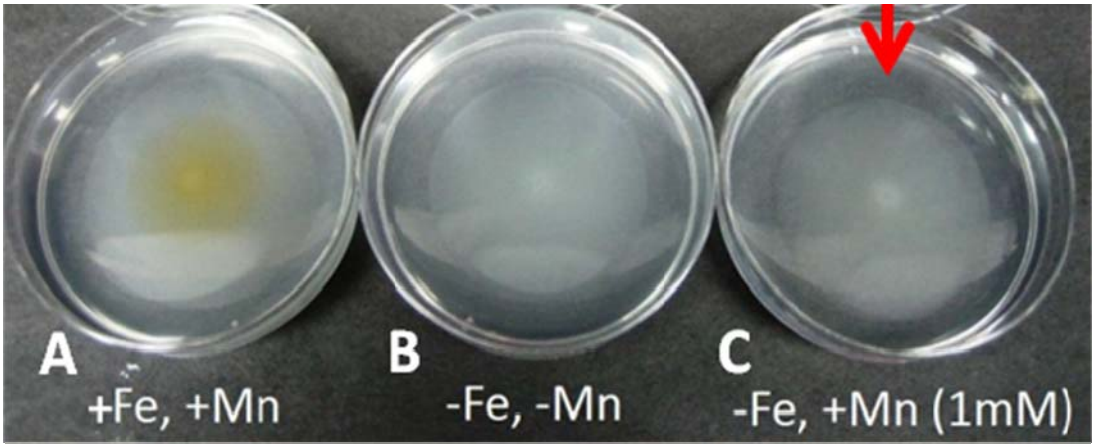

Figure C.1 Motility and Mn(II) oxidation of *P. putida* GB-1 in different Mn(II) and Fe(III) condition. Plates were incubated at room temperature for 24 hours.
A. Regular amount of Fe(III) (0.037 μM) and Mn(II) (200 μM) was added. B. No additional Fe(III) and Mn(II) was added. C. No additional Fe(III) was added, but 2 μl of 1 mM Mn(II) spotted where red arrow is pointing.

C.4 References

Anjem A, Varghese S, and Imlay JA. 2009. Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. *Mol. Microbiol.* 72:844-858

Parker DL, Morita T, Mozafarzadeh ML, Verity R, McCarthy JK, and Tebo BM. 2007. Inter-relationships of MnO_2 precipitation, siderophore-Mn(III) complex formation, siderophore degradation, and iron limitation in Mn(II)-oxidizing bacterial culture. *Geochim. Cosmochim. Acta.* 71:5672-83

Biographical Sketch

I was born in Shimane-ken, Japan on May 10, 1983. Besides the two years spent in the United States, I lived in my hometown until I was18-years old. Then, I moved to the United States to study English at Berkeley High School for one year. Following graduation from high school, I decided to go to Diablo Valley College, where I majored in Environmental Science. After two years, I transferred to the University of California, San Diego, where I got my bachelor's degree in Environmental Systems in Evolution, Behavior, and Ecology track, and a minor in Chemistry. During my senior year, I did research with Dr. Milton H. Saier on phylogenetic relationships of mercury transporter systems, which was published in the journal of Water, Air, and Soil Pollution. This research project was also awarded as the Dean's Award for Outstanding Environmental systems Senior Projects. In 2007, I started my graduate study at Oregon Graduate Institute, which is now the Department of Science & Engineering within Oregon Health and Science University, majoring in Biochemistry and Molecular Biology. All of my work is presented in this thesis, and I am working to publish part of it in a journal. After graduation, I will be working in the Manufacture Division at Merck in Japan.