UNDERSTANDING THE MOLECULAR MECHANISM OF MANGANESE OXIDATION IN *LEPTOTHRIX DISCOPHORA* SS-1

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Daniela Bocioaga

August, 2013

Understanding the molecular mechanism of Mn oxidation in *Leptothrix discophora* SS-1

Daniela Bocioaga, Ph.D.

Cornell University 2013

The purpose of this research is to understand the molecular mechanism of manganese oxidation in *Leptothrix discophora* SS1 which until now has been hampered by the lack of a genetic system. *Leptothrix discophora* SS1 is an important model organism that has been used to study the mechanism and consequences of biological manganese oxidation. In this study we report on the development of a genetic system for *L. discophora.* First, the antibiotic sensitivity of *L. discophora* was characterized and a procedure for transformation with exogenous DNA via conjugation was developed and optimized, resulting in a maximum transfer frequency of $5.2*10^{-1}$ (transconjugant/donor). Genetic manipulation of *Leptothrix* was demonstrated by disrupting *pyrF* via chromosomal integration of a plasmid with an R6Kɣ ori through homologous recombination. This resulted in resistance to fluoroorotidine which was abolished by complementation with an ectopically expressed copy of *pyrF* cloned into pBBR1MCS-5. This genetic system was further used to disrupt five genes in *Leptothrix discophora* SS1, which were considered to be the best candidates for the enzyme encoding the manganese oxidizing activity in this bacterium. All of the disrupted mutants continued to oxidize manganese, suggesting that these genes may not play a role in manganese oxidation, as hypothesized. MofA a putative muticopper oxidase, identified from the oxidizing fraction of *Leptothrix discophora* SS1 supernatant to encode the manganese oxidizing activity, was deleted from the genome and the cells lacking *mofA* did not lose the ability to oxidize manganese. This finding suggests that *mofA*

is dispensable to Mn oxidation in *Leptothrix*. Transposon mutagenesis performed on a Δ*mofA* *Leptothrix* strain resulted in the isolation of white, non-manganese oxidizing mutants. Mapping of the transposon insertions identified insertions in 4 genes located in 2 regions on the chromosome. One of the genes, named *mnxG2* is a putative multicopper oxidase similar to the manganese oxidizing enzyme in *Bacillus sp* SG-1. The other three genes with insertions encode a hypothetical protein, a putative cytochrome c next to a putative copper metallochaperone (Sco1/SenC/PrrC) involved in the biogenesis of cytochrome oxidase. Further analysis of the non-manganese oxidizing mutants identified through transposon mutagenesis coupled with the draft genome sequence of *Leptothrix discophora* SS1 should provide information about the number and the nature of proteins involved in manganese oxidation in this bacterium. The draft genome of *Leptothrix discophora* SS1 contains 4.2Mb with 3,791 identified protein coding sequences. In contrast with previous information of *Leptothrix* as an obligate aerobic heterotroph, functional analysis of the draft genome revealed the potential for a diverse metabolism such as fermentation, anaerobic respiration with nitrate and arsenate, sulfur oxidation and carbon fixation. The information provided by the draft genome about the metabolism of *L. discophora* SS1 as well as genomic context information about the genes identified to be important in manganese oxidation represent an important addition to the genetic system developed for *Leptothrix*, and together with the new metabolic information should expand our understanding of the manganese oxidation in *Leptothrix discophora* SS1.

BIOGRAPHICAL SKETCH

Daniela Bocioaga was born and grew up in Tulcea, Romania, by the shores of the beautiful Danube river, where she spent her peaceful years of childhood and the intense years of high school. After she received her bachelor in science in biology from the University of Bucharest, she travelled to the United States to study bioremediation of heavy metals from mining pits using unicellular algae under the guidance of a remarkable advisor and mentor, Dr. Grant Mitman, at the University of Montana in Butte. She spent two years, made many good friends and developed an interest for the application of microbiology to solve environmental problems. She continued her graduate education by pursuing her Ph.D. at Cornell University, under the guidance of Dr. Anthony Hay and Dr. Wiliam C. Ghiorse.

ACKNOWLEDGEMENTS

First, I want to thank my main advisor, Dr. Anthony Hay, for believing in me and for standing by me on this journey. He continues to be a source of inspiration not only as a scientist, but as an individual in the real life. I would like to thank Dr. Bill Ghiorse, who inspired me throughout the years with his true passion for (good) teaching and his dedication to the undergraduate students. My thanks extend to my committee members Dr. John Helmann and Dr. Len Lion for their contribution to my project that brought insight and helped keep things into perspective. I would like to thank all the entities that provided funding for my research (NSF and The Department of Microbiology).

As a graduate student I feel that I grew not only to become a better scientist, but I also learned to be a better teacher, for which I want to thank Sue Merkel for all I learned from her about teaching and for being my inspiration in this field.

Dr. Grant Mitman was the one who planted the first seed for my pursuing of a Ph.D. and I would like to thank him for believing in me and for being an amazing mentor and friend.

Many thanks to the administrative staff of the department of Microbiology (Shirley, Patty, Cathy). My sincere thanks to Shirley, for her support in the darkest hours of editing.

Thanks to my lab colleagues, past and present (Andreas, Gabi, Karl, Hanh and many more) who have been good friends, teachers and sources of inspiration.

I will always be thankful to my parents for instilling in me a love and respect for knowledge and education and for continuing to support me with their unconditional, endless love; to my best friend, my dear sister, for quietly extending her help when I needed the most and to my own little family.

DEDICATION

To my parents, who made me strong and taught me unconditional love

TABLE OF CONTENTS

CHAPTER ONE: INTRODUCTION

Background and significance of biological manganese oxidation

1.1 Manganese in the environment

Manganese is ubiquitous in the environment. It comprises about 0.1% of the Earth's crust which makes it the second most abundant transition metal in the Earth's crust, after iron (1). In nature it occurs as a component of more than 100 minerals, including various sulfides, oxides, carbonates, silicates, phosphates, and borates (2). In these (and other) compounds Mn can occur in 11 oxidation states, from -3 to +7, but the most important environmentally are II, III and IV. Oxidation state is important since it controls the solubility and bioavailability of Mn to most forms of life which require Mn in trace amounts as an essential micronutrient (3). Mn(II) (manganous) cation is the most important soluble form of manganese in nature (4), while Mn (IV) forms highly insoluble oxides, hydroxides and oxyhydroxides (4). Mn(III) is unstable in neutral environments and readily disproportionates to Mn(II) and Mn(IV). The distribution of Mn among these three oxidation states in the environment is controlled by pH, oxygen concentration and microbial activity (2). Environments with low pH such as acid mine drainage or low oxygen concentrations (such as anoxic regions of lakes and other bodies of water, deep-sea hydrothermal vents, waterlogged soils or anaerobic sediments) will favor the presence of Mn (II), whereas at high pH and/or increased oxygen concentration Mn(IV) will dominate.

Manganese is an essential trace element for all organisms. It is a component of some metalloenzymes, such as superoxide dismutase, arginase, and some phosphate-transferring enzymes (5). In plants, manganese atoms are involved in the light-mediated oxidation of H_2O to

O_2 in photosystem II. High intracellular concentrations of Mn^{2+} have been reported to function as a protectant against superoxide toxicity in a microaerophilic bacterium, *Lactobacillus plantarum* (6).

1.2 Abiotic and biological Mn oxidation. Mn oxides

Oxidation of Mn(II) to Mn(IV) in natural environments can occur as an abiotic process or a biological process and results in a mixture of manganese oxides, hydroxides and oxyhydroxides. Reports of biological manganese oxidation in nature have been made almost a century ago (7) and there has been a long debate as to whether manganese oxidation is an abiotic or biological process. It is now generally agreed that the majority of naturally occurring Mn oxides are biogenic in nature, derived directly from biological Mn oxidation processes or from the subsequent alteration of biogenic oxides (8).

Abiotic oxidation of aqueous Mn(II) is energetically favorable under neutral aerobic conditions, however the reaction rates are very small and this reaction is not significant for pH values smaller than 8 or 9. Measured values of Mn oxidation in many marine and fresh water environments show that Mn (II) is oxidized at much higher rates (as much as 5 orders of magnitude higher) than those predicted by the thermodynamic conditions of the environment (9). The difference between the expected Mn oxidation rates from thermodynamic calculations and the measured rates for a particular environment was attributed to oxidation of Mn by microorganisms. Microorganisms can mediate the oxidation of Mn in a direct or indirect fashion. During indirect oxidation, microorganisms can locally increase the pH or the concentration of oxygen in their surrounding environments; this localized change in environmental conditions will in turn favor the oxidation of Mn. Direct oxidation involves a bacterial–produced enzyme as

a catalyst and it is mostly a cell associated process with Mn oxides deposited on the cell surfaces (10).

Mechanistically, chemical manganese oxidation occurs as two sequential one-electron reactions: Mn(III) oxides are formed first and then converted to Mn(IV) oxides (4). In abiotic manganese oxidation, Mn(III) represents a distinct intermediate step followed by slow disproportionation and protonation reactions to form Mn(IV) oxides. Studies of the manganese oxidation in *Bacillus sp*. spores show that biological manganese oxidation also proceeds as a sequence of two enzymatically mediated one-electron transfer reactions, however Mn(III) intermediate is transient and short lived; it could be soluble or enzyme-complexed and does not occur as a solid intermediate as it does in abiotic Mn(II) oxidation (11). These findings are interesting when linked with the multicopper oxidases (MCO), the enzymes proposed to catalyze the biological oxidation of Mn(II) which are known to catalyze the transfer of one electron only during oxidation-reduction reactions (12). This suggests that either more than one MCO catalyzes the oxidation of Mn(II) to Mn(IV) or that a novel MCO is involved that is capable of catalyzing a two-electron transfer reaction (11). Preliminary experiments with manganese oxides produced by *Leptothrix discophora SS1* also had an average oxidation state of 3.32, while aged manganese oxides reached an oxidation state close to 4, suggesting that manganese oxidation in *Leptothrix* could also proceed as a two-step process including a Mn(III) intermediate as in *Baccilus*. Also, manganese oxidation in *Leptothrix discophora SS1* was microscopically observed as large aggregates of membranous particles ("blebs")(13). Purification of such aggregates to yield a single protein greatly decreased the manganese oxidizing activity of this protein, suggesting that the process might be catalyzed by a complex of proteins or proteins and polysaccharides rather than a single protein. More recent data about enhanced Mn oxidation in

the presence of light in planktonic Roseobacter-like bacterium also supports the idea that an additional metabolite may be involved in the oxidation process (14).

1.3 Mn oxidation by *Leptothrix discophora* SS-1

L. discophora SS1, the model organism for studding Mn oxidation, is a gram negative, sheathed, rod-shaped bacterium that can oxidize both iron and manganese. It was isolated by Dr. Bill Ghiorse from a fresh water, swamp-like environment in the Sapsucker Woods, Ithaca, NY (15). In this environment it exists either as a Mn oxidizing surface film, with the appearance of an oil "sheen", or associated with the roots of *Lemna* plants, located a few millimeters under the surface. It has a *"swarmer"* life stage, with motile flagellated single cells which eventually start growing into a chain. In natural environments the chains are surrounded by a "sheath" containing heteropolysaccharides which is encrusted with Mn and Fe oxides (16). For this reason it was initially believed that the sheath is the site for Mn oxidation activity. However, in a strain of *L. discophora* SS1 which lost its ability to form a sheath upon culturing under laboratory conditions, the Mn oxidizing ability was unaffected (17). On medium with less glucose (PYG), most of the activity is secreted in the supernatant, while when more glucose is available, most of the activity remains cell associated (13). Initial approaches to study Mn oxidation in *L. discophora* SS1 employed biochemical methods (17–19). A Manganese Oxidizing Factor (MOF) was isolated and partially purified from culture supernatant of *L. discophora* SS1. This factor oxidized manganese and staining with periodic acid/Schiff's reagent suggested that polysaccharides material was associated with MOF. It was therefore proposed that the Mn(II) oxidation factor *in Leptothrix discophora* SS1 may be an oxidizing complex consisting of glycoprotein(s) associated with anionic polysaccharides that resides in membranous "blebs" (13).

Further investigation using the biochemical approach was challenging, due to the difficulty of purifying MOF as well as to the loss in activity upon purification.

A genetic approach for studying this process resulted in finding the putative sequence of *mofA*, the gene proposed to encode the manganese oxidizing activity protein (20). Antibodies raised against purified MOF were used to screen an expression library of *L. discophora* SS1 and α-MOF positive clones were used to isolate the corresponding gene, *mofA*. Further sequencing downstream of *mofA* revealed two more open reading frames, *mofB* and *mofC*, which are suggested to form an operon with *mofA.* The derived amino acid sequence of *mofA* indicates a molecular weight of 174 kDa. Further attempts to characterize the role of *mofA, B* and *C* were unsuccessful (21) due to the lack of genetic tools amenable to *L. discophora* SS1. Initial heterologous expression of *mofA* alone in *E. coli* resulted in a protein without Mn oxidizing activity while the expression of the entire operon was toxic to the cells (22). Upon further optimization, the *mof* operon could successfully be expressed in *E.coli*, however the Mn oxidizing activity could not be recovered (23). Chemical and UV mutagenesis methods did not render any mutants that did not oxidize manganese and no DNA transfer system could be established for *L. discophora* SS1 so far (24). Tandem mass spectrometry of manganese oxidizing band from polyacrylamide gel electrophoresis of concentrated *Leptothrix* supernatant did not identify MofA as one of the proteins present in these band (Daniela Bocioaga, unpublished results). Although a variety of methods, such as biochemistry, microscopy, proteomics, molecular biology and genetics have been used (with varying levels of success) to gather knowledge about the manganese oxidation process in *Leptothrix discophora SS1*, the development of a genetic system for this bacterium seems imminent, in order to make further progress in understanding this process.

1.4 Manganese oxidation by other microorganisms: the state of knowledge

Observation and study of bacterial Mn oxidation for over a century revealed that a wide variety of phylogenetically diverse microorganisms can oxidize Mn and that these manganese oxidizers inhabit very diverse environments. Knowledge about this process accumulated at a slow pace, due to the difficulty of isolating and culturing the Mn oxidizing bacteria under laboratory conditions as well as the low amenability of many of these bacteria to genetic techniques. Although a variety of bacteria that can oxidize manganese have been isolated from diverse environments, a more detailed study of Mn oxidation has been limited to *L. discophora* SS1, *Pseudomonas putida* strains GB-1 and MnB1, *Bacillus sp*. strain SG-1, *Pedomicrobium sp.ACM 3067* and *Aurantimonas sp. SI185-9A1*.

The manganese oxidizing activity for *Bacillus sp* SG-1, isolated from marine sediment in a manganese enrichment culture, was identified in the spore coat of dormant spores (25). Transposon mutants that did not oxidize manganese had insertions in 7 genes (*mnx*A-G), presumably in an operon (26, 27). Further tandem mass spectrometry of manganese oxidizing bands identified peptides of one of the proteins predicted to be a multicopper oxidases, MnxG (28, 29). While heterologous expression of *mnxG* in *E.coli* did not result in a manganese oxidizing protein, co-expression of *mnxEFG* was sufficient to recover manganese oxidation (30). *Pseudomonas putida MnB1*, an isolate from a Mn oxide-encrusted pipeline and *Pseudomonas putida GB-1* both oxidize manganese in the early stationary phase. Mutants that did not oxidize manganese were obtained when 2 genes encoding putative multicopper oxidases, *mcoA* and *mcoB* were deleted simultaneously in *Pseudomonas putida GB-1* (31). Also, a two component regulatory pathway MnxS1/MnxS2 and MnxR is essential for Mn(II) oxidation in *P. putida GB-1* (32). In a separate experiment, transposon mutants that did not oxidize manganese had

insertions in genes involved in the general secretion pathway, the cytochrome c maturation pathway or the tryptophan biogenesis pathway (33–36).

A transposon insertion in *moxA*, encoding a putative multicopper oxidase as in the case of the aforementioned *Bacillus and Pseudomonas*, eliminated the manganese oxidation activity in *Pedomicrobium sp.ACM 3067*; in addition, two more genes flanking *moxA* were identified, *moxB* and *moxC*, and proposed to have some involvement in Mn oxidation as part of a *moxBAC* operon (37, 38).

Aurantimonas sp. SI185-9A1 was isolated from the oxic/anoxic interface (120m deep) of the Saanich inlet, based on its ability to oxidize manganese. A heme type peroxidase was identified by tandem mass spectroscopy as the dominant protein in a manganese oxidizing band; genome mining of the draft genome of *Aurantimonas sp.* identified two more proteins, putative multicopper oxidases, MoxA/B that have been proposed to have a role in manganese oxidation, however their role was not confirmed with tandem mass spectroscopy (39, 40).

One aspect that appears to be consistent in most of the manganese oxidizers for which manganese oxidation was studied at the molecular level is the involvement of multicopper oxidases as the enzymes that potentially catalyze the manganese oxidation process.

1.5 Multicopper oxidases and their potential for enzymatic manganese oxidation

Multicopper oxidases (MCO) constitute a large family of proteins with approximately 500 homologs found in all three domains(41) and one of the most diverse groups of proteins, both in terms of their sequences, functions, and distribution in organisms. These proteins are involved in a wide range of functions, such as oxidation of different organic substrates (laccases, phenoxazinone synthase, ascorbate oxidase, bilirubin oxidase), copper resistance (CueO in *E.*

coli, CopA and C in *Pseudomonas syringae* pv. *tomato*), oxidation and acquisition of iron (Fet3p in yeast, ceruloplasmin in humans) (42) and melanin production and UV protection (43, 44). Other multicopper proteins lacking known functions have been recently postulated from direct sequencing of genomes, such as the *Aquifex aeolicus* periplasmic cell division protein (45). A wide diversity also exists in terms of substrate specificity: many of the MCO's such as the plant and fungal laccases, have broad substrate specificity**,** being able to oxidize multiple substrates including both metals and organics (46), while others have more defined substrate specificity like the ascorbate oxidase and ceruloplasmin (47). A multicopper oxidase that can oxidize manganese directly has not yet been described in bacteria (48). Therefore MofA, MnxG, McoA, McoB, MoxA would be the first ones in this family with this function.

Multicopper oxidases are a class of Cu enzymes which utilize the redox property of the copper ion as a cofactor for the oxidation of different substrates (41). In these proteins Cu ions are coordinated to three or four ligands donated by the amino acid residues, usually histidines (but other residues, too), which are the conserved residues of the Cu binding sites. Four types of copper-binding sites exist based on the number of Cu ions that participate in the binding site and their spectral behavior: type I (one Cu ion), type II (one Cu ion, different coordination) type III (2 Cu ions) and type IV (3 Cu ions). In general, MCO's contain four copper atoms distributed between one type I Cu site, and a trinuclear cluster of three Cu ions coming from a type II and a type III sites (but MCO's with 5 and 6 copper atoms also exist (41, 47). Type I Cu is involved in the oxidation of substrate by four subsequent one-electron oxidations. These electrons are further used in the trinuclear cluster in a four-electron reduction of oxygen to water (49). The quaternary structure of the proteins and the positioning of the domains provide the functional proximity

between the type I Cu and the trinuclear cluster to facilitate transfer of electrons from the type I Cu to the trinuclear cluster (12, 47).

The assignment of MofA, MnxG, McoA, McoB and MoxA to the MCO class of proteins was made based on their sequence characteristics, particularly the presence of conserved Cu binding motifs. Even if these proteins are proposed to catalyze the same reaction, there is little homology between MofA, MnxG, McoA, McoB and MoxA as well as between these putative manganese oxidizing MCO's and other multicopper oxidases, except for the copper binding motifs. However, it is not uncommon for different multicopper oxidases to share very little sequence similarity (41). The multicopper class of proteins is known to require Cu as a cofactor. The sequence of *mofA, mcoA, mcoB, mnxG* and *moxA* contains the Cu binding motifs and preliminary experiments showed that addition of Cu increased Mn(II) oxidation when added to the growing cultures.

Due to the wide range of functions and substrates that MCO's can have, more experimental evidence is necessary to unequivocally prove the manganese oxidizing role of these multicopper oxidases. For exemple, CumA, a putative multicopper oxidase initially proposed to be the manganese oxidizing enzyme in *Pseudomonas putida GB-1* appears to be widely distributed within the genus *Pseudomonas*, occurring in both Mn(II) oxidizing and non-Mn(II) oxidizing strains (48, 50). Recently it was shown that this putative MCO does not catalyze manganese oxidation in *Pseudomonas.*

1.6 Significance of manganese oxidation to bacteria and the environment

Manganese is an essential trace element for all organisms. It is a component of some metalloenzymes, such as superoxide dismutase, arginase, some phosphate-transferring enzymes (5) as well as part of the complex involved in photosysthesis. Since most organisms can only use soluble manganese, oxidation of Mn(II) can cause this ion to become limiting and affect growth. This can be of practical importance in agriculture and plant diseases caused by manganese deficiency are known (7). Biogenic Mn oxides resulting from biological manganese oxidation are highly reactive mineral phases in soils, sediments and waters, are strong oxidants and have high sorptive capacities and ion exchange capacities (4). They have large surface areas with high negative charges and can adsorb a variety of cations on their surfaces and incorporate metal like Cu, Co, Cd, Ni, Sn, Zn, Pb in their crystal structure. This can further decrease the dissolved trace metals and radionuclides concentration in the environment by orders of magnitude (3). On a more practical side the sorption properties of (biogenic) manganese oxides could make available an *in situ* method for removing toxic metals from water (51).

As some of the strongest oxidizing agents in the environment after oxygen, Mn oxides can promote the degradation of a wide array of complex organics including humic substances, PCB's, phenols, chlorinated anilines (52, 53). Degradation of humic and fulvic acids by manganese oxides results in a variety of biologically available organic compounds, possibly making available to microbial communities a source of carbon from otherwise biologically refractory pools (54). Thus, by mediating the production of manganese oxides, the manganese oxidizing bacteria also could play an important role in the biogeochemical cycling of organic carbon (55).

The possible involvement of one or more enzymes in manganese oxidation suggests that this process must impart some selective advantages to microorganisms, although the present state of knowledge about this process cannot demonstrate a specific function. It is possible that manganese oxidation could function as a defense mechanism against toxic metals, reactive oxygen species, UV light or predation or as a scavenging mechanism for trace metals. Manganese oxides could play a role as storage of an electron acceptor for use in anaerobic respiration or when oxygen concentrations are low (56). Such a scenario is not unlikely for *Leptothrix discophora SS1* considering its environment, an oxic/anoxic interface for which variations in the concentration of oxygen are expected (57). Cells coated with manganese oxides tend to adhere more strongly to surfaces, so that manganese-oxidizing cells might benefit from the "biofilm effect", concentrating nutrients from nutrient-poor liquid environments (7). The highly reactive manganese oxides can break down fulvic acids and provide a carbon source otherwise unavailable to bacteria (54), which may impart benefits to the microbial community. It is less likely that, for some species, manganese oxidation may provide no benefit to the cell at all, but could be only a by-product of some other function.

The presence of the Mn(III) intermediate in the reaction sequence may add to the environmental importance of biological manganese oxidation. Mn(III) is a strong oxidant, implicated in the degradation of lignin by fungi and the oxidation of sulfur and nitrogen compounds, while Mn(III) organic complexes are labile with respect to other ligands. Thus manganese oxidizing bacteria can be an important source of this powerful oxidant in many aquatic and terrestrial settings (11, 58, 59).

REFERENCES

1. **P.L. Howe, H.M. Malcolm, S. Dobson**. 2004. Manganese and its compounds: environmental aspects. Concise International Chemical Assessment Document. World Health Organization. Geneva.
2. **Ehrlich HL, Newman DK**. 2008. Geomicrobiology, Fifth Edition. CRC Press.
3. **Tebo BM, Johnson HA, McCarthy JK, Templeton AS**. 2005. Geomicrobiology of manganese(II) oxidation. Trends Microbiol. **13**:421–428.
4. **Tebo BM, Bargar JR, Clement BG, Dick GJ, Murray KJ, Parker D, Verity R, Webb SM**. 2004. BIOGENIC MANGANESE OXIDES: Properties and Mechanisms of Formation. Annu. Rev. Earth Planet. Sci. **32**:287–328.
5. **Dismukes G.C.** 1996. Manganese enzymes with binuclear active sites. Chem. Rev. **96**:2909–2926.
6. **Archibald FS, Fridovich I**. 1981. Manganese and Defenses against Oxygen Toxicity in Lactobacillus plantarum. J. Bacteriol. **145**:442–451.
7. **DePalma SR**. 1993. Manganese oxidation by Pseudomonas putida. Harvard University.
8. **Geszvain K, Butterfield C, Davis RE, Madison AS, Lee S, Parker DL, Soldatova A, Spiro TG, Luther GW, Tebo BM**. 2012. The molecular biogeochemistry of manganese(II) oxidation. Biochem. Soc. Trans. **40**:1244–1248.
9. **Nealson KH, Tebo BM, Rosson RA**. 1988. Occurrence and Mechanisms of Microbial Oxidation of Manganese, p. 279–318. *In* Allen I. Laskin (ed.), Advances in Applied Microbiology. Academic Press.
10. **Tebo B. M., He L. M.** 1999. Microbially Mediated Oxidative Precipitation Reactions, p. 393–414. *In* Mineral-Water Interfacial Reactions. American Chemical Society.
11. **Webb SM, Dick GJ, Bargar JR, Tebo BM**. 2005. Evidence for the presence of Mn(III) intermediates in the bacterial oxidation of Mn(II). Proc. Natl. Acad. Sci. U. S. A. **102**:5558–5563.
12. **G. J. Brouwers, E. Vijgenboom, P. L. A. M. Corstjens, J. P. M. de Vrind EW de VJ**. 2000. Bacterial Mn2+ Oxidizing Systems and Multicopper Oxidases: An Overview of Mechanisms and Functions. Geomicrobiol. J. **17**:1–24.
13. **Adams LF**. 1986. Physiology, Cytology, and manganese oxidizing activityof Leptothrix discophora SS1. Cornell University.

14. **Hansel CM, Francis CA**. 2006. Coupled photochemical and enzymatic Mn(II) oxidation pathways of a planktonic Roseobacter-Like bacterium. Appl. Environ. Microbiol. **72**:3543–3549.
15. **Adams LF, Ghiorse WC**. 1986. Physiology and ultrastructure of Leptothrix discophora SS-1. Arch. Microbiol. **145**:126–135.
16. **Emerson D, 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**:4001–4010.
17. **Adams LF, Ghiorse WC**. 1987. Characterization of extracellular Mn2+-oxidizing activity and isolation of an Mn2+-oxidizing protein from Leptothrix discophora SS-1. J. Bacteriol. **169**:1279–1285.
18. **Boogerd FC, Vrind JP de**. 1987. Manganese oxidation by Leptothrix discophora. J. Bacteriol. **169**:489–494.
19. **Corstjens PL, Vrind JP de, Westbroek P, Jong EW de V**. 1992. Enzymatic iron oxidation by Leptothrix discophora: identification of an iron-oxidizing protein. Appl. Environ. Microbiol. **58**:450–454.
20. **Corstjens PLAM, de Vrind JPM, Goosen T, Jong EW de V**. 1997. Identification and molecular analysis of the Leptothrix discophora SS-1 mofA gene, a gene putatively encoding a manganese-oxidizing protein with copper domains. Geomicrobiol. J. **14**:91–108.
21. **G. J. Brouwers, P. L. A. M. Corstjens, J. P. M. de Vrind, A. Verkamman, M. de Kuyper EW de VJ**. 2000. Stimulation of Mn2+ Oxidation in Leptothrix discophora SS-1 by Cu2+ and Sequence Analysis of the Region Flanking the Gene Encoding Putative Multicopper Oxidase MofA. Geomicrobiol. J. **17**:25–33.
22. **Browers G-J**. 1999. Molecular Aspects of Microbial Manganese Oxidation. Thesis. Leiden University, Netherlands.
23. **El Gheriany IA**. 2010. Manganese oxidation by Leptothrix discophora SS1. Cornell University.
24. **Siering PL**. 1996. Application of molecular approaches to investigate the role of microorganisms in manganese cycling in wetland environments. Cornell University.
25. **Waasbergen LG van, Hoch JA, 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.

26. **Waasbergen LG van, Hildebrand M, Tebo BM**. 1996. Identification and characterization of a gene cluster involved in manganese oxidation by spores of the marine Bacillus sp. strain SG-1. J. Bacteriol. **178**:3517–3530.
27. **Francis CA, Tebo BM**. 2002. Enzymatic Manganese(II) Oxidation by Metabolically Dormant Spores of Diverse Bacillus Species. Appl. Environ. Microbiol. **68**:874–880.
28. **Francis CA, Casciotti KL, Tebo BM**. 2002. Localization of Mn(II)-oxidizing activity and the putative multicopper oxidase, MnxG, to the exosporium of the marine Bacillus sp. strain SG-1. Arch. Microbiol. **178**:450–456.
29. **Dick GJ, Torpey JW, Beveridge TJ, Tebo BM**. 2008. Direct Identification of a Bacterial Manganese(II) Oxidase, the Multicopper Oxidase MnxG, from Spores of Several Different Marine Bacillus Species. Appl. Environ. Microbiol. **74**:1527–1534.
30. **Butterfield CN, Soldatova AV, Lee S-W, Spiro TG, Tebo BM**. 2013. Mn(II,III) oxidation and MnO2 mineralization by an expressed bacterial multicopper oxidase. Proc. Natl. Acad. Sci. **110**:11731–11735.
31. **Geszvain K, McCarthy JK, Tebo BM**. 2013. Elimination of Manganese(II,III) Oxidation in Pseudomonas putida GB-1 by a Double Knockout of Two Putative Multicopper Oxidase Genes. Appl. Environ. Microbiol. **79**:357–366.
32. **Geszvain K, Tebo BM**. 2010. Identification of a Two-Component Regulatory Pathway Essential for Mn(II) Oxidation in Pseudomonas putida GB-1. Appl. Environ. Microbiol. **76**:1224–1231.
33. **Brouwers GJ, Vrind JPM de, Corstjens PL a. M, Jong EW de V**. 1998. Involvement of genes of two-step protein secretion pathway in the transport of the manganese-oxidizing factor across the outer membrane of Pseudomonas putida strain GB-I. Am. Miner. **83**:1573–1582.
34. **Caspi R, Tebo BM, Haygood MG**. 1998. c-type cytochromes and manganese oxidation in Pseudomonas putida MnB1. Appl. Environ. Microbiol. **64**:3549–3555.
35. **De Vrind JPM, Brouwers GJ, Corstjens PLAM, den Dulk J, de Vrind-de Jong EW**. 1998. The Cytochrome c Maturation Operon Is Involved in Manganese Oxidation in Pseudomonas putida GB-1. Appl. Environ. Microbiol. **64**:3556–3562.

36. **De Vrind J, De Groot A, Brouwers GJ, Tommassen J, De Vrind-de Jong E**. 2003. Identification of a novel Gsp-related pathway required for secretion of the manganese-oxidizing factor of Pseudomonas putida strain GB-1. Mol. Microbiol. **47**:993–1006.
37. **Larsen EI, Sly LI, McEwan AG**. 1999. Manganese(II) adsorption and oxidation by whole cells and a membrane fraction of Pedomicrobium sp. ACM 3067. Arch. Microbiol. **171**:257–264.
38. **Ridge JP, Lin M, Larsen EI, Fegan M, McEwan AG, Sly LI**. 2007. A multicopper oxidase is essential for manganese oxidation and laccase-like activity in Pedomicrobium sp. ACM 3067. Environ. Microbiol. **9**:944–953.
39. **Dick GJ, Podell S, Johnson HA, Rivera-Espinoza Y, Bernier-Latmani R, McCarthy JK, Torpey JW, Clement BG, Gaasterland T, Tebo BM**. 2008. Genomic Insights into Mn(II) Oxidation by the Marine Alphaproteobacterium Aurantimonas sp. Strain SI85-9A1. Appl. Environ. Microbiol. **74**:2646–2658.
40. **Anderson CR, Johnson HA, Caputo N, Davis RE, Torpey JW, Tebo BM**. 2009. Mn(II) Oxidation Is Catalyzed by Heme Peroxidases in "Aurantimonas manganoxydans" Strain SI85-9A1 and Erythrobacter sp. Strain SD-21. Appl. Environ. Microbiol. **75**:4130–4138.
41. **Solomon EI, Sundaram UM, Machonkin TE**. 1996. Multicopper Oxidases and Oxygenases. Chem. Rev. **96**:2563–2606.
42. **Silva D de, Davis-Kaplan S, Fergestad J, Kaplan J**. 1997. Purification and Characterization of Fet3 Protein, a Yeast Homologue of Ceruloplasmin. J. Biol. Chem. **272**:14208–14213.
43. **Claus H**. 2003. Laccases and their occurrence in prokaryotes. Arch. Microbiol. **179**:145–150.
44. **Taylor AB, Stoj CS, Ziegler L, Kosman DJ, Hart PJ**. 2005. The copper-iron connection in biology: Structure of the metallo-oxidase Fet3p. Proc. Natl. Acad. Sci. U. S. A. **102**:15459–15464.
45. **Deckert G, Warren PV, Gaasterland T, Young WG, Lenox AL, Graham DE, Overbeek R, Snead MA, Keller M, Aujay M, Huber R, Feldman RA, Short JM, Olsen GJ, Swanson RV**. 1998. The complete genome of the hyperthermophilic bacterium Aquifex aeolicus. Nature **392**:353–358.

46. **Höfer C, Schlosser D**. 1999. Novel enzymatic oxidation of Mn2+ to Mn3+ catalyzed by a fungal laccase. FEBS Lett. **451**:186–190.
47. **Nakamura K, GO N**. 2005. Function and molecular evolution of multicopper blue proteins. Cell. Mol. Life Sci. CMLS **62**:2050–2066.
48. **Brouwers G-J, Vrind JPM de, Corstjens PLAM, Cornelis P, Baysse C, Jong EW de V**. 1999. cumA, a Gene Encoding a Multicopper Oxidase, Is Involved in Mn2+ Oxidation in Pseudomonas putida GB-1. Appl. Environ. Microbiol. **65**:1762–1768.
49. **Stoj CS, Kosman DJ**. 2006. Copper Proteins: OxidasesEncyclopedia of Inorganic Chemistry. John Wiley & Sons, Ltd.
50. **Francis CA, Tebo BM**. 2001. cumA Multicopper Oxidase Genes from Diverse Mn(II)-Oxidizing and Non-Mn(II)-OxidizingPseudomonas Strains. Appl. Environ. Microbiol. **67**:4272–4278.
51. **Nelson YM, Lion LW, Ghiorse WC, Shuler ML**. 1999. Production of Biogenic Mn Oxides by Leptothrix discophora SS-1 in a Chemically Defined Growth Medium and Evaluation of Their Pb Adsorption Characteristics. Appl. Environ. Microbiol. **65**:175–180.
52. **Tebo BM, Ghiorse WC, Waasbergen LG van, Siering PL, Caspi R**. 1997. Bacterially mediated mineral formation; insights into manganese(II) oxidation from molecular genetic and biochemical studies. Rev. Miner. Geochem. **35**:225–266.
53. **Forrez I, Carballa M, Verbeken K, Vanhaecke L, Michael Schlüsener, Ternes T, Boon N, Verstraete W**. 2010. Diclofenac Oxidation by Biogenic Manganese Oxides. Environ. Sci. Technol. **44**:3449–3454.
54. **Sunda WG, Kieber DJ**. 1994. Oxidation of humic substances by manganese oxides yields low-molecular-weight organic substrates. Nature **367**:62–64.
55. **Nealson KH, Myers CR**. 1992. Microbial reduction of manganese and iron: new approaches to carbon cycling. Appl. Environ. Microbiol. **58**:439–443.
56. **Ghiorse WC**. 1984. Biology of Iron-and Manganese-Depositing Bacteria. Annu. Rev. Microbiol. **38**:515–550.
57. **Brune A, Frenzel P, Cypionka H**. 2000. Life at the oxic–anoxic interface: microbial activities and adaptations. FEMS Microbiol. Rev. **24**:691–710.

58. **Villalobos M, Toner B, Bargar J, Sposito G**. 2003. Characterization of the manganese oxide produced by pseudomonas putida strain MnB1. Geochim. Cosmochim. Acta **67**:2649–2662.
59. **Klewicki JK, Morgan JJ**. 1998. Kinetic Behavior of Mn(III) Complexes of Pyrophosphate, EDTA, and Citrate. Environ. Sci. Technol. **32**:2916–2922.

CHAPTER TWO

DEVELOPMENT OF A GENETIC SYSTEM FOR A MODEL MN-OXIDIZING PROTEOBACTERIUM, *LEPTOTHRIX DISCOPHORA* SS1

2.1 ABSTRACT

Though many bacteria can oxidize manganese, the genetic determinants controlling this process are not well understood. *Leptothrix discophora* SS1 is an important model organism that has been used to study the mechanism and consequences of biological manganese oxidation especially as it relates to the cycling of carbon and heavy metals. Understanding the molecular underpinnings of manganese oxidation in *L. discophora* SS1 has been hampered by the lack of a genetic system. In this study we report on the development of a genetic system for *L. discophora* SS1. First, the antibiotic sensitivity of *L. discophora* SS1 was characterized and a procedure for transformation with exogenous DNA via conjugation was developed and optimized, resulting in a maximum transfer frequency of $5.2*10^{-1}$ (transconjugant/donor). Genetic manipulation of *Leptothrix* was demonstrated by disrupting *pyrF* via chromosomal integration of a plasmid with an R6Kɣ ori through homologous recombination. This resulted in resistance to fluoroorotidine which was abolished by complementation with an ectopically expressed copy of *pyrF* cloned into pBBR1MCS-5. This genetic system should be amendable to a systematic analysis of the genes involved in manganese oxidation in *L. discophora* SS1.

2.2 INTRODUCTION

Leptothrix discophora SS1 is a filamentous gram negative β proteobacterium best known for its ability to oxidize soluble manganese (II) to insoluble manganese oxides (III and IV). When it was isolated, these oxides encrusted the sheath that surrounded the filaments making the cells clearly visible via light microscopy and gave rise to its early classification as a "sheathed" bacterium (1). In its native habitat, rafts of sheathed *Leptothrix* impregnated with the brown-black ferro-manganese oxides are also visible to the naked eye as a metallic surface film that refracts light much like a sheen of oil (2). It was probably the macroscopic manifestation of this microscopic process that led scientist to the discovery and characterization of *Leptothrix* and the closely related genus *Sphaerotilus* which were described in the scientific literature as early as 1797. In fact, *Leptothrix* was among the first microorganisms to be described in detail by early microbiologists (3).

The result of biological manganese oxidation is a mixture of Mn(III) and Mn(IV) oxides. These products are some of the strongest oxidants in the environment after oxygen and have great adsorption capacities, binding a wide variety of cations including heavy metals to their surfaces (4). These properties emphasize the geochemical importance of manganese oxides in fresh water and marine systems and attract the interest of a community of scientists such as microbiologists, geochemists and water scientists. While the importance of microbial manganese oxidation in biogeochemical cycling has become increasingly evident since it's discovery more than 200 years ago (3, 5-7), the basic questions about the physiological importance of this process to the organisms that perform it, how it happens, and what proteins or cofactors are involved have only recently begun to be addressed.

Although several enzymes responsible for manganese oxidation in other bacteria have been reported (8-10), the molecular determinants of manganese oxidation by *Leptothrix discophora* are poorly characterized. Our understanding of manganese oxidation by *Leptothrix* has been hampered by the lack of a genetic system. Although studies of morphology, physiology and biochemistry that do not necessarily require tractable genetic systems, have increased our knowledge about the biology of *Leptothrix discophora* (11) and it's so called manganese oxidizing factor (MOF), attempts to study MOF by cloning and heterologously expressing the genes thought to be involved did not result in a protein that oxidized manganese (12). Despite previous efforts to develop a genetic system in *Leptothrix* SS1 (13), no successful introduction of exogenous DNA in to *L. discophora* SS1 by transformation, conjugation or transduction has been reported. To move forward in the study of manganese oxidation in this organism, it seems imperative that a genetic system be developed.

Here we report on the development for the first time of a genetic system for *Leptothrix discophora SS1*. We show that the transfer of exogenous DNA to *L. discophora* via conjugation is possible at rates comparable to those of other gram negative bacteria. We demonstrate that a pVIK165 derivative, containing the widely-used R6Kγ conditional origin of replication, was able to integrate into the *Leptothrix* chromosome via homologous recombination thus allowing us to insertionally inactivate *pyrF*, whose product is required for uracil auxotrophy and 5-fluorouracil sensitivity (14). Finally we complemented the *pyrF* insertion mutant *in trans* with a plasmid-born copy of *pyrF* that restored uracil auxotrophy and 5-Fluorouracil sensitivity. This system should allow for a more systematic analysis of the genetic underpinnings of *L. discophora* biology including manganese oxidation as described in chapter 3 of this dissertation.

2.3 MATERIALS AND METHODS

Culture conditions and growth media

Leptothrix discophora SS1 was maintained on 2XPYG medium (15) (per liter: 0.5 g peptone, 0.5 g yeast, 0.5 g glucose, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.07g $CaCl_2$ and 3.57g Hepes, adjusted to pH 7.2 with 1M NaOH) either on plates or poured tubes. Transfers to fresh plates were made every 2 weeks. Cultures for most experiments were grown in 60 mL glass tubes containing with 20 mL 2xPYG medium with antibiotics amended as needed and shaken at 120 rpm. When larger volumes were needed, cells were grown in 50 mL of 2xPYG medium in 250 mL glass flasks. Minimum Salts Vitamin Glucose medium (MSVG, per liter 0.24g $(NH_4)_2SO_4$, 0.06g $MgSO_4 \cdot 7H_2O$, 0.07g $CaCl_2$, 0.02g KH_2PO_4, 0.03 Na_2HPO_4 and 2.38g Hepes, 0.5 glucose and adjusted to pH 7.2 with 1M NaOH) (15) was also used and amended with 1% casamino acids and/or 50 μgmL^{-1} uracil when needed. Antibiotics were used as needed at the following concentrations: 50 μgmL^{-1} kanamycin (Km), 15 μgmL^{-1} nalidixic acid (Nal), 50 μgmL^{-1} rifampicin (Rif), 50 μgmL^{-1} streptomycin, 10 μgmL^{-1} tetracycline, 10 μgmL^{-1} gentomicin, 12.5 μgmL^{-1} chloramphenicol, 100 μgmL^{-1} ampicilin. 5-FOA (5-fluororotidine) was prepared as 1000x DMSO stock solution and used at a concentration of 50 μgmL^{-1} when needed.

Assessment of *L. discophora* SS1 sensitivity to antibiotics and isolation of spontaneous antibiotic resistant mutant

A 10 mL aliquot of an actively growing culture of *L. discophora* SS1 was inoculated into 40 mL of 2xPYG. This culture was allowed to reach logarithmic phase (approximately 2 days) at which point Nal was added to a concentration of 15 μgmL^{-1}. This culture was shaken at 120 rpm for about 7 days, after which it was spun down, the supernatant was removed and the cells were

inoculated in 50 mL of fresh 2xPYG with 15 µgmL^{-1} Nal. This culture was allowed to grow for 7 days at which point 5 mL were removed, concentrated to 100 microliters and spread onto solid 2xPYG medium containing 15 µgmL^{-1} Nal. Colonies that appeared after approximately one week were re-streaked onto fresh antibiotic-containing plates and upon confirmation of their resistance were used for further experiments. Subsequently, a similar approach was used to isolate rifampicin and nalidixic double mutants as well as rifampicin, nalidixic and streptomycin triple resistant mutants from Nal^{+} and Rif^{+} Nal^{+} *L. discophora* derivatives respectively.

Table 2.1: Strains and plasmids used

Species or plasmid	**Relevant characteristics**	**Source or reference**
L. discophora SS1	Wild type	(2)
L. discophora SS1 Nal+	Spontaneous Rif and Nal resistant mutant	This work
L. discophora SS1:pJSpyrF	*pyrF* interrupted mutant	This work
E. coli S17-1 λ pir	*λ pir* lysogen	(34)
pBBR1MCS2	Broad host range vector, Km resistant	(20)
pBBR1MCS	Broad host range vector, Cm resistant	(20)
pBBR1MCS3	Broad host range vector, Tet resistant	(20)
pBBR1MCS4	Broad host range vector, Amp resistant	(20)
pBBR1MCS5	Broad host range vector, Gm resistant	(20)
pVIK165		(29)
pJSpyrF	pVIK165 cloned with internal *pyrF* used for homologous recombination	This work
pBBR1MCSp	pBBR1-MCS cloned with *pyrFpurB*, complementation plasmid	This work

Routine bacterial mating

For routine matings, 20 mL of *L. discophora* SS1 cultures from mid logarithmic to stationary growth stage and 5 mL of an overnight *E. coli* donor were centrifuged separately and washed with 20 and 5 mL of 2xPYG respectively. The suspension was centrifuged again, the supernatant was removed and donor and recipient cells were mixed and placed on a 2XPYG plate without antibiotics. Mating was allowed to occur for 16 to 24 hours, after which the mating mix was scraped off the plate into 1mL of 2xPYG medium. Serial dilutions ($10^{-1}$to -10^{-5}) of this cell suspension were plated onto 2xPYG plates containing the respective selective antibiotic and manganese. Most of the conjugations were selected on media with and without manganese in parallel to asses any effect manganese might have on conjugation outcome. Dilutions of the recipient before and after mating were also plated to assess the survival of *Leptothrix* during the mating process.

Optimization of conjugation

Age of the recipient

For experiments that assessed how age of recipient affected conjugation, *L. discophora* SS1 was grown in 10 replicates (20 mL each). Two replicates were used for each time point (early log phase, mid log phase, late log phase, stationary phase and late stationary phase). The donor culture was refreshed at appropriate intervals such that each mating mix contained donor of the same OD and age.

Donor to recipient ratio

To assess the effect of donor to recipient ratio on conjugation, both donor (*E coli S17*) and recipient (*L. discophora* SS1*)* were grown to mid log phase and late log phase respectively

(OD=0.4 for *E coli* and OD= .25 for *L. discophora* SS1) and the cells numbers/mL were determined by plating serial dilutions. Next, 10 mL of *E coli* and 100 mL of *L. discophora* SS1 grown to OD=0.4 and 0.25 respectively were concentrated 100 times for *Leptothrix* and 10 times for *E. coli* and the thick suspensions were used to obtain the ratios indicated in a final volume of 500µL.

Mating time

Six identical mating mixes were prepared as described in section 3) and were allowed to mate (in duplicate) for 6 hours, 12 hours and 18 hours, after which time they were plated as described above.

Recovery after mating

Two identical mating mixes were set up and mated for the same amount of time and under the same conditions, after which one of the mating mixtures was re-suspended in 1mL 2XPYG and dilutions plated immediately. The other replicate was re-suspended in 20 mL of liquid 2xPYG without antibiotics, and allowed to recover for 6 to 8 hours by shaking on a rotary shaker at 120 RPM. Cells were then collected by centrifugation, re-suspended in 1mL 2xPYG and dilutions were plated as in the case of mating without recovery.

Manganese effect on conjugation

Mating mixes were always plated in parallel on plates containing manganese and plates without manganese for all of the factors assessed.

Plasmid construction/DNA manipulations

A fragment of 640 bp was PCR amplified from the internal portion of the *pyrF* gene of *L. discophora* SS1 using primers designed to add SacI and XbaI restriction sites to each end of the

fragment (nucleotides encoding restriction sites are underlined in the primer sequence) pyrF int ForSacI and pyrF int RevXbaI (table 2); the resulting fragment was cloned into the SacI/XbaI sites of pVIK165 carrying a Km marker, to create plasmid pJSpyrF. This plasmid was transformed into *E.coli S17* and one transformed colony that up took the plasmid was selected and used to mate with *L. discophora* Nal^+. Transconjugant *L. discophora* SS1 colonies were selected onto 2xPYG Nal, Km, Mn plates and checked by PCR for the integration of plasmid into the chromosome. To create the complementation plasmid, a 3Kb fragment of DNA containing both *pyrF* and *purB* genes as well as their native promoter located upstream of *purB* were amplified from *L. discophora* SS1 using primers pyrF upKpnI and purB RevSacI. The PCR product was cleaned, digested and ligated into the Kpn and SacI sites of pBBR1MCS generating plasmid pBBR1MCSp. This plasmid was transformed into *E. coli* S17 and one clone harboring the plasmid was further used as a donor in a mating to create *L. discophora* pBBR1MCSp.

Plasmid stability in *L. discophora* SS1

L. discophora pBBR1-MCS2 and *L. discophora* pJSpyrF were initially scraped from plates, inoculated into 20 mL 2xPYG with appropriate antibiotics and allowed to grow to late exponential phase, when 5mL from each culture were transferred to 15mL of fresh medium containing antibiotics. When this culture reached late exponential growth, 2mL were used to inoculate 2 tubes of 18mL of 2XPYG medium with and without Km and allowed to grow for 2 days after which time 100µL were removed and dilutions were spread onto plates with and without Km. Similar transfers of 2mL from the culture grown without antibiotics to media with and without antibiotics were performed every two days, for 6 transfers spanning 18 days. This experiment was performed in triplicate and each triplicate was spread on two plates. Colony

forming units were averaged and compared to assess plasmid stability with and without antibiotic pressure.

Interruption of *pyrF*, assessment of 5-Fluororotidine (FOA) sensitivity/resistance and Uracil auxotrophy/prototrophy

pJSpyrF was introduced into *Leptothrix* by conjugation and the mating mix was plated on three media in parallel: nalidixic acid/kanamycin and nalidixic acid/kanamycin /5-FOA to select for integrants that abolished the activity of PyrF and on nalidixic acid/5-FOA to account for spontaneous 5-FOA mutants. The cultures were first grown to late logarithmic phase under, using antibiotics when needed. For 5-FOA resistance/sensitivity 500 µL of each culture to be tested were inoculated in 19.5 mL of fresh 2PYG amended with appropriate antibiotics and 5-FOA when needed. For uracil prototrophy/auxotrophy, 500 µL were removed and centrifuged to remove the supernatant. Cells were washed with 500µL of MSVG prior to inoculation into fresh medium with or without 50 µg/mL uracil.

2.4 RESULTS AND DISCUSSION

Characterization of antibiotic sensitivity of *L. discophora* and isolation of several spontaneous antibiotic resistant mutants

Antibiotic markers are the building blocks of a genetic toolbox (16); to develop a genetic system for *L. discophora* SS1 we therefore first analyzed its susceptibility to antibiotics. Initial results were gathered from a disc assay, by spreading 100ul of a stationary phase culture of *L. discophora* SS1 on solid medium, on top of which we applied sterile filter paper discs soaked in antibiotic solution of the tested concentration (table 1). *L. discophora* SS1 showed zones of inhibition for all 11 antibiotics tested. Next, we determined minimum inhibition concentrations (MICs) in liquid cultures, by testing growth in several dilutions of each antibiotic. MICs in liquid cultures were as follows: rifampicin (5µg/mL), ampicillin (1 µg/mL), kanamycin (1 µg/mL), tetracycline (0.5 µg/mL), gentamicin (1 µg/mL), chloramphenicol (0.5 µg/mL), streptomycin/spectinomycin (1 µg/mL), and nalidixic acid (0.5 µg/mL). These results indicate that several different antibiotic markers could be used in downstream genetic manipulations. When antibiotic resistance was acquired by *Leptothrix*, either by spontaneous mutation or provided on a plasmid, the concentration of antibiotics was adjusted as to not interfere with the growth pattern, but to still be effective as selection after genetic manipulations, as seen in table 2.2.

Table 2.2 Sensitivity, MIC and working concentrations of several antibiotics tested on *Leptothrix discophora* SS1

Antibiotic tested	Disc quantity (µg/mL)	Inhibition zone (mm)	MIC liquid (µg/mL)	Working concentrations (µg/mL)
Kanamicin	30	26	1	50
Ampicilin	100	40	1	75
Rifampicin	5	0	5	50
Nalidixic acid	n/a	n/a	0.5	15
Streptomicin	10	34	1	50
Gentomicin	10	21	0.5	8
Tetracycline	30	21	0.5	10
Chloramphenicol	5	12	0.5	8
Novobiocin	30	10	Not tested	Not used
Penicilin	10	30	Not tested	Not used
Spectinomycin	100	32	Not tested	Not used

The growth pattern of the antibiotic resistant mutants was similar to that of the wild type for the concentrations tested (Figure 2.1)

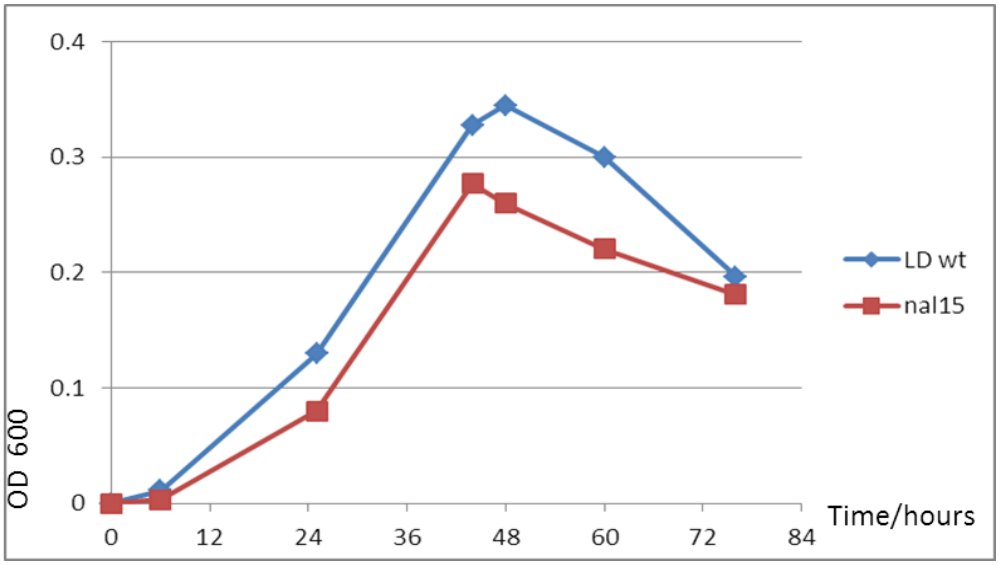


Figure 2.1: Nalidixic acid resistant *L.discophora* SS1 grows like the wild type

Optimization of efficient DNA transfer into *L. discophora* SS1 by conjugation

Previous attempts to establish a DNA transfer protocol by electroporation or chemical competence were not successful (13), most likely due to the low survival of *Leptothrix* cells after the washes required by these methods. Moreover, repeated inconsistencies in the growth of *Leptothrix* when transferred from liquid to solid medium raised a question about the viability of the cells that undergo plating and added another layer of uncertainty. DNA transfer by conjugation is a natural event that happens often in the environment (17). The minimal manipulation of cells required for this process would eliminate some of the problems encountered with electroporation or chemical competency and in turn could increase the probability of DNA transfer. Other potential advantages that conjugation might have are related to the possible restriction system incompatibilities between the donor and recipient as well as the size of the vector transferred. The incompatibilities between the restriction systems of the donor and *Leptothrix* cells is overcome by the fact that in conjugation, transferred DNA enters the recipient as a single strand and is methylated following second strand synthesis, thus bypassing the restriction system of the cells (18). Also, while the size of the transferred DNA is a limitation for electroporation, it does not affect conjugation, as large vectors and genomes have been successfully transferred (19).

An initial conjugation using a *Leptothrix* culture from mid log phase and the broad host range vector pBBR1MCS2 carried by *E. coli S17* host yielded only a single transconjugant colony (approximately 10^{-9} efficiency). Plasmid pBBR1MCS2 is a broad-host-range vector with a small size (5144 bp) and compatibility with IncQ, P and W which has made it a good candidate for many bacterial systems relying on conjugation (20). While this proved to us that conjugation was possible, the development of a functional genetic system requiring much higher levels of

conjugation prompted the testing of a number of parameters to optimize the efficiency of plasmid transfer.

Efficiency of transfer (frequency of transfer) is defined in multiple ways in the literature (21). The most common way of defining it is as transconjugants/donors, although other definitions are used as well, such as transconjugants/recipients and transconjugants/surviving recipients or donors (22). We initially considered using the latter definition in our case, given the poor survival of *Leptothrix* to washing and plating established previously (13) and to account for the loss of cells. We reasoned that washing of cells prior to mating and keeping them at very high densities, with possibly reduced access to oxygen during the mating period, could challeng cells and decrease survival. If this was the case, calculating the efficiency of transfer as transconjugants/surviving recipients would provide the most accurate result. To assess this we plated dilutions of the mating mix and determined *Leptothrix's* viability after the mating period. In general, we did not see significant loss of viability after mating compared to the initial cultures and adopted trasnconjugants/donors as our definition of efficiency of mating.

Ratio of donor to recipient

This is often the factor that is first tested and optimized in efforts to obtain better transfer efficiency. Although it was initially thought that a ratio of donor to recipient of 1:1 would result in the highest frequency of plasmid transfer, reports on the development of genetic systems for other organisms have shown that ratios that favor either the donor or the recipient produce better results in a strain specific manner. For example, while a ratio of 1:1 gave a maximum number of transconjugants for conjugation between *E. coli* strains (23) or between lactococcal species (24), a ratio that favored *E.coli* donors 10^5:1 yielded the best efficiency for transfer to Bifidobacterium strains (22). Another study, however, found that results varied depending on

the kinds of donors, recipients and the mobile element used (25). We tested a range of ratios that favored either *Leptothrix* (the recipient) or *E. coli S17* (the donor) and found out that *Leptothrix* seemed to follow the general rule, with highest plasmid transfer (0.52) and plasmid integration (0.008) rates observed when the ratio of donor to recipient was close to 1:1 (Table 2.3). In general, when ratios favored the *Leptothrix* 1:3, 1:7 or 1:10, the efficiency of transfer dropped rapidly three to six orders of magnitude, with no transconjugants observed when the ratio favored *Leptothrix* 1:100. The change in efficiency was less dramatic when the ratio changed in favor the donor: we saw 30 times fewer transconjugants for ratios that favored the donor 12:1 and 500 times fewer for ratios that favored *E. coli* 50 to 1. 100,000 fewer transconjugants were obtained at ratios that favored the donor 1000:1. This pattern is not particularly surprising, since it is hard to imagine how increasing the number of recipients while maintaining the number of donors could increase the number of transconjugants unless secondary transfer between recipients was occuring. The failure of increased donor numbers, which should have increased the possibility of cell-to-cell contact and hence the transfer of plasmid DNA from donor to recipient was unexpected.

Table 2.3 Frequency of transfer of replicative and non-replicative plasmids from *E.coli S17* to *L. dischophora* SS1. The frequency of transfer varied for different ratios of donor to recipient, being the highest at an approximately 1 to 1 ratio. Ratios that favor the donor (*E. coli)* 10 times or more resulted in the lowest efficiency, with no transconjugants for ratio of 1:100; The results are the mean of two biological replicates, with each replicate plated on two plates. a, b, c: data in each of the a, b, c groups was obtained from three independent experiment

Plasmid used	**Donor total cfu #**	**Recipient total cfu #**	**Transconjugant total cfu #**	**Ratio Donor/ recipient**	**Frequency of transfer (donor/ transconjugant)**
Replicative plasmids					
	$3*10^{10}$	$2.6*10^{12}$	0	**1 : 100[a]**	0
	$3*10^{11}$	$2.6*10^{12}$	$1.8*10^{6}$	**1 : 10[a]**	$6*10^{-6}$
pBBR1MCS2 (Km^R)	$2*10^{11}$	$1.5*10^{12}$	$5*10^{6}$ cells	**1 : 7[a]**	$2.5*10^{-5}$
	$5*10^{11}$	$1.5*10^{12}$	10^{8} cells	**1 : 3[a]**	$2*10^{-4}$
	$5*10^{7}$	$3.6*10^{7}$	$2.6*10^{7}$	**1.4 : 1[b]**	0.52
	$2.5*10^{8}$	$2*10^{7}$	$4.5*10^{6}$	**12 : 1[b]**	$1.8*10^{-2}$
	10^{12}	$5*10^{10}$	10^{9}	**50 : 1[c]**	10^{-3}
	$4.5*10^{8}$	$4*10^{6}$	$1.4*10^{6}$	**112 : 1[b]**	$3.11*10^{-3}$
	$4*10^{11}$	4*108	$5*10^{6}$	**1000 : 1**	$1.25*10^{-5}$
pBBR1MCS3 (Tet^R)	10^{12}	$5*10^{10}$	$5*10^{8}$	**50 : 1[c]**	5810^{-4}
Non-replicative plasmids and plasposoms					
pJS:mofA250 (Km^R)	$2.5*10^{8}$	$2*10^{7}$	$2.5*10^{5}$	**12 : 1[b]**	10^{-3}
	$5*10^{7}$	$3.6*10^{7}$	$4*10^{5}$	**1.4 : 1[b]**	$8*10^{-3}$
	10^{12}	$5*10^{10}$	$5*10^{8}$	**50 : 1[c]**	$5*10^{-4}$
	$4.5*10^{8}$	$4*10^{6}$	$1.5*10^{5}$	**112 :1[b]**	3.3810^{-4}
pJS:mofA330 (Km^R)	10^{12}	$5*10^{10}$	$7*10^{8}$	**50 : 1[c]**	$7*10^{-4}$
pUTminiTn5tet	10^{12}	$5*10^{10}$	10^{8}	**50 : 1[c]**	10^{-4}

Growth stage of recipient

The impetus for testing this factor was the assumption that cells harvested at different growth stages would have different susceptibilities to conjugation. Conjugation is an energetic expense both for the donor as well as for the recipient. It was hypothesized that logarithmically growing cells would have more resources to spend on conjugation as opposed to cells in stationary phase and that this difference would affect the number of transconjugants. By assessing the number of transconjugants from recipient cultures kept identical except for their age, we wanted to evaluate whether there were other growth stage dependent factors, besides energetic considerations, that could affect the outcome of conjugation. Similar numbers of transconjugants were obtained for early log phase and mid log phase and these values were an order of magnitude lower than transconjugants obtained with *Leptothrix* from either late logarithmic, stationary or late stationary phase (Data not shown).

Time of mating

The amount of mating time has been reported to have an effect on the number of transconjugants for some bacteria but not others (17). We wanted to determine the optimal time of mating for *Leptothrix* that would allow sufficient time for the plasmid transfer to occur, but not too long as to energetically deplete and stress the cells. Mating times of 6 and 12 hours resulted in similar number of transconjugants whereas the number was an order of magnitude lower for matings that lasted for 18 hours (Data not shown).

Recovery versus no recovery after mating

We tested this parameter to determine if cells exposed to mating conditions experience stress. A period of time without the antibiotic pressure was used to allow transconjugants to recover before they were plated on antibiotic selective plates. We had already observed, independently of the conjugation experiments, that plating the same number of cells under the same conditions could sometimes result in different numbers of colony forming units (CFUs) and hypothesized that plating itself may constitute a stress for *Leptothrix* cells. Giving mated cells a recovery period would allow them to better confront this stress. To this end, the mated mixture was re-suspended in 20 mL of liquid 2xPYG without antibiotics, and allowed to recover for six hours (with shaking). After that the cells were collected by centrifugation, re-suspended in 1mL 2xPYG and treated identically to the non-recovered cells. The generation time for *Leptothrix* is three hours under optimal, logarithmic growth conditions, therefore six hours was expected to not result in more than a doubling, and this should not require any adjustments to the calculation of efficiency. The same number of transconjugants was obtained for both recovered and non-recovered mating mixes, indicating that recovery of mated cells in liquid media before plating was not needed (Data not shown).

Selecting on Manganese versus no Mn plates

The role that manganese oxidation plays in the life of a manganese oxidizing bacterium is still in question (26). In the light of this we wanted to determine if the presence of manganese in the selection plates would have an effect on the number of transconjugants obtained. Observations in our lab and other reports (27), suggest that some antibiotics such as kanamycin interfere with manganese oxidation. However, there was not a significant difference between

transconjugant numbers obtained on plates with manganese compared to the plates without manganese.

Based on the results obtained from testing these parameters, an optimized protocol was established that was used thereafter for routine *Leptothrix* conjugation: 20 mL of cultures grown in liquid medium to late log phase (OD=0.25) were mixed with the *E. coli* recipient in a in a 1 to 1 ratio and allowed to mate for 6 to 12 hours on solid 2XPYG medium after which the mating mixture was diluted and plated directly on plates containing manganese, without recovery. It was imperative that 2 or 3 dilutions of the mating mixture be plated, since a very dense or dilute mating mixture often did not yield any transconjugants. Dilutions in the range of 10^{-2} and 10^{-3} typially resulted in tens or hundreds of well separated transconjugant colonies per plate. In general visible colonies of transconjugants transformed with replicative plasmids would appear after 6-7 days, while transconjugants in which the plasmid integrated into the chromosome would only become visible after 10-12 days. Importantly, while transconjugants were obtained from most of the conjugations, it was difficult to obtain an identical number of transconjugants even from identical replicates of the same experiments inoculated from the same culture. This suggests that there are variations within each culture of *Leptothrix* that affect the viability and readiness of cells to undergo conjugation.

Plasmid stability in *L. discophora*

Some plasmids can be stably maintained in a host even without the selective pressure (antibiotics, in most cases) while other plasmids are easily lost once the selective pressure is removed (28). The stability or instability of a plasmid in a host can therefore be used to

advantage when developing genetic systems that rely on recombination so long as they contain appropriate selection and counter selection markers. Because certain antibiotics (Km) interact with manganese oxidation in *Leptothrix discophora* SS1 and some assays to quantify manganese oxidation would require removal of antibiotics from the medium, we assessed the stability of both the broad host range vector pBBR1MCS2 (20) and of the suicide vector pJSmofA250, the latter having a conditional ori that replicates only in hosts containing the Pir protein (29). In this case, our pJS derivative contained a 250bp portion of the gene encoding the putative manganese oxidizing factor *mofA* that allowed it to integrate into the chromosome via homologous recombination (described in chapter 3). Loss of plasmids from the cells was detectable in both cases when the antibiotic selection was removed (Figure 2.2). In the case of pBBR1MCS2, after growing a culture for 3 days (first transfer) without antibiotic selection (approximately 18 generations) 20% fewer CFUs were obtained on plates with the antibiotic as compared to plates without antibiotic. When an aliquot from the first transfer was grown again for 3 days without antibiotics (second transfer) and plated on selective and non-selective plates, a further decrease in CFU was observed, suggesting that without the antibiotic the plasmid continued to be lost. After 6 transfers and approximately 100 generations, only 20% of the cells that grew on non-selective media also grew on kanamycin plates, suggesting that the plasmid had been lost from the other 80%. The integrated pJS plasmid followed a similar trend, albeit with a less abrupt slope. . The loss of pBBR1MCS at approximately 1% per generation was small enough to allow this plasmid to be valuable as a tool for complementing mutations that will be generated. By comparison, the same plasmid experienced 100% loss within 12 generations in a *Geobacter sulfurreducens host* (28).

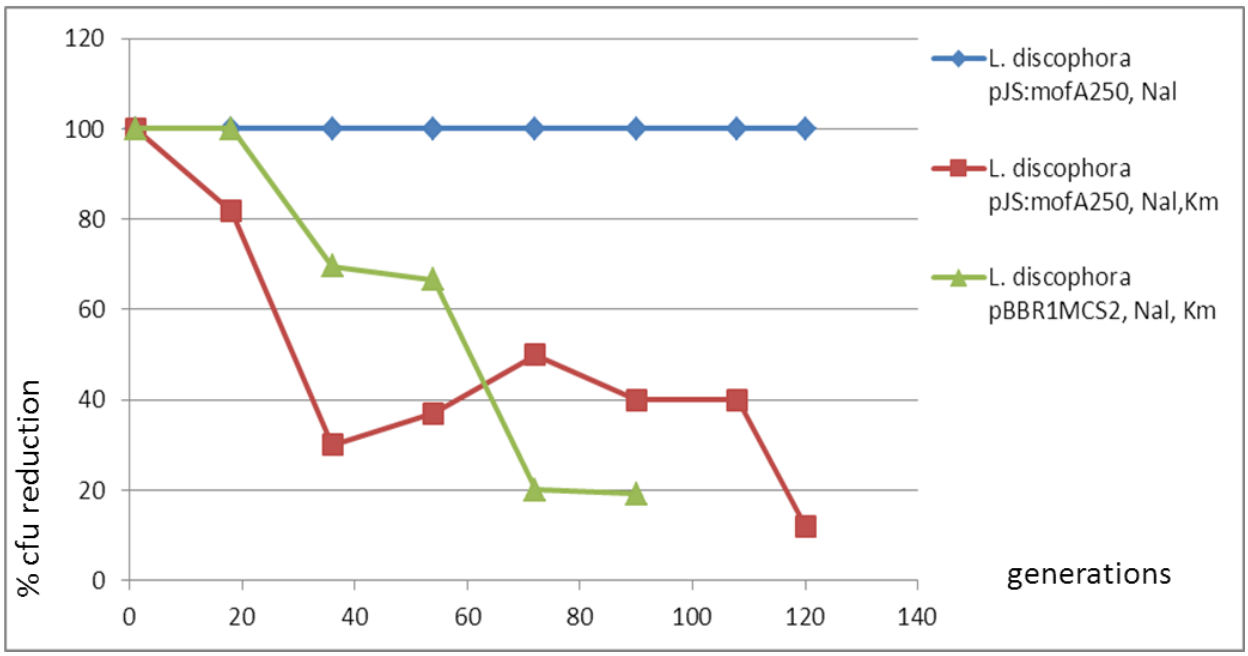


Figure 2.2: Loss of replicative plasmid pBBR1-MCS2 (triangles) and chromosome integrated plasmid pJSmofA250 (squares) from *Leptothrix discophora* SS1 cells respectively, over more than 100 generations (7 transfers over 18 days) in the absence of antibiotic selection.

Construction of a 5-fluroorotic acid resistant uracil auxotroph by disruption of orotidine 5' phosphate decarboxylase (*pyrF*) and restoration of function upon complementation

a) Identification of a potential gene to disrupt

To demonstrate the efficiency of the genetic system we developed for *L.discophora* SS1 we disrupted *pyrF* which is predicted to encode orotidine 5' phosphate decarboxylase, an enzyme involved in the *de novo* synthesis of pyrimidines (30). Insertion of pJS:pyrF by homologous recombination resulted in resistance to 5-FOA and uracil auxotrophy (Figure 6a and

6b). When a wild type copy of the gene was provided in *trans* on pBBR1MCS-5 (a gentamicin resistant derivative of pBBR1MCS-2), the activity was restored (Figure 6a and 6b).

This gene was chosen because it lends itself to both positive and negative selection strategies. Its ortholog (*URA3)* has been widely used in yeast genetic systems and more recently in bacteria, especially to create markerless mutations in strains with multiple natural antibiotic resistance determinants (31)(32). *pyrF* codes for the last of the five enzymes found in the pathway for *de novo* production of pyrimidines (Figure 2.3). Under normal circumstances, PyrE orthologs (orotate phosphoribosyl transferase) convert orotate to orotidine monophosphate (OMP) which is further transformed to UMP (uridine monophosphate) by PyrF. UMP is the precursor of all pyrimidine nucleotides. When 5-FOA is added to the medium, it is converted to 5-F-OMP (5-fluoro-orotylidate) by PyrE and then to 5-F-UMP by PyrF. While 5-FOA and to 5-F-OMP are not toxic, the accumulation of 5-F-UMP leads to inhibition of macromolecular synthesis (RNA) and cell death. Disruption of *pyrF* stops the activity of orotidine 5' phosphate decarboxylase, preventing the production of toxic 5-F-UMP essentially rendering them resistant to 5-FOA. The other consequence of *pyrF* interruption is the loss of *de novo* uracil production, rendering the cells uracil auxotrophs. This combination of resistance/sensitivity to 5-FOA and uracil auxotrophy/prototrophy allows this strategy to be employed as both positive and negative selection (33).

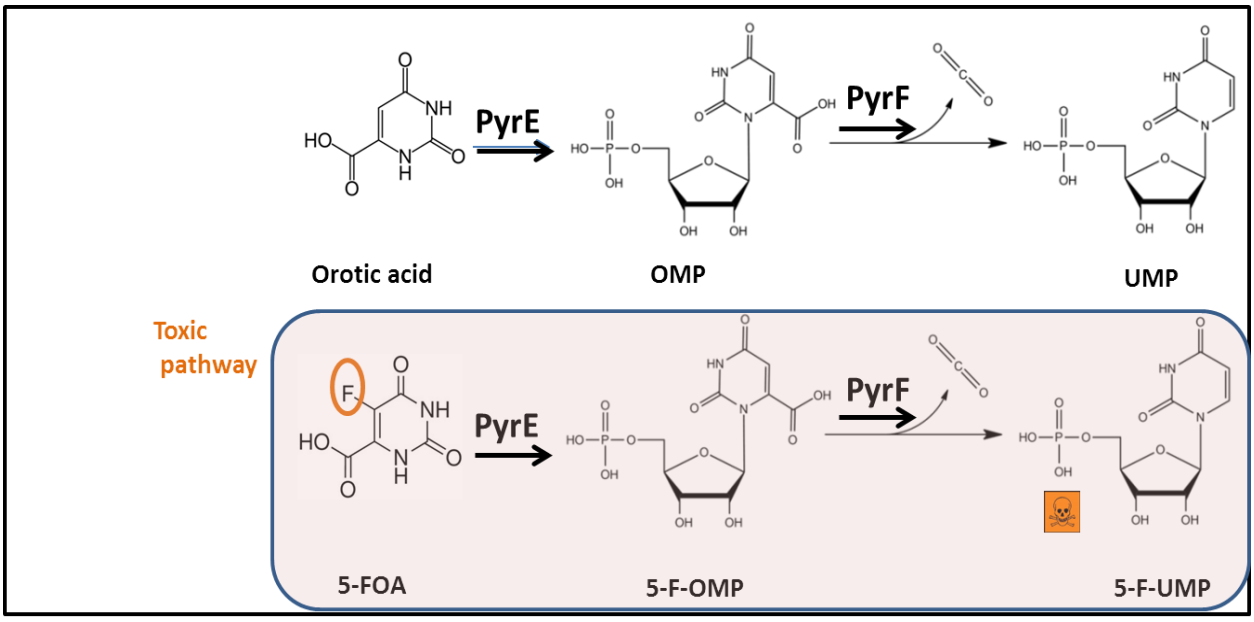


Figure 2.3 (top row) *De novo* pyrimidine biosyntetic pathway in *Leptothrix discophora* SS1 is predicted to include 5 enzymes and culminates with production of UTP; the enzymes and substrates are as follows: PyrB, aspartate transcarbamoylase PyrC-Dihydroorotase, PyrD-Dehydroorotate dehydrogenase, PyrE-Orotate phosphoribosyl transferase and PyrF- Orotidine 5' monophosphate (OMP) decarboxylase; OMP- orotidin 5-phosphate, UMP- uridine 5-phosphate.(bottom row) The same pathway will produce toxic compounds deadly to the cell when 5-FOA enters the pathway as an orotate analog at the PyrE step and is further transformed by PyrF to toxic 5-F-UMP that accumulates in the cell and causes death.

We chose to disrupt *pyrF* for several reasons: a) in searching the draft genome of *L. discophora* SS1 we identified putative *pyrF* homolog whose upstream and downstream regions contained open reading frames similar to the other four genes specific for the *de novo* pyrimidine synthesis (*pyrB, pyrC, pyrD and pyrE*); b) we were able to isolate spontaneous 5-FOA resistant *Leptothrix* mutants (data not shown), we thus had preliminary evidence that this was an efficient selection system that resulted in an easily identified and tested phenotype; c) *pyrF* has potential

to be developed into a more refined genetic tool allowing for the construction of markerless second deletions in a marked first deletion background.

b) Gene disruption and *in trans* complementation

After mating, hundreds of *Leptothrix* transconjugants were obtained on both nalidixic acid/kanamycin and nalidixic acid/kanamycin /5-FOA, while zero to one or two colonies were obtained on nalidixic acid/5-FOA, confirming that the majority of 5-FOA resistant mutants arose due to insertion of the plasmid at the *pyrF* locus and not to the acquisition of spontaneous resistance to 5-FOA.

Single-crossover integration at the *pyrF* locus would result in the generation of two partial copies of the *pyrF* gene in the chromosome, one truncated at the 3'end the other at the 5' end, separated from one another by 5000kb of plasmid. This scenario was confirmed by PCR (Figure 2.5). While the internal *pyrF* fragment could be amplified from both the wild type *Leptothrix* SS1 and the *pyrF* mutant (Figure 2.5, panel a), the full gene could not be amplified from the *Leptohtrix* SS1:pJSpyrF mutant (Figure 2.5, panel b). When the extension time was increased to 3 minutes, an approximately 6 kb PCR product resulted, consistent with the amplification of the entire plasmid that disrupted pyrF (data not shown). A primer pair designed to anneal to the region upstream of *pyrF* and to the GFP encoding gene on the plasmid only amplified a product from the integration mutant and not the wild type, confirming the presence of the plasmid in the chromosome at the *pyrF* locus (Figure2.5, panel c).

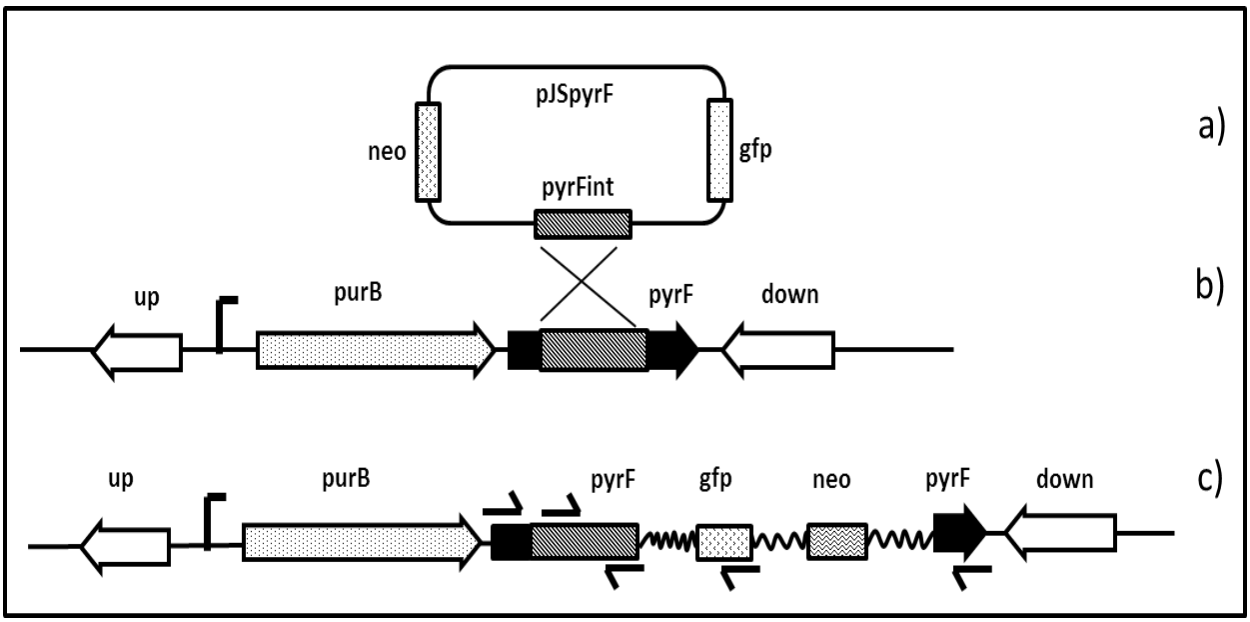


Figure 2.4: Schematic representation of *pyrF* disruption by integration of the plasmid pJSpyrF into the chromosome of *L. discophora* SS1 by homologous recombination at the *pyrF* locus; a) an internal fragment of the *pyrF* gene was cloned in to the multicloning site of plasmid pVIK165 to generate pJSpyrF, which also carries the genes for Km resistance and GFP expression; b) because pJSpyrF requires the pir protein to replicate, it can only persist in the cell by integration into the chromosome which is facilitated by the presence of the cloned fragment of *pyrF*; c) integration of the plasmid into the chromosome interrupted the chromosomal copy of *pyrF* resulting in two nonfunctional *pyrF* fragments.

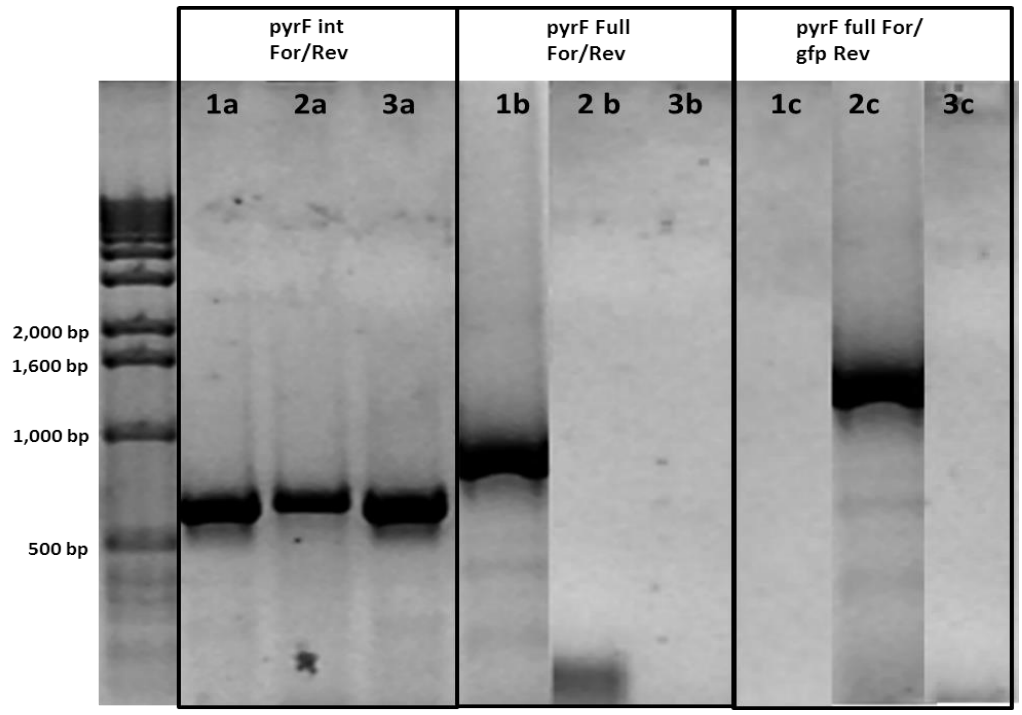


Figure 2.5: Electropherogram of PCR products from the *L. discophora pyrF* mutant confirms disruption of the gene. a) pyrF int For/Rev amplify the *pyrF* internal fragment used for homologous recombination from both the wild type (1), the insertion mutant (2) and the plasmid pJSpyrF (3) b) pyrF full For/Rev located upstream and downstream of pyrF amplify the entire *pyrF* gene*;* as expected, PCR product is only seen for the wild type (1), but not the insertion mutant (2) or the plasmid (3) c) pyrF full For/GFP rev *pyrF* can only amplify from the insertion mutant (2), but not the wild type (1) due to no annealing site for GFPrev primer or the plasmid (3), due to no annealing site for the pyrF full primer

To confirm that the resistance to 5-FOA was a result of disrupting the activity of orotidine 5' monophosphate (OMP) decarboxylase (PyrF) and not to unexpected secondary mutation or some unanticipated effect on downstream gene expression, a wild type copy of *pyrF* with its native promoter was provided on plasmid pBBR1MCS resulting in plasmid pBBR1MCSp. An *in silico* search of the upstream region of *pyrF* for its native promoter revealed

that the gene *purB* (involved in the synthesis of purines) was located only 11 nucleotides upstream of *pyrF* and that the nearest easily identifiable promoter was upstream of *purB*. Although *purB* should not be required for complementation, the complementation construct included the putative promoter upstream of *purB*, as well as *pyrF* because efforts to artificially clone the *purB* promoter immediately adjacent to *pyrF* failed consistently. *L. discophora* SS1:pJSpyrF was transformed with the replicative pBBR1MCSp and transconjugants were readily obtained on nalidixic acid, kanamycin and chloramphenicol. If *pyrF* was successfully expressed from the complementation plasmid, then transconjugant *Leptothrix* SS1:pJSpyrFpBBR1MCSp cells should have an active PyrF that would convert 5-FOA to its toxic compounds and the cells would not be able to grow in the presence of 5-FOA. Restoring PryF activity also means restoring the *de novo* production of uracil, hence the cells would return to uracil prototrophy and should be able to grow in minimal medium without uracil. As seen in Figure 2.6.a), the complemented strain behaved in a similar manner to wild type *Leptothrix* SS1 and could not grow in medium with 5-FOA. In contrast, 5-FOA did not have a toxic effect on SS1:pJSpyrF with or without the vector control. When assessed for uracil requirements, the *pyrF* interrupted mutant with or without the empty vector could not grow in medium without uracil, confirming a loss of *de novo* uracil production in the growth of these mutants. Growth of the mutant, however, was indistinguishable from the wild if uracil was provided in the medium (Figure 2.6.b). Again, *Leptothrix* SS1:pJSpyrF pBBR1MCSp showed wild type uracil prototrophy and 5-FOA sensitivity providing clear evidence of complementation.

Overall these assays confirm that the genetic system described here enabled successful manipulation of a gene of interest in *L. discophora* SS1.

Although our mutant retained its antibiotic marker this work suggests that a clean deletion of *pyrF* could be constructed with the same vector by flanking the *neo gene* on pVIK165 with sequences upstream of *pyrF* on one side and downstream of *pyrF* on the other. The first round of selection on Km would select for plasmid integration upstream or downstream of *pyrF*, while a second round of selection on 5-FOA would select for deletion of *pyrF*. Use of a mutant with a clean *pyr*F deletion together with a second pVIK165 derivative that contained a wild type copy of *pyrF* , would allow for selection of transconjugants into other chromosomal locations based on the restoration of de novo uracil biosynthesis and therefore growth on minimal medium. In theory, such a system could be used again and again to generate multiple markerless mutations in the same background.

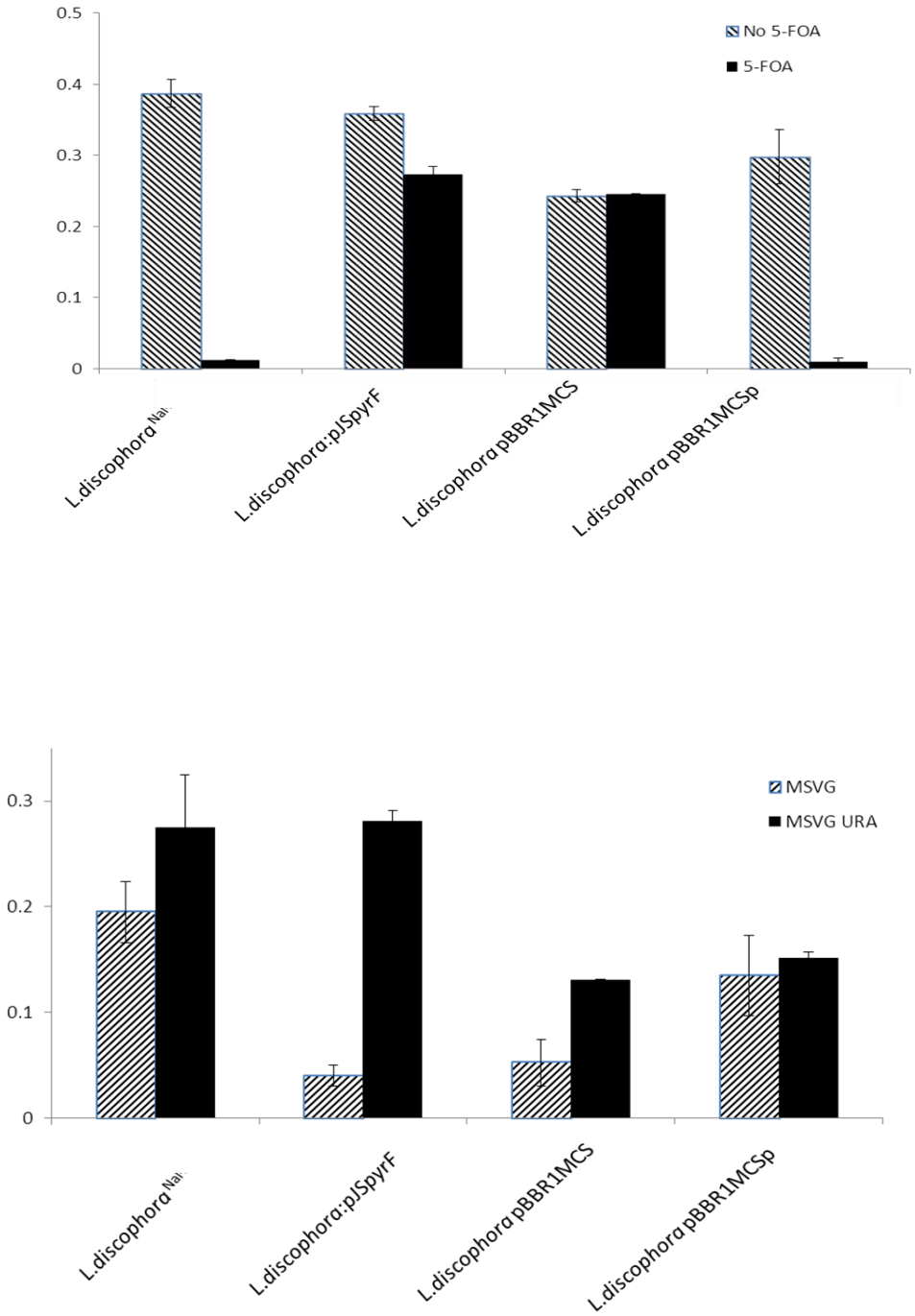


Figure 2.6 a (top) and b (bottom) Complementation of *L. discophora* SS1:*pyrF* mutant a with a wild type copy of *pyrF* located on pBBR1MCS restores the wild type phenotype, rendering the cells sensitive to 5-FOA and uracil prototrophs; a) comparison of maximum growth in the absence (light bars) and presence (dark bars) of 5-FOA; b) maximum growth in the absence and presence of uracil of the uracil.

2.5 CONCLUSION

Overall, we were able, for the first time, to develop a genetic system for *L. discophora* and to confirm its effectiveness by inactivating a gene of interest (*pyrF*), showing loss of function, then complement in *trans* with a wild type copy on a plasmid and recovery of the function of the interrupted gene. We have shown that plasmid DNA can be introduced into *Leptothrix* cells by conjugation at high frequencies of transfer that are comparable with other microbial systems and most importantly enable further genetic manipulations such as isolation of chromosomal integrants. Moreover, we have demonstrated that we can also deliver plasmids encoding transposons to *Leptothrix* SS1, which should open the way to random insertional inactivation. Together these techniques should help us identify previously uncharacterized genes that may have a role in manganese oxidation while also scrutinizing the role that previously implicated genes such as *mofA* play in this important process. Lastly, the deletion/complementation of *pyrF* provides proof of concept that *pyrF* has potential as a selectable marker in *Leptothrix* and could be further developed into a more powerful tool for creating multiple deletions in the same strain.

2.6 REFERENCES

1. **Ghiorse WC**. 1984. Biology of Iron-and Manganese-Depositing Bacteria. Annu. Rev. Microbiol. **38**:515–550.
2. **Adams LF, Ghiorse WC**. 1986. Physiology and ultrastructure of Leptothrix discophora SS-1. Arch. Microbiol. **145**:126–135.
3. **Schleifer K-H**. 2006. The Prokaryotes: A Handbook on the Biology of Bacteria. Springer.
4. **Tebo BM, Bargar JR, Clement BG, Dick GJ, Murray KJ, Parker D, Verity R, Webb SM**. 2004. BIOGENIC MANGANESE OXIDES: Properties and Mechanisms of Formation. Annu. Rev. Earth Planet. Sci. **32**:287–328.
5. **Dondero NC**. 1975. The Sphaerotilus-Leptothrix Group. Annu. Rev. Microbiol. **29**:407–428.
6. **Kielemoes J, Bultinck I, Storms H, Boon N, Verstraete W**. 2002. Occurrence of manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water. FEMS Microbiol. Ecol. **39**:41–55.
7. **Ghiorse WC**. 1984. Biology of Iron-and Manganese-Depositing Bacteria. Annu. Rev. Microbiol. **38**:515–550.
8. **Ridge JP, Lin M, Larsen EI, Fegan M, McEwan AG, Sly LI**. 2007. A multicopper oxidase is essential for manganese oxidation and laccase-like activity in Pedomicrobium sp. ACM 3067. Environ. Microbiol. **9**:944–953.
9. **Anderson CR, Johnson HA, Caputo N, Davis RE, Torpey JW, Tebo BM**. 2009. Mn(II) Oxidation Is Catalyzed by Heme Peroxidases in “Aurantimonas manganoxydans” Strain SI85-9A1 and Erythrobacter sp. Strain SD-21. Appl. Environ. Microbiol. **75**:4130–4138.
10. **Francis CA, Tebo BM**. 2002. Enzymatic Manganese(II) Oxidation by Metabolically Dormant Spores of Diverse Bacillus Species. Appl. Environ. Microbiol. **68**:874–880.
11. **Adams LF, Ghiorse WC**. 1987. Characterization of extracellular Mn2+-oxidizing activity and isolation of an Mn2+-oxidizing protein from Leptothrix discophora SS-1. J. Bacteriol. **169**:1279–1285.
12. **El Gheriany IA**. 2010. Manganese oxidation by Leptothrix discophora SS1. Cornell University.

13. **Siering PL**. 1996. Application of molecular approaches to investigate the role of microorganisms in manganese cycling in wetland environments. Cornell University.
14. **Thia-Toong T-L, Roovers M, Durbecq V, Gigot D, Glansdorff N, Charlier D**. 2002. Genes of De Novo Pyrimidine Biosynthesis from the Hyperthermoacidophilic Crenarchaeote Sulfolobus acidocaldarius: Novel Organization in a Bipolar Operon. J. Bacteriol. **184**:4430–4441.
15. **Adams LF**. 1986. Physiology, Cytology, and manganese oxidizing activityof Leptothrix discophora SS1. Cornell University.
16. **Bitan-Banin G, Ortenberg R, Mevarech M**. 2003. Development of a Gene Knockout System for the Halophilic Archaeon Haloferax volcanii by Use of the pyrE Gene. J. Bacteriol. **185**:772–778.
17. **Chen I, Christie PJ, Dubnau D**. 2005. The Ins and Outs of DNA Transfer in Bacteria. Science **310**:1456–1460.
18. **Szostková M, Horáková D**. 1998. The effect of plasmid DNA sizes and other factors on electrotransformation of Escherichia coli JM109. Bioelectrochem. Bioenerg. **47**:319–323.
19. **Isaac JH, Holloway BW**. 1968. Control of Pyrimidine Biosynthesis in Pseudomonas aeruginosa. J. Bacteriol. **96**:1732–1741.
20. **Kovach ME, Elzer PH, Steven Hill D, Robertson GT, Farris MA, Roop II RM, Peterson KM**. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166**:175–176.
21. **Hao L, Liu X, Wang H, Lin J, Pang X, Lin J**. 2012. Detection and validation of a small broad-host-range plasmid pBBR1MCS-2 for use in genetic manipulation of the extremely acidophilic Acidithiobacillus sp. J. Microbiol. Methods **90**:309–314.
22. **Dominguez W, O'Sullivan DJ**. 2012. Developing an efficient and reproducible conjugation-based gene transfer system for bifidobacteria. Microbiology **159**:328–338.
23. **Fernandez-Astorga A, Muela A, Cisterna R, Iriberri J, Barcina I**. 1992. Biotic and abiotic factors affecting plasmid transfer in Escherichia coli strains. Appl. Environ. Microbiol. **58**:392–398.
24. **Lampkowska J, Feld L, Monaghan A, Toomey N, Schjørring S, Jacobsen B, van der Voet H, Andersen SR, Bolton D, Aarts H, Krogfelt KA, Wilcks A, Bardowski J**. 2008.

A standardized conjugation protocol to asses antibiotic resistance transfer between lactococcal species. Int. J. Food Microbiol. **127**:172–175.

25. **Willetts N, Wilkins B**. 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. **48**:24–41.
26. **Nealson KH, Tebo BM, Rosson RA**. 1988. Occurrence and Mechanisms of Microbial Oxidation of Manganese, p. 279–318. *In* Allen I. Laskin (ed.), Advances in Applied Microbiology. Academic Press.
27. **Rosson RA, Tebo BM, Nealson KH**. 1984. Use of Poisons in Determination of Microbial Manganese Binding Rates in Seawater. Appl. Environ. Microbiol. **47**:740–745.
28. **Coppi MV, Leang C, Sandler SJ, Lovley DR**. 2001. Development of a Genetic System forGeobacter sulfurreducens. Appl. Environ. Microbiol. **67**:3180–3187.
29. **Kalogeraki VS, Winans SC**. 1997. Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene **188**:69–75.
30. **Haugaard L e., West T p.** 2002. Pyrimidine biosynthesis in Pseudomonas oleovorans. J. Appl. Microbiol. **92**:517–525.
31. **Galvao TC, de Lorenzo V**. 2005. Adaptation of the Yeast URA3 Selection System to Gram-Negative Bacteria and Generation of a ?betCDE Pseudomonas putida Strain. Appl. Environ. Microbiol. **71**:883–892.
32. **Schneider JC, Jenings AF, Mun DM, McGovern PM, Chew LC**. 2005. Auxotrophic markers pyrF and proC can replace antibiotic markers on protein production plasmids in high-cell-density Pseudomonas fluorescens fermentation. Biotechnol. Prog. **21**:343–348.
33. **Takeno S, Sakuradani E, Murata S, Inohara-Ochiai M, Kawashima H, Ashikari T, Shimizu S**. 2004. Cloning and sequencing of the ura3 and ura5 genes, and isolation and characterization of uracil auxotrophs of the fungus Mortierella alpina 1S-4. Biosci. Biotechnol. Biochem. **68**:277–285.
34. **Kolter R, Helsinki DR**. 1979. Regulation of Initiation of DNA Replication. Annu. Rev. Genet. **13**:355–391.

CHAPTER THREE

ISOLATION OF *LEPTOTHRIX DISCHOPHORA* SS1 Δ*MOFA* TRANSPOSON MUTANTS THAT CANNOT OXIDIZE MANGANESE

3.1 ABSTRACT

The recent development of a genetic system for *Leptothrix discophora* SS1 coupled with the availability of a draft genome sequence has opened up the possibility of identifying the genetic determinants of manganese oxidation by this important model organism. Currently, two classes of proteins, multicopper oxidases and heme peroxidases, have been shown to catalyze manganese oxidizing activity in bacteria. In strain SS1, MofA, a putative multicopper oxidase, has long been thought to be responsible for manganese oxidation. We report here, however, that SS1 still oxidized manganese like wild type colonies on plates even when 94% of *mofA* was deleted. In addition, interruption of two other putative multicopper oxidases, *mcoA* and *mcoB* and two putative heme peroxidases, *hemA* and *hemB* that were similar to genes known to encode manganese oxidation in other organisms, did not affect manganese oxidation by SS1. Screening of a transposon mutant library of SS1 also failed to yield any manganese-oxidation deficient mutants. Transposon mutagenesis of the Δ*mofA* mutant, however, resulted in the isolation of more than 60 mutants that show no or altered manganese oxidation activity on plate. None of the non-manganese oxidizing mutants characterized had insertions in genes initially considered as candidates likely to encode manganese oxidation (*mofA, mcoA, mcoB, hemA, hemB*) or to *mofA2*

which shares 85% nucleotide identity with *mofA.* The insertions in manganese deficient mutants were located in four genes, in two distinct regions on the chromosome. One of the genes, named *mnxG2* (region 2) was annotated as a putative multicopper oxidase similar to the manganese oxidizing enzyme in *Bacillus sp* SG-1. The other three genes all appeared to be in the same operon (region 1) and are predicted to encode a hypothetical protein, a putative cytochrome c, and a putative copper metallochaperone (Sco1/SenC/PrrC) for the biogenesis of cytochrome oxidase. Further analysis of these genes should help elucidate the mechanism of manganese oxidation in *Leptothrix discophora* SS1 and address the possibility of redundant manganese oxidation systems (*mofA* and *mnxG2*), as was recently reported for *Pseudomonas putida GB-1.*

3.2 INTRODUCTION

Leptothrix discophora SS1 (SS1) is a model organism for the study of manganese oxidation in bacteria. Other well-studied manganese oxidizers include *Pseudomonas putida MnB-1, Bacillus sp SG-1 and Pedomicrobium sp. ACM 3067* (1–4). Although the study of manganese oxidation in the laboratory has focused primarily on these organisms, there is abundant evidence that this process is wide spread in nature, both in terms of the phylogenetic diversity of the bacteria involved (3, 5, 6) as well as of the environments in which it can occur (7–9). The ubiquity of this process coupled with the fact that it is enzymatically driven is somewhat surprising given that the selective benefits to the cells performing it are unclear (10, 11). Why would microorganisms invest energy to catalyze a process with no apparent immediate

benefit? It is hoped that by learning how the process happens, what enzymes and genes are involved, that we may learn more about the biological context of the process and further understand why it happens.

There is unequivocal evidence that manganese oxidation at circumneutral pH is enzymatic (7). While initial experiments were focused on proving the enzymatic nature of this process within bacteria (1, 12–14), more recent publications have reported on efforts to identify the genes and proteins responsible for this process (4, 15). The manganese oxidizing proteins/enzymes fall in two groups: multicopper oxidases (16, 17) and manganese peroxidases with Ca^{2+} binding domains (4) (Table 3). Besides these proteins which have been reported to perform manganese oxidation itself, transposon mutagenesis experiments in *Pseudomonas putida GB-1* and *Bacillus sp SG-1* identified a number of other genes which, when disrupted, abolished manganese oxidation. These were genes encode proteins involved in the cytochrome c maturation pathway (*ccm*) (18), in the general secretory pathway (*ssh* genes) (19) and in the production of tryptophan. In addition to transposon mutagenesis, a variety of other techniques such as proteomics, classic molecular biology and reverse genetics have been used to try and identify the genes coding for manganese oxidation (table 3). The multicopper oxidase MnxG of *Bacillus sp. SG-1* was identified by transposon mutagenesis (20, 21) while the MCO MoxA *of Pedomicrobium* was discovered using a primer walking approach (17).

Although the involvement of some of these genes (PputBG-1_2447 and PputGB-1_2665, *mnxS1, mnxS2 and mnxR* in *P. putida GB-1, moxA* in *Pedomicrobium*) is supported by evidence that satisfies Koch's molecular postulates (16, 17, 22), evidence for the role of *mofA*, the putative multicopper oxidase reportedly involved in manganese oxidation by SS1 does not. This gene was initially identified by raising antibodies against a manganese oxidizing factor, which were then

used to screen an expression library of SS1 in *E. coli* (23). Although this preliminary evidence was important, the protein encoded by *mofA* was of a different size than that which was used to raise the antibodies. Heterologous expression of *mofA* in *E. coli* failed to yield manganese oxidation activity (3, 24) and we failed to find any *mofA*-encoded peptides in the manganese oxidizing fraction of SS1 supernatant (data not shown). In addition, the lack of a genetic system for SS1 has meant that it was not possible to interrupt or delete this gene in order to establish its role in manganese oxidation.

The recent development of a genetic system for SS1 (Chapter 2), however, coupled with a draft genome sequence (Chapter 4), has opened up the possibility of determining the role of *mofA* and other candidate genes utilizing traditional genetics techniques. Here we show that disrupting *mofA* as well as other four candidates genes (*mcoA, mcoB, hemA and hemB*) related to known manganese oxidizing factors had no effect on the ability of *Leptothrix discophora* SS1 to oxidize manganese. To avoid confounding results that might have occurred due to polar effects we even went so far as to completely delete *mofA* and still found that *Leptohrix* was able to perform manganese oxidation. Finally, transposon mutagenesis, which had failed to abolish manganese oxidation in the wild type, was undertaken in the *mofA* deletion since a recent report suggests that manganese oxidation may be encoded by redundant systems in some bacteria (16). Importantly, this effort resulted in the isolation of scores of manganese oxidation deficient mutants. Sequencing of the transposon insertions revealed two regions (four genes) in SS1 which appear to play a role in manganese oxidation. Further analysis of these genes should help elucidate the mechanism of manganese oxidation in *Leptothrix discophora* SS1.

3.3 MATERIALS AND METHODS

Bacterial strains and culture conditions

Leptothrix discophora SS1 was maintained on solid 2XPYG medium (25) (per liter of deionized water: 0.5 g peptone, 0.5 g yeast, 0.5 g glucose, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.07g $CaCl_2$ and 3.57g Hepes, adjusted to pH 7.2 with 1M NaOH) either on plates or poured tubes. Cultures on plates had to be refreshed every 10 days to 2 weeks to ensure culture viability. When needed, a few colonies were removed from the plate and inoculated into 20 mL of liquid medium in 60 mL glass tubes. This culture was allowed to grow at room temperature, shaking at 120 rpm, to late-logarithmic phase (OD= 0.25) and was used as an inoculum as necessary. Antibiotics were used as needed at the following concentrations: 50 μgmL^{-1} kanamycin, 15 μgmL^{-1} nalidixic acid, 50 μgmL^{-1} streptomycin, 10 μgmL^{-1} tetracycline, 10 μgmL^{-1} gentamicin, ampicillin 80 μgmL^{-1}. Manganese was added to media after autoclaving from a stock of 100mM of sterile $MnSO_4 \cdot 7H_2O$ to a final concentration of 100µM. The *E. coli* strains used in this study were grown in lysogeny broth (LB) medium (per liter of deionized water: 5g NaOH, 10g yeasts extract and 10g peptone) at 37^0C, with antibiotics when necessary at the following concentrations: 50 μgmL^{-1} kanamycin, 50 μgmL^{-1} streptomycin, 10 μgmL^{-1} tetracycline, 25 μgmL^{-1} gentomicin, ampicillin 150 μgmL^{-1}.

Table 3.1: Strains and Plasmids used in this study

Species or plasmid	**Relevant characteristics**	**Source or reference**
SS1 *SS1*	Wild type	1
SS1 SS1 Nal+	Spontaneous Rif and Nal acid resistant	This work
SS1 SS1 Nal pJSmofA250int	Mutant with an insertion in *mofA*	This work
SS1 SS1 Nal pJSmcoIIAint	Mutant with an insertion in mco*A*	This work
SS1 SS1 Nal pJSmcoIIBint	Mutant with an insertion in *mcoB*	This work
L. discophora SS1 Nal^+pLD55hemAint	Mutant with an insertion in *hemA*	This work
L. discophora SS1 Nal^+pLD55hemBint	Mutant with an insertion in *hemB*	This work
L. discophora SS1 Nal^+ΔmofA	Mutant with deleted *mofA*	This work
L. discophora SS1 Nal^+ΔmofATn5	Mutant with deleted *mofA* and Tn5 insertions	This work
E. coli S17-1 ʎ pir	*ʎ pir* lysogen	
pVIK165	Suicide vector, Km resistant	22
pJS:mofA250int	pVIK165 cloned with internal *mofA* used for homologous recombination	This work
pKNG101orf-gm-mofB	pKNG101 cloned with a cassette of gm and flanking regions of *mofA*	This work
pLD55	Suicide vector, Tet and Amp resistant	
pLD55hemAint	pLD55 cloned with an internal fragment of *hemA*	This work
pLD55hemBint	pLD55 cloned with an internal fragment of *hemB*	This work
pJSmcoAint	pLD55 cloned with an internal fragment of *mco*A	This work
pJSmcoBint	pLD55 cloned with an internal fragment of *mcoB*	This work
pUTminiTn5 Tet	Transposon delivery plasmid, Tet resistant	

Sequence analyses

A draft genome sequence of *Leptothrix discophora SS1* was used to create a database for local BLAST using BioEdit software. This was then interrogated to identify manganese oxidation candidate genes for insertional mutagenesis and to also identify the genes interrupted in manganese-oxidation deficient transposon mutants. Nucleotide and amino acid sequences of manganese-oxidizing factors from *Pseudomonas putidaGB-1* (Accession number NC_010322), *Bacillus sp.SG-1 (Accession number* U31081), *Pedomicrobium sp* ACM 3067 (Accession number AM049177) *and Aurantimonas* sp. strain SI85-9A1 (Accession number NZ_AAPJ00000000) were downloaded from NCBI and used as queries for the BLAST search. In addition, an automatically annotated version of the draft genome was interrogated using searches for "multicopper oxidases" and "hemolysin-type calcium binding proteins". Rapid Annotation Subsystem Technology (RAST) and National Microbial Pathogen Database Resource (NMPDR) were used to determine the location of transposon insertions (26).

Plasmids constructs for disruption of genes

Suicide vectors used for constructing plasmids for recombination were either pVIK165 (Km^R) (27) for disruption of *mofA*, *mcoA* and *mcoB* genes, or pLD55 (Amp^R, Tet^R) for disruption of *hemA* and *hemB* genes. For each construct, an internal fragment of approximately 600 bp of each gene was amplified by PCR with primers that had restriction sites corresponding to the target vector (see table 2), the PCR products were cleaned, digested and then cleaned again after digestion. Target vectors for each construct were digested with the corresponding enzymes, cleaned and ligated with the digested internal PCR fragments as explained above. This ligation mixture was then transformed into *E. coli S17-1 λ pir* and transformants were selected on plates

containing Km for pVIK165 constructs (*mofA, mcoA* and *mcoB*) or Tet and Amp for pLD55 constructs (*hemA* and *hemB*). After confirming that the transformants harbored the plasmid with the correct *Leptothrix* gene fragment they underwent conjugation with SS1. Conjugations between *E. coli S17-1 λ pir pBBR1MCS2* (Km^R) *and E.coli S17-1 λ pir pBBR1MCS3* (Tet^R) were set up in parallel, as controls for the efficiency of conjugation.

Confirmation of plasmid integration and gene disruption

PCR was used to confirm that the plasmids recombined into the chromosome of SS1 at the correct site. Each strain was compared the wild type and the purified plasmid (from *E. coli*). Primer combinations were designed to amplify the internal fragments (positive control) or the entire gene.

Conjugation

All plasmids were transferred into SS1 by conjugation, since it is the only means of DNA transfer presently available for this bacterium. For routine matings, 20 mL of SS1 cultures from mid logarithmic to stationary growth stage and 5 mL of an overnight *E. coli* donor were centrifuged separately and washed with 20 and 5 mL of 2xPYG respectively. The suspension was centrifuged again, the supernatant was removed and donor and recipient cells were mixed and placed on a 2XPYG plate without antibiotics. Mating was allowed for 16 to 24 hours, after which the mating mix was scraped off the plate into 1mL of 2xPYG medium. Dilutions ($10^{-1}$to 10^{-4}) of this cell suspension were plated onto 2xPYG plates containing the respective selective antibiotic and manganese. Most of the conjugations were selected on media with and without manganese in parallel to asses any effect manganese might have on the conjugation outcome.

Dilutions of the recipient before and after mating were also plated to assess the survival of SS1 during the mating process.

Determination of optimal (toxic) sucrose concentration for counter-selection

The optimal sucrose concentration to be used in the selection of mutants that had undergone a second round of recombination and lost the integrated plasmid was determined based on growth results in liquid and on solid 2XPYS (S=sucrose) medium for the following range of concentrations: 0.05%, 0.5%, 1%, 2%, 3%, 4%, 5% sucrose. To make the media, a solution of 0.5g/L peptone, 0.5g/L yeast extract, 0.06g/L $MgSO_4 \cdot 7H_2O$, 0.07g/L $CaCl_2 \cdot 2H_2O$ and 3.4g/L Hepes buffer was first adjusted with 1M NaOH to pH=7.3 and then autoclaved. The appropriate amount of sucrose was then added from a sterile 40% stock to the hot media. Growth tests in liquid were done in 20mL volumes in glass tubes. For growth on solid substrates, plates with different concentrations of 2XPYS were spread with 100µL of a fresh *Leptothrix* culture. Growth was assessed by reading the optical density (OD) of liquid cultures and by counting the SS1 colonies that grew on plates for solid medium.

Arbitrary PCR for transposon mapping

Arbitrary PCR was used to obtain products flanking the site of insertion (28). For each transposon mutant a colony was scraped from the plate and re-suspended in 500 µL of 15mM Hepes buffer, centrifuged and re-suspended in 50 µL PCR water and maintained at -20^{o}C. Primer sets were Arb2/TetRP2for the first PCR and Arb1/TetRP1 for the second PCR (primer sequences are found in table 2). The first PCR reaction contained the following in a total volume of 20 µL: 5 µL of 2xPCR Buffer (New England Biolabs), 1 µL each of forward and reverse primer, 1 µL of

the cell suspension, 2µL H2O. The thermocycling conditions for the first reaction were as follows: 1) 95^0C, 3 min 2) 95^0C, 30 seconds 3) 42^0C for 30 seconds 4) 68^0C for 3 minutes, repeat steps 2 through 4, 10 times 5) 95^0C for 30 seconds 6) 52^0C for 30 seconds 7) 68^0C for 3 minutes, repeat steps 5 through 7, 30 times. These PCR products were brought to 100 µL by adding 80 µL PCR-grade H_2O and were used as a template for the second PCR. The 100 µL reaction of the second PCR included: 50µL of 2xPCR Buffer, 10µL each of forward and reverse primer, 10µL of diluted (1:5, as described) first PCR product for each corresponding clone, 20µL H2O. The second PCR included a touch-down step for the temperature interval 60^0C to 50^0C in the following program: 1) 95^0C, 3 min 2) 95^0C, 30 seconds 3) 60^0C for 30 seconds, minus 1^0C per cycle 4) 68^0C for 3 minutes, repeat steps 2 through 4, 10 times 5) to 95^0C for 30 seconds 6) 50^0C for 30 seconds 7) 68^0C for 3 minutes, repeat steps 5 through 7, 30 times. A 10µL aliquot from each reaction was analyzed by gel electrophoresis for the presence and quality of PCR product. The remaining of each reaction was then cleaned using an ethanol precipitation method and submitted for sequencing.

3.4 RESULTS AND DISCUSSION

Interruption of orthologs of known Mn oxidation factors does not alter Mn oxidation in SS1

Identification of candidate genes

In addition to *mofA,* five other genes were identified in the *Leptothrix discophora SS1* draft genome by BLASTp analysis that shared sequence similarity with genes implicated in manganese oxidation in other bacteria (Table 3.2). Among these was *mofA2,* which shares 85%

nucleotide identity with *mofA* and was located approximately 1Mb downstream from *mofA*. The protein encoded by *mofA2* is predicted to be 1,697 amino acid (aa) long (MofA is 1,661 aa).

Given that multicopper oxidases have been reported to be involved in manganese oxidization in many bacteria (Table 3.2) (29), we searched the draft genome of SS1 for genes encoding other putative multicopper oxidases. This resulted in the identification of two other genes, ORF00945 and ORF01827, annotated as type II multicopper oxidases and predicted to be 946 and 973 AA respectively. For ease of discussion, we renamed these *mcoA* and *mcoB* respectively. They are 82% identical to one another over 98% of their sequence, but are separated by approximately 1Mb. None of the neighboring genes shared any sequence similarity.

Another recent study identified heme peroxidases as a new class of enzymes involved in manganese oxidation (Table 3.2) (4). A search of the annotated SS1 genome for genes encoding enzymes resulted in two hits, specifically ORF03379 annotated as hemolysin-type calcium binding region and ORF03380, a rhizobiocin/RTX toxin and hemolysin-type calcium binding protein which we renamed *hemA* and *hemB*. The corresponding proteins are 1450 and 2557 aa long respectively and the genes overlap by 3 nucleotides, suggesting that these two genes might be co-transcribed. Local BLAST analysis of the SS1 genome using the aa sequence of MnxG, the proposed manganese oxidizing enzyme from *Bacillus sp. SG-1*, did not result in any significant matches (18 identities over 7,039 nucleotide query).

Table 3.2 Proteins known or suggested to be involved in manganese oxidation in different bacteria.

Strain	Protein type	Gene	Evidence for involvement in Mn oxidation	Reference
L. discophora	Multicoper oxidase (MCO)	*mofA*	Biochemical evidence of activity	(25)
Pedomicrobium	Multicoper oxidase (MCO)	*moxA*	Genetic (gene interruption) Biochemical evidence of enzymatic activity	(14)
Aurantimonas sp.	Heme binding peroxidases with hemolysin type Ca^{2+} binding domains	*mopA (mn peroxidase)*	Proteomics: tandem mass spectrometry	(4)
Erythrobacter sp.	Heme binding peroxidases with hemolysin type Ca^{2+} binding domains	*mopA*	Proteomics: tandem mass spectrometry	(4)
P. putida	Multicoper oxidase (MCO)	PputGB-1_2447	Genetic/deletion/complementation	(16)
		PputGB-1_2665	Genetic/deletion/complementation	(16)
	Cytochrome c biogenesis operon	*ccm* operon	Tn mutagenesis	(18, 36)
	General secretory pathway	*xcp* genes	Tn mutagenesis	(19)
	TCR (two component regulatory pathway) Putative sensor histidine kinase Putative sensor histidine kinase Response regulator	*mnxS1 mnxS2 mnxR*	Genetic/(deletion/complementation)	(22)
	TCA cycle (trichloroacetic acid)		Tn mutagenesis	(36)
	Tryptophan biosynthetic pathway		Tn mutagenesis	(36)
Bacillus SG-1	Multicoper oxidase (MCO)	*mnxG*	Genetic, biochemical, Proteomics	(37, 38)
	Spore coat proteins	*mnxA-F*	Tn mutagenesis	(20, 21)

We therefore considered *mofA*, *mcoA, mcoB, hemA and HemB* (Table 3.3) to be the best candidates for encoding manganese oxidation activity in SS1 (although we mentioned *mofA2* as a potential candidate above, it was not included in the interruption experiments since it was discovered later, after additional genome sequencing and assembly refinements). We disrupted each of them to determine if they were involved in manganese oxidation. Recombinants were obtained for all 5 genes at a frequency of 10^{-3} to 10-^{5} after 9 days. There was no difference in the numbers of transconjugants observed on the plates with or without manganese. All transconjugants still oxidized manganese although a delay in manganese oxidation and a decrease in brown color formation were observed when compared to the wild type.

Table 3.3 Genes identified *in silico* in *L. discophora* as potential candidates for encoding a protein that catalyzes manganese oxidation

Gene name in *L. discophora*	**AA**	**Gene Annotation (Craig Venter)**	**Gene annotation (Rast)**
mofA	1665	Fibronectin type III domain protein	Putative protein
hemA	1450	Heme peroxidase	Alkaline phosphatase
hemB	2557	Heme peroxidase	Alkaline phosphatase
mcoIIA	946	Multicopper oxidase typeII	Glycoprotein gp2
mcoIIB	973	Multicopper oxidase typeII	Glycoprotein gp2

Manganese oxidation activity occurs when cells enter stationary phase (12) . It is possible that the small difference observed in the manganese oxidizing activity of the mutants was a consequence of an altered growth rate causing a delay in reaching stationary phase. Additionally,

the less intense brown color of the transconjugants colonies was likely due at least in part, to interference by the antibiotics used in the medium for manganese oxidation (30). It has been reported (17) that kanamycin and rifampicin inhibited manganese oxidation of *Pedomicrobium sp.* ACM 3067 cultures, grown without manganese. The same authors presented supporting evidence that this inhibition is due to the ability of antibiotics to block Mn(II) adsorption onto preformed manganese oxides, rather than a direct inhibition of the enzymatic Mn(II) oxidation. To verify that the decrease in brown color in the transconjugants was due to the presence of the antibiotics, the color intensity of the transconjugant colonies was compared to that of two control strains SS1 *pBBR1MCS2* and SS1 *pBBR1MCS3*. These strains harbor replicative plasmids (that do not interrupt any genes) but encode resistance to kanamycin (on pBBR1MCS2) and tetracycline (on pBBR1MCS3)(31). These strains also had colonies that were less brown than the wild type and of similar brown color to the transconjugants.

It is also possible that even if the candidate genes were involved in Mn oxidation, the translated product of the two fragments could somehow retain some activity or reconstitute a functional protein, although this is unlikely, especially for the putative multicopper oxidase MofA, since the homologous internal fragment for the interruption of *mofA* was designed so as to disrupt two of the four putative copper binding centers of this predicted multicopper oxidase. Although the resulting MofA fragment would still have one of the copper binding centers intact, it has been shown that all of the copper centers have to be present to ensure an active multicopper oxidase (32).

Although interrupting these candidate genes did not eliminate manganese oxidation activity in SS1, these results cannot unequivocally disprove the involvement of the gene products in manganese oxidation. Given the lack of a clean deletion and the ambiguous results from the

observed attenuation in manganese oxidation, we undertook optimization of a cleaner gene deletion strategy and also considered the possibility that *mofA2* which shares 85% nucleotide identity with *mofA* may have a redundant role in manganese oxidation.

Deletion of *mofA* does not eliminate Mn oxidation

To eliminate some of the ambiguity raised by merely interrupting the genes as discussed above we used a double recombination strategy with sucrose counter-selection to completely delete *mofA*. Sucrose counter-selection relies on the enzyme levansucrase (encoded by *sacB*) to induce toxicity to cells expressing this enzyme when exposed to sucrose (33). Numerous gentamycin and streptomycin resistant colonies were obtained when pKNG101orf-gm-mofB, which also encodes *sacB*, was transformed into SS1 by conjugation. PCR analyses of these colonies confirmed that the plasmid had recombined into the chromosome of these transconjugants in either orf or *mofB*, both of which flank *mofA* (data not shown). Initial attempts, however, using previously described conditions (5% sucrose) (34) to obtain sucrose resistant derivatives of *Leptothrix*::pKNG101orf-gm-mofB tranconjugants failed. We therefore undertook an assessment of the effects of sucrose on growth of the wild type since high concentrations of sucrose can result in significant osmotic stress to cells. Importantly, 5% sucrose in the medium prevented growth of wild type *Leptothrix*, possibly explaining why no double recombinants were initially obtained. Sucrose at 3%, however, was tolerated although it still reduced growth rate (Figure 3.1).

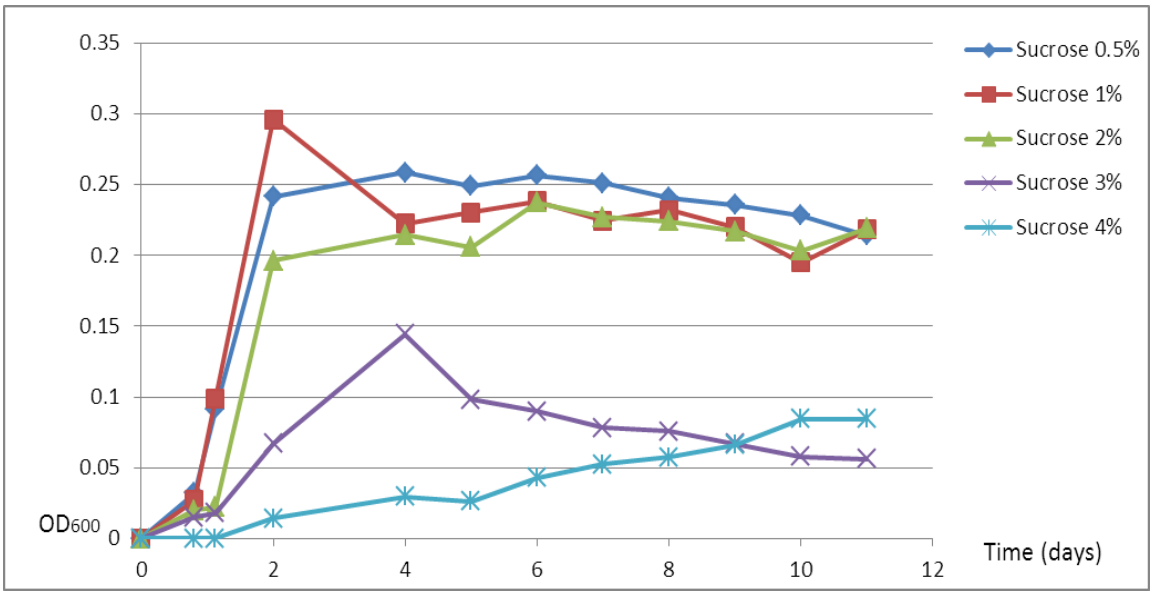


Figure 3.1: Determination of the effects of sucrose concentration on growth of wild type *Leptothrix:* sucrose concentration above 3% greatly reduce the growth of *Leptothrix discophora*

Growth of *Leptothrix*::pKNG101orf-gm-mofB on medium containing gentamycin and 3% sucrose led to the isolation of hundreds of streptomycin sensitive colonies. PCR analyses confirmed that in approximately 5% of these colonies, a second recombination event resulted in deletion of 94% of *mofA* (Figure 2). Surprisingly, these Δ*mofA* mutants still oxidized manganese and no difference in the brown color was distinguishable between the wild type, *Leptothrix*::pKNG101orf-gm-mofB and *Leptothrix* Δ*mofA* mutant grown on solid media. These results clearly demonstrate that although *mofA* has been reported to encode the manganese oxidizing enzyme in *Leptothrix discophora* SS1, deleting it does not eliminate the ability of *Leptothrix* cells to oxidize manganese.

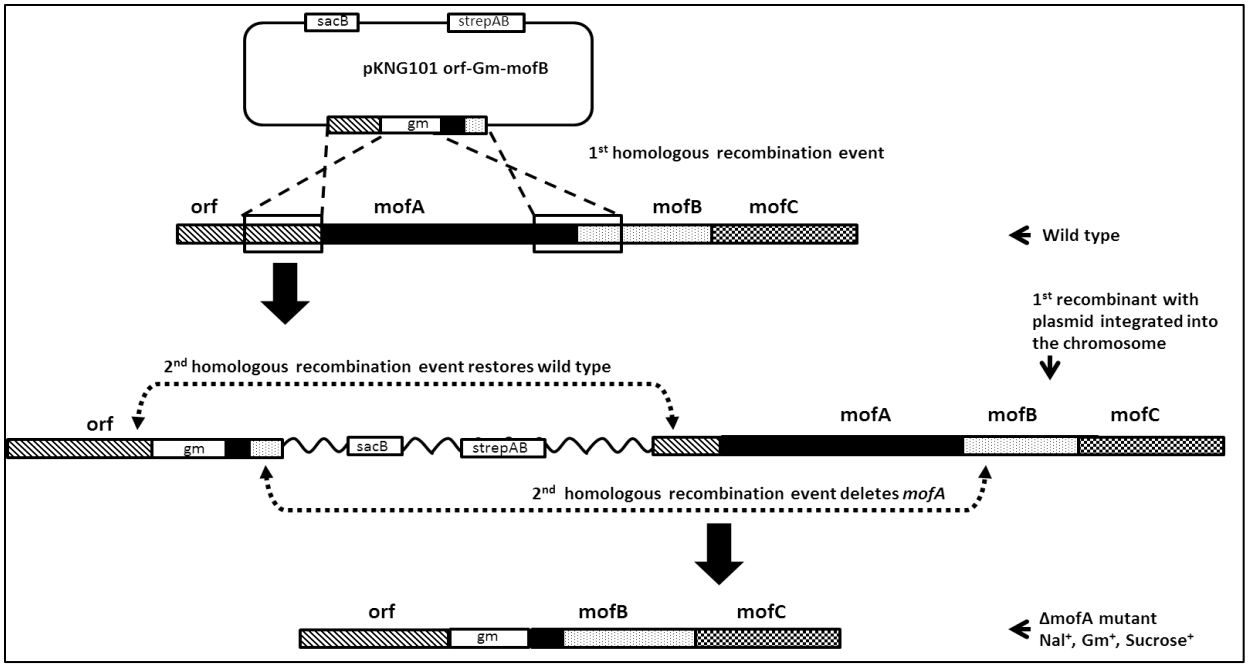


Figure 3.2 a) Schematic of first and second recombination events leading to the deletion of *mofA:* A first recombination event should result in integration of the pKNG101-orf-Gm-mofB plasmid at either orf ormofB (not shown) locations in the chromosome of SS; a second recombination event would either remove the plasmid and reconstitute the wild type (not shown) or remove the *mofA* fragment, resulting in a deletion of *mofA.*

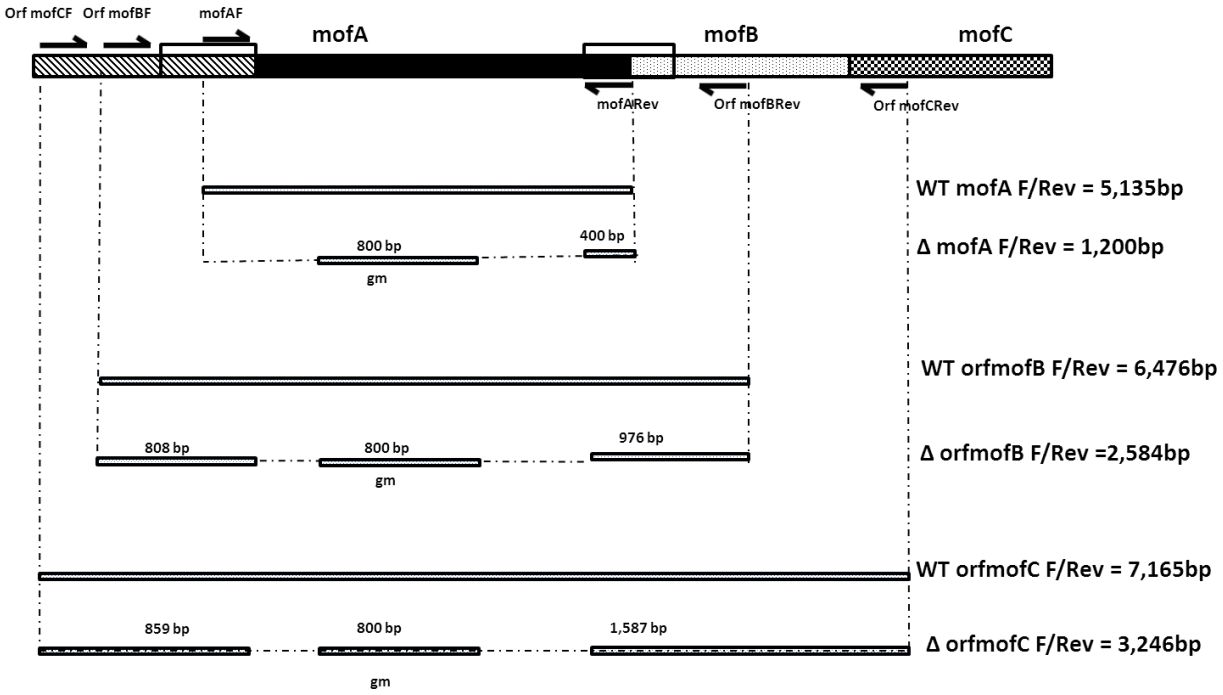


Figure 3.2 b) Schematic showing the expected PCR product in the wild type *Leptothrix* and the Δ*mofA* mutant

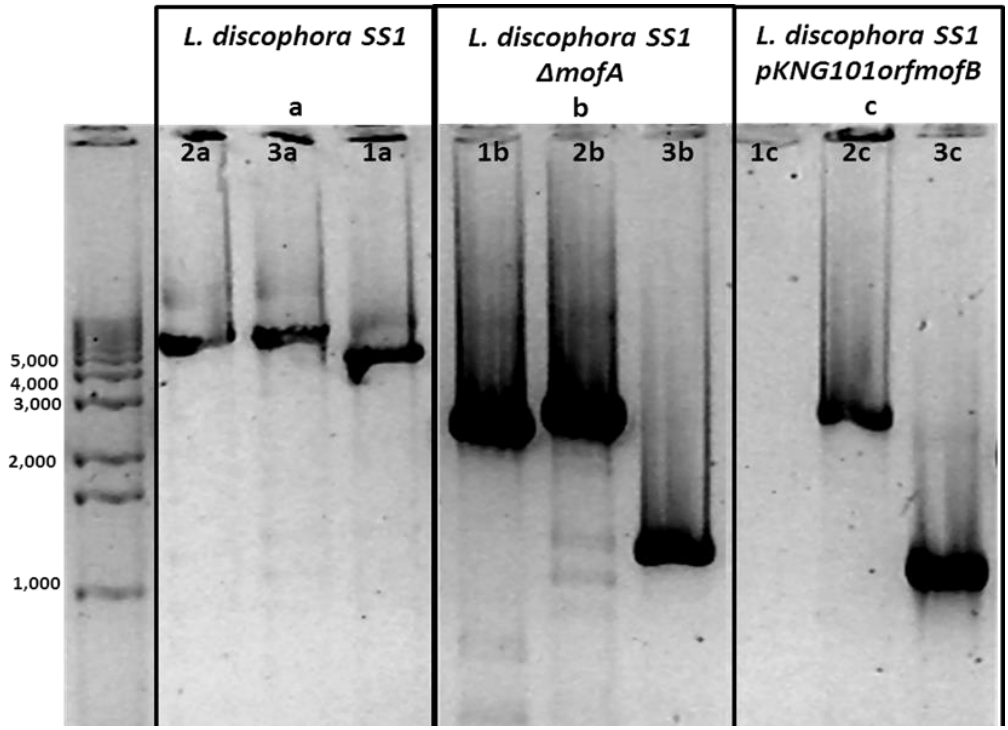


Figure 3.2 c) Electropherogram of the PCR products obtained from amplification of *mofA* and upstream and downstream regions in the wild type, the Δ*mofA* mutant and the pKNG101orfmofB integrant; a) *L. discophora* wild type, b) *L. discophora* ΔmofA c) *L. discophora* pKNG101-orf-gm-mofB; primer sets used are 3a, 3b, 3c= mofA For/Rev, 1a,1b, 1c=orfmofB For/rev , 2a, 2b, 2c=orfmofC For/Rev.

While we saw no obvious decrease in the level of manganese oxidation by the mutant, one way to rationalize a role for MofA given the carefully conducted, though incomplete previous results (23) is to hypothesize that it is a dispensable part of a redundant multi-enzyme complex. This possibility was suggested initially, before the identification of *mofA* and in fact was a reason that manganese oxidation activity was attributed to a Manganese Oxidizing Factor (MOF) rather than a single enzyme (12, 25). Given the presence of *mofA2*, which is closely related to *mofA* and the other genes interrupted above, the possibility of redundancy cannot be ignored. This is especially true given the recent finding of redundant manganese oxidizing

systems in *Pseudomonas putida GB*-1 (16) and our own failure to identify manganese oxidation-deficient transponson mutants, despite screening more than 4 x 10^5 colonies (data not shown).

Transposon mutagenesis of a Δ*mofA* mutant results in a non-oxidizing phenotype

One of the obvious candidates for a redundant manganese oxidation system was *mofA2*. The most direct way of investigating the role of *mofA2* in manganese oxidizing process would have been be to create a *mofA2* deletion in the SS1 Δ*mofA* background. We reasoned, however, that transposon mutagenesis of SS1 Δ*mofA* was a better first approach since it did not make any a priori assumptions about the nature of any putative redundant manganese oxidation system and would thus allow us determine if other enzymes played a role in manganese oxidation.

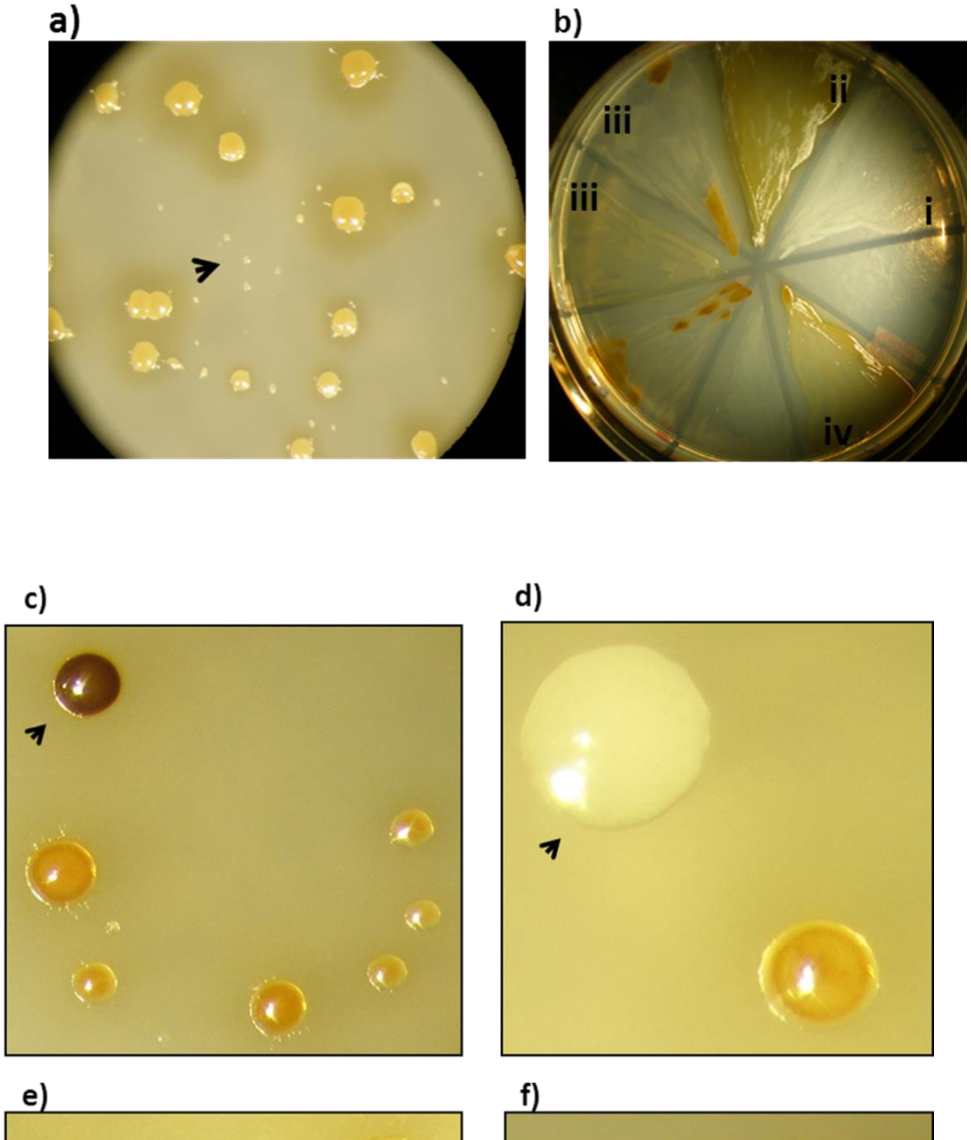
Table 3.4 Outcome of transposon mutagenesis of *L. discophora* Δ*mofA*: 4.9x10^4 transposon mutants were screened, of which 70% were large colonies and 30% small colonies; 60 transposon mutants mutant colonies were either white or off white, while 12 colonies were darker than the average brown colonies.

	Transposon mutants	**Frequency**
Total	4.9 x 10^4 (33 plates x 1500 colonies/ plate	1
Normal size	3.4x10^4	0.7
Mutants with a visible change in color	Dark brown mutant colonies 12	2.4 x 10^{-4}
	White mutant colonies 29	5.9 x 10^{-4}
	Off –white mutant colonies 31	

Transposon mutants first became visible as pinpoint colonies by the 8th day after plating and continued to grow for another week. Similarly to the wild type, and in agreement with the manganese oxidizing activity occurring towards stationary growth phase, the colonies were transparent-white when they first appeared with most of them slowly turned brown within a week. Interrogation of the plates for white, putatively manganese oxidation deficient colonies was made by day 14 (and thereafter) during which time almost all of the colonies (98.8%) on the plates turned brown. Interestingly, two general classes of transposon mutant colonies were initially observed (Table 3.4). Normal size colonies (70% of the total) were the first to appear and reached a typical diameter of 2-3mm at maturity, whereas small colonies only became visible 2 to 3 days later. These were evenly distributed on the plates among the larger colonies so their small size was not likely caused by nutrient limitation due to high colony density (Figure 3.3a). An obvious explanation for the cause of this variation is lacking, but different colony morphologies on the same plate (with cells coming from the same culture) were often seen with *Leptothrix* cultures (both wild type and mutant strains). Although they do not appear brown in Figure 3.3, these colonies eventually turned brown, suggesting that the delay in manganese oxidation was likely due to growth related defects and was not investigated further.

Figure 3.3 Colony distribution and appearance of different categories of *Leptothrix* Δ*mofA* transposon mutants mutants a) Sector of an original selection plate showing *normal* (brown halo) and *small* colonies (arrow) of transposon mutants on initial selection plates; b) Re-streak plate indicating the oxidation ability of the transposon mutants from the four categories identified: white(I), dark brown++(ii) light-brown (ii) patched white and brown growth (iii)

c), d), e), f) Close-up of individual colonies of the different transposon mutants dark brown++ (c), white (d) and off white (e, f).


a)
b)
ii
iii
iii
i
iv
c)
d)
e)
f)

In total, approximately 4.9 x10^5 transposon mutants were screened (> 10X coverage of the genome) (Table 3.4). Based on coloration, at least four different types of colonies could be identified: a) white colonies, b) darker than normal colonies (brown++); c) whitish colonies with streaks or slight coloration from manganese oxides, either in the center of the colony or dispersed in the shape of a star towards the edges and d) brown colonies, with the latter making up the majority of transposon mutants (Table 3.4, Figure 3.3). 60 white and whitish colonies and 12 darker brown colonies were lifted off the plates and re-streaked to confirm their manganese oxidation status. 29 of the 60 white colonies remained white after re-streaking and thereafter, while the remaining 31 either turned various shades of brown or grew in a patched white and brown lawn when re-streaked (Figure 3.3b). Further experiments were done with the 29 mutants that remained white as well as with the 12 darker brown mutants.

Identification of the transposon insertion site

Arbitrary PCR products yield sequence for 23 of the 29 white mutants and 8 out of 12 dark++ mutants. The sequences were blasted against the draft genome of SS1, as well as against the NCBI database. The sites of insertion are summarized in Table 3.5. The 23 white transposon mutants mapped in two regions of the chromosome, with 14 mutants inserted in region 1, and 9 mutants in region 2. Region 1 contains the following genes: Orf1593 annotated as a surface antigen (6 mutants), Orf1594, a cytochrome oxidase biogenesis protein Sco1/SenC/PrrC, putative copper metallochaperone (one mutant), Orf1595 annotated as a putative cytochrome c family protein (four mutants) and Orf1596 annotated as Glycoprotein gp2 (Table 3.5).

Table 3.5 Genes identified by transposon mutagenesis of a Δ*mofA mutant* that produced a white, non-manganese oxidizing phenotype; Insertions were identified in 2 regions of the chromosome, region one at Orf1593 region and region two at Orf 2655. Although no insertions were identified in Orf1592, this gene is 85% identical to Orf2655, for which most insertions were mapped (9). Orf1592 and Orf1593 were annotated as hypothetical by RAST, but as MnxG (multicopper oxidase responsible for Mn oxidation in *Bacillus SG-1)* by Craig Venter Institute pipeline.

Gene # (RAST)	**aa**	**# mutants**	**Gene annotation (RAST)**	**Alternative Annotation (Craig Venter Institute)**	**Blast against (NCBI) *Leptothrix cholodni* (% identity)**
Orf 1592	1597	0	hypothetical	MnxG	Putative MCO, 85%
Orf 1593	663	6	surface antigen gene -	40-residue yvtn family beta-propeller repeat protein	40-residue YVTN family beta-propeller repeat , 82%
Orf 1594	448	1	Cytochrome oxidase biogenesis protein Sco1/SenC/PrrC, putative copper metallochaperone	electron transport protein SCO1/SenC	Electron transport protein SCO1/SenC cytochrome c, putative 88%
Orf 1595	525	4	cytochrome c family protein, putative	cytochrome c, putative	Cytochrome c putative SP6 69 %
Orf 1596	472	0	Glycoprotein gp2	hypothetical protein	Hypothetical 75%
Orf 2665	1591	9	Hypothetical protein	MnxG	Putative MCO, 84%
1594-1595		2	n/a	n/a	n/a
1595-1596		1	n/a	n/a	n/a

Three other mutants had transposons inserted between two genes, with 2 mutants' insertions mapping between Orf1594 and Orf1595 and one mapping between Orf1595 and Orf1596. A search for promoters using BPROM identified a putative promoter in the 398 nucleotides intergenic region upstream of Orf1593, but no potential promoters were found in the

67 nucleotide intergenic region upstream of Orf1594 or in the 46nt upstream of Orf1595, which suggests that these three genes might form an operon transcribed from the promoter identified upstream of Orf1593 (Figure 3.4).

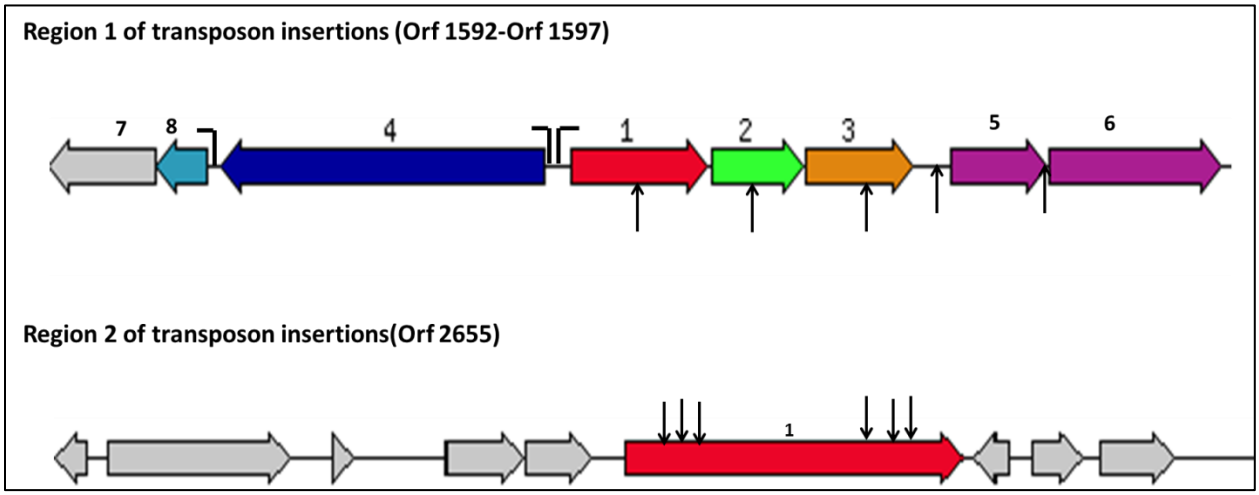


Figure 3.4: Schematic showing the two genomic regions in which the transposon mutants inserted in the non-manganese oxidizing phenotypes of *L. discophora* Δ*mofA* mutants. Small arrows indicate transposon insertion. Genes in region one are annotated as follows:

Region 1: 1) Orf 1593, Surface antigen gene; 2) Orf 1594, Cytochrome oxidase biogenesis protein Sco1/SenC/PrrC, 3) Orf 1595, Putative cytochrome c family protein, 4) Orf 1592, Hypothetical, putative multicopper oxidase 5) Orf 1596 Glycoprotein gp2 6) Orf 1597 Hypotethical, 7) Orf 1591 Two component system response regulator QseB, 8) Orf 1590 Sensory histidine kinase, QseC

Region 2: 1) Orf 2655, hypothetical protein, putative multicopper oxidase

Although there were no mutants identified with insertions in Orf1592, this gene drew our attention because it has 85% homology (97% query coverage) with Orf2655 from region 2, for which all 9 insertions of region two were mapped. Orf1592 is oriented in opposite direction

from Orf1593 and it seems to be transcribed from a second promoter identified in the intergenic region between Orf1593 and Orf1592. Orf1592 is annotated as a hypothetical protein in RAST. An alternative annotation obtained from TIGR, however, identifies it as an MnxG homolog, the protein responsible for manganese oxidation in *Bacillus sp. SG-1*, although there is only 28% similarity at the amino acid level between MnxG of *Bacillus sp. SG1* and Orf1592. This poor homology explains why it was not identified in our original homolog-mutagenesis approach. Orf1592 was named *mnxG1* and Orf2655 was named *mnxG2* thereafter.

A BLASTp of *mnxG1* against the NCBI protein database identified a number of multicopper oxidases from *Nitrosomonas europaea* (obligate chemolithoautotroph, ammonia oxidizer), *Solangium cellulosum* (a cellulose degrading saprophyte) and *Geobacter metallireducens* (an anaerobic respirer) with a 40% similarity (over 96% of the sequence) with Orf1592. Immediately upstream from *mnxG1*, there is a predicted two component system response regulator QseB (Orf1590) and sensory histidine kinase, QseC (Orf1591). It is important to note that a multicomponent regulatory system comprised of the sensor histidine kinases MnxS1 and MnxS2 and the response regulator MnxR were shown to each be required for manganese oxidation in *P. putida GB-1* (22).

None of the 23 non-oxidizing clones sequenced mapped in Orf1590 or Orf1591, but it is possible that some of the 6 clones that were not mapped might contain an insertion in these genes. This will be pursued further by designing gene specific primers for this region to determine if an insertion occurred there in any of the uncharacterized mutants. Finally, three proteins upstream of Orf1590 are annotated as type II secretion proteins, likely to be involved in the type II secretion machinery. The organization of the genes in region one is conserved in *L. cholodni*, which seems to have two copies of this region (Figure 3.5), as well as in a number of

other bacteria, such as Pseudomonads, *Geobacter sulfurreducens* and *Sorangeum cellulosum*, except for the orientation of *mnxG1* (Orf1592).

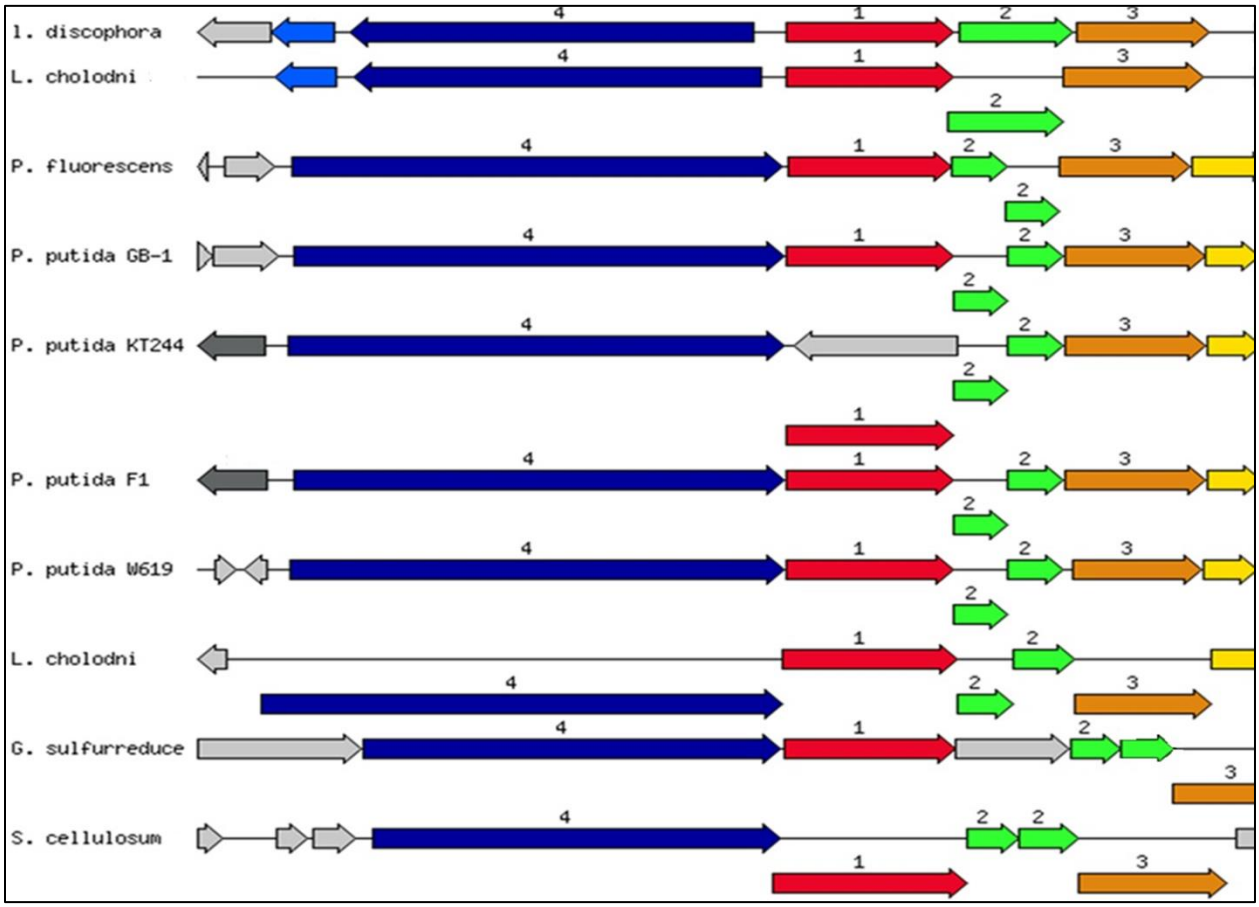


Figure 3.5: The genes in region one (at Orf1593) in which 14 of the non-manganese oxidizing transposons were mapped are conserved for several other species, including. *L.cholodni, P. putida GB-1, Geobacter sulfurreducens, S. cellulosum;* the genes of interest are: 1) Orf 1593 Surface antigen gene 2) Orf 1594, Cytochrome oxidase biogenesis protein Sco1/SenC/PrrC, 3) Orf 1595, Putative cytochrome c family protein, 4) Orf 1592, Hypothetical, putative multicopper oxidase

Annotation of the region two transposon mutants revealed that all nine insertions mapped to Orf2655 (*mnxG2*). BLAST analysis against the NCBI protein database revealed *mnxG2* to be similar to two putative multicopper oxidases from *L. cholodni.* It also showed weak similarity to PputGB1_2447 (32% over 94% of the sequence). Interestingly, deletion of both PputGB1_2447 and another gene (PputGB_2665) encoding a putative multicopper oxidase was required to eliminate manganese oxidation activity in *P. putida* GB-1; deletion of a single gene at a time was not sufficient. Our data are consistent with these results and suggest a redundant Mn oxidation system in SS1.

3.5 CONCLUSION

The data we have presented here provides new insights into manganese oxidation by SS1, but also raises new questions. Although it was believed for a long time that MofA was the enzyme required for manganese oxidation in this bacterium, we have unambiguously determined that MofA is dispensable for this process. Interestingly, however, we were only able to obtain non manganese-oxidizing mutants by further mutagenizing a Δ*mofA* mutant, since transposon mutagenesis of the wild type did not result in any non-manganese oxidizing mutants. Contrary to expectation, none of the manganese deficient Δ*mofA* transposon mutants, however, mapped to *mofA2*, which is 85% similar to *mofA*. Transposon insertions affecting manganese oxidation were not found in any of the other candidate genes initially suspected of being important for manganese oxidation either. Surprisingly, other genes including a putative multicopper oxidase, a surface antigen, a putative cytochrome c and a cytochrome oxidase biogenesis protein were identified as playing a role in manganese oxidation in a Δ*mofA* background. Given the fact that

three of the four genes identified above appear to reside in a single operon, the possibility of polar effects should be investigated, though clearly this region is important.

Recently is has been shown that while expression of the multicopper oxidase responsible for manganese oxidation in *Bacillus sp* SG-1 (*mnxG)* alone, in *E. coli,* did not result in a protein with manganese oxidation activity, co-expression of *mnxDEFG* operon resulted in manganese oxidation activity (35), suggesting that MnxG requires MnxD,E,F and that they probably function as a complex. This finding is not surprising, since early observations of the manganese oxidizing activity in SS1 also suggested the existence of an oxidizing complex (MOF= Manganese Oxidizing Factor) consisting of one or more glycoproteins associated with anionic polysaccharides that resides in membranous "blebs", observed microscopically as large aggregates of membranous particles (25) . Under such a scenario it is possible that Orf 1594 and Orf1595 could play a similar role in SS1 as do MnxD,E,F in *Bacillus,* by assisting the putative MCO MnxG2 in the process of Mn oxidation.

The potential redundancy of *mofA* and *mnxG2* needs to be further investigated, by assessing the manganese oxidizing activity of both a Δ*mnxG2* mutant as well as that of a Δ*mofA* Δ*mnxG2* double mutant. If the Δ*mnxG2* mutant maintains its manganese oxidation activity, then an Δ*mnxG2*Δ*mofA* double knock-out mutant should be created and manganese oxidation assessed to investigate the role of *mofA.* Lack of manganese oxidation in this strain would suggest a role for *mofA* in the manganese oxidation process and restoration of this activity by complementation with a wild copy of *mofA* should solidify this argument.

Addendum in proof

The day before this dissertation was due to be handed in, the Cornell Biotech Center was able to reanalyze the identity of peptides extracted from the manganese oxidizing band of a denaturing polyacrylamide gel containing a 1000x concentrate of SS1 supernatant. Comparison with the updated draft genome sequence showed that the band contained peptides unique to MnxG1, and to two glycoproteins (orfs 1920 and 1921) located just upstream of *mnxG1* (orf 1926). These new data suggest that the role of *mnxG1* needs further investigation since it is possible that the arbitrary PCR sequences thought to be unique to *mnxG2* could actually come from *mnxG1* in some cases, given the similarity between the genes. This should be followed up on by designing primers that are unique for *mnxG1* and *mnxG2* and confirming that transposition only occurred in *mnxG2* as predicted from the sequence of the arbitrary PCR products.

3.6 REFERENCES

1. **Francis CA, Tebo BM**. 2002. Enzymatic Manganese(II) Oxidation by Metabolically Dormant Spores of Diverse Bacillus Species. Appl. Environ. Microbiol. **68**:874–880.
2. **Browers G-J**. 1999. Molecular Aspects of Microbial Manganese Oxidation. Thesis. Leiden University, Netherlands.
3. **Corstjens PL**. Bacterial Oxidation of Iron and Manganese: a molecular-biological approach.
4. **Anderson CR, Johnson HA, Caputo N, Davis RE, Torpey JW, Tebo BM**. 2009. Mn(II) Oxidation Is Catalyzed by Heme Peroxidases in "Aurantimonas manganoxydans" Strain SI85-9A1 and Erythrobacter sp. Strain SD-21. Appl. Environ. Microbiol. **75**:4130–4138.
5. **Ghiorse WC**. 1984. Biology of Iron-and Manganese-Depositing Bacteria. Annu. Rev. Microbiol. **38**:515–550.
6. **Dondero NC**. 1975. The Sphaerotilus-Leptothrix Group. Annu. Rev. Microbiol. **29**:407–428.
7. **Nealson KH, Tebo BM, Rosson RA**. 1988. Occurrence and Mechanisms of Microbial Oxidation of Manganese, p. 279–318. *In* Allen I. Laskin (ed.), Advances in Applied Microbiology. Academic Press.
8. **Ehrlich HL, Newman DK**. 2008. Geomicrobiology, Fifth Edition. CRC Press.
9. **Tebo BM, Johnson HA, McCarthy JK, Templeton AS**. 2005. Geomicrobiology of manganese(II) oxidation. Trends Microbiol. **13**:421–428.
10. **Tebo BM, Geszvain K, Lee S-W**. 2010. The Molecular Geomicrobiology of Bacterial Manganese(II) Oxidation, p. 285–308. *In* Barton, LL, Mandl, M, Loy, A (eds.), Geomicrobiology: Molecular and Environmental Perspective. Springer Netherlands.
11. **Schleifer K-H**. 2006. The Prokaryotes: A Handbook on the Biology of Bacteria. Springer.
12. **Adams LF, Ghiorse WC**. 1987. Characterization of extracellular Mn2+-oxidizing activity and isolation of an Mn2+-oxidizing protein from Leptothrix discophora SS-1. J. Bacteriol. **169**:1279–1285.
13. **Okazaki M, Sugita T, Shimizu M, Ohode Y, Iwamoto K, Jong EW de V, Vrind JP de, Corstjens PL**. 1997. Partial purification and characterization of manganese-oxidizing factors of Pseudomonas fluorescens GB-1. Appl. Environ. Microbiol. **63**:4793–4799.
14. **Larsen EI, Sly LI, McEwan AG**. 1999. Manganese(II) adsorption and oxidation by whole cells and a membrane fraction of Pedomicrobium sp. ACM 3067. Arch. Microbiol. **171**:257–264.
15. **Brouwers G-J, Vrind JPM de, Corstjens PLAM, Cornelis P, Baysse C, Jong EW de V**. 1999. cumA, a Gene Encoding a Multicopper Oxidase, Is Involved in Mn2+ Oxidation in Pseudomonas putida GB-1. Appl. Environ. Microbiol. **65**:1762–1768.

16. **Geszvain K, McCarthy JK, Tebo BM**. 2013. Elimination of Manganese(II,III) Oxidation in Pseudomonas putida GB-1 by a Double Knockout of Two Putative Multicopper Oxidase Genes. Appl. Environ. Microbiol. **79**:357–366.
17. **Ridge JP, Lin M, Larsen EI, Fegan M, McEwan AG, Sly LI**. 2007. A multicopper oxidase is essential for manganese oxidation and laccase-like activity in Pedomicrobium sp. ACM 3067. Environ. Microbiol. **9**:944–953.
18. **De Vrind JPM, Brouwers GJ, Corstjens PLAM, den Dulk J, de Vrind-de Jong EW**. 1998. The Cytochrome c Maturation Operon Is Involved in Manganese Oxidation in Pseudomonas putida GB-1. Appl. Environ. Microbiol. **64**:3556–3562.
19. **De Vrind J, De Groot A, Brouwers GJ, Tommassen J, De Vrind-de Jong E**. 2003. Identification of a novel Gsp-related pathway required for secretion of the manganese-oxidizing factor of Pseudomonas putida strain GB-1. Mol. Microbiol. **47**:993–1006.
20. **Waasbergen LG van, Hoch JA, 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.
21. **Waasbergen LG van, Hildebrand M, Tebo BM**. 1996. Identification and characterization of a gene cluster involved in manganese oxidation by spores of the marine Bacillus sp. strain SG-1. J. Bacteriol. **178**:3517–3530.
22. **Geszvain K, Tebo BM**. 2010. Identification of a Two-Component Regulatory Pathway Essential for Mn(II) Oxidation in Pseudomonas putida GB-1. Appl. Environ. Microbiol. **76**:1224–1231.
23. **Corstjens PLAM, de Vrind JPM, Goosen T, Jong EW de V**. 1997. Identification and molecular analysis of the Leptothrix discophora SS-1 mofA gene, a gene putatively encoding a manganese-oxidizing protein with copper domains. Geomicrobiol. J. **14**:91–108.
24. **El Gheriany IA**. 2010. Manganese oxidation by Leptothrix discophora SS1. Cornell University.
25. **Adams LF**. 1986. Physiology, Cytology, and manganese oxidizing activityof Leptothrix discophora SS1. Cornell University.
26. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O**. 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics **9**:75.
27. **Kalogeraki VS, Winans SC**. 1997. Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene **188**:69–75.

28. **Das S, Noe JC, Paik S, Kitten T**. 2005. An improved arbitrary primed PCR method for rapid characterization of transposon insertion sites. J. Microbiol. Methods **63**:89–94.
29. **G. J. Brouwers, E. Vijgenboom, P. L. A. M. Corstjens, J. P. M. de Vrind EW de VJ**. 2000. Bacterial Mn2+ Oxidizing Systems and Multicopper Oxidases: An Overview of Mechanisms and Functions. Geomicrobiol. J. **17**:1–24.
30. **Rosson RA, Tebo BM, Nealson KH**. 1984. Use of Poisons in Determination of Microbial Manganese Binding Rates in Seawater. Appl. Environ. Microbiol. **47**:740–745.
31. **Kovach ME, Elzer PH, Steven Hill D, Robertson GT, Farris MA, Roop II RM, Peterson KM**. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166**:175–176.
32. **Nakamura K, GO N**. 2005. Function and molecular evolution of multicopper blue proteins. Cell. Mol. Life Sci. CMLS **62**:2050–2066.
33. **Pelicic V, Reyrat JM, Gicquel B**. 1996. Expression of the Bacillus subtilis sacB gene confers sucrose sensitivity on mycobacteria. J. Bacteriol. **178**:1197–1199.
34. **Pelicic V, Reyrat JM, Gicquel B**. 1996. Generation of unmarked directed mutations in mycobacteria, using sucrose counter-selectable suicide vectors. Mol. Microbiol. **20**:919–925.
35. **Butterfield CN, Soldatova AV, Lee S-W, Spiro TG, Tebo BM**. 2013. Mn(II,III) oxidation and MnO2 mineralization by an expressed bacterial multicopper oxidase. Proc. Natl. Acad. Sci. **110**:11731–11735.
36. **Caspi R, Tebo BM, Haygood MG**. 1998. c-type cytochromes and manganese oxidation in Pseudomonas putida MnB1. Appl. Environ. Microbiol. **64**:3549–3555.
37. **Dick GJ, Torpey JW, Beveridge TJ, Tebo BM**. 2008. Direct Identification of a Bacterial Manganese(II) Oxidase, the Multicopper Oxidase MnxG, from Spores of Several Different Marine Bacillus Species. Appl. Environ. Microbiol. **74**:1527–1534.
38. **Francis CA, Casciotti KL, Tebo BM**. 2002. Localization of Mn(II)-oxidizing activity and the putative multicopper oxidase, MnxG, to the exosporium of the marine Bacillus sp. strain SG-1. Arch. Microbiol. **178**:450–456.

CHAPTER FOUR

IN SILICO EVIDENCE FOR THE METABOLIC VERSATILITY OF *LEPTOTHRIX DISCOPHORA* SS1

4.2 INTRODUCTION

Leptothrix discophora SS1 is a gram negative, manganese oxidizing *beta*proteobacterium, which together with members from the genus *Sphaerotilus*, form the sheathed group of bacteria (1, 2). SS1 was isolated from the ferromanganese metallic surface film of a swamp near Ithaca NY, however *Leptothrix* species are widely distributed in environments that are characterized by a circumneutral pH, low concentrations of easily degradable organic nutrients, an oxygen gradient and a source of reduced iron or manganese minerals (3, 4). In such environments *Leptothrix discophora SS1* and other manganese oxidizers readily oxidize manganese which precipitates as a brown mass outside the cells. Manganese oxides are important chemical species in the environment, particularly due to their properties as strong oxidants and effective adsorptive surfaces (5, 6)). From this perspective, the scientific community that studies biological manganese oxidation wants to know to what extent and in which ways the manganese oxidizing bacteria impact the geochemical cycles in their habitats.

Progress in this field was slow, particularly because many of the model organisms used to study this process were refractory to genetic manipulations (7–9). In this work we report insights gained about *Leptothrix's* genetic potential as revealed by the draft genome. Together with recent advances in developing a genetic system for *L. discophora*, the availability of a genome creates a foundation for further exploratory work to understand the molecular biology of manganese oxidation in *Leptothrix* and the possible role this organism plays in diverse environments. We present here general insights gained about *Leptothrix's* physiological potential as suggested by the draft genome, including genes likely to have a contribution to manganese oxidation in this bacterium

4.3 MATERIAL AND METHODS

Bacterial strains

Leptothrix discophora was grown in 2XPYG media. Genomic DNA was isolated using standard protocols for bacteria (10).

Sequencing

Shotgun cloning and sequencing were performed at the Memorial Sloan Kettering Cancer Center using 454 technology in a combination of pair end and single end reads. Additional sequencing was performed at Weil Medical Center, Cornell University, using a Single Molecule Teal Time sequencing technology (SMRT) with correction based on 100 bp Illumina reads. Assembly of reads was performed by the Computational Biology Service Unit, Cornell University using Newbler assembly software and with assistance from Russell Durrant. The draft genome of *Leptothrix discophora* SS1 was 4.2 Mb assembled in 32 scaffolds. Automated annotation was performed using the automated prokaryotic annotation pipeline at J. Craig Venter Institute and RAST (Rapid Annotation Subsystems Technology).

Growth in soft agar tubes

A low melting temperature, soft agar (0.5%), 2xPYG (11) medium was used for assessing growth in tubes. 10 mL soft agar medium was first allowed to cool, after which it was quickly mixed homogeneously with 0.5mL of *Leptothrix discophora* SS1 cell suspension. Tubes were capped and growth was observed over a period of several months.

4.4 RESULTS AND DISCUSSION

General characteristics of the genome

Sequence information was obtained from a combination of 454, PacBio, and Illumina sequencing. Though closure was not complete, the 32 scaffolds appear to assemble into one chromosome of approximately 4.2 Mb. No plasmids were identified by Newbler assembler, although Celera assembler (J Craig Venter Institute) identified sequence behavior that could indicate the presence of extra-chromosomal DNA, such as a plasmid (there are many more mates from degen-to-degen than degen-to-scaffold). The draft genome sequence of *L. discophora* is approximately 15% smaller than that of *L. cholodni* (4.9Mb, Table 4.1), which is currently the closest relative with a sequenced genome. As expected, the genome has a high G+C content (67%), though numerous AT rich regions are present suggesting a history of horizontal gene transfer. RAST annotation of the chromosome identified 3734 protein encoding sequences and 57 RNA genes. The genome includes 793 genes (21%) predicted to encode hypothetical proteins, 167 (4.4%) putative proteins and 15 proteins of unknown function (0.4%).

Table 4.1: General characteristics of the draft genome of *L. discophora SS1* as compared to the genome of *L. cholodni*

General features of the genome	*L. discophora SS1*		*L. cholodni*	
	Number	**% of total**	**Number**	**% of total**
Size bp	4.2×10^6	N/A	4909403	100%
DNA coding bases		N/A	4520554	92%
G+C content	67.39%	N/A	68.9%	N/A
Coding density %		NA	92.08%	N/A
Total genes	3734	100%	4420	100%
Protein coding genes	3677	98.47%	4363	98.71%
RNA genes	57	1.52%	57	1.29%
Protein coding genes with function	2959	73.03%	3372	76.29%
Protein coding genes without function	975	25.45%	991	22.42%

Organic carbon utilization

All the genes required for classical glycolysis (Embden-Meyerhof pathway) are present. The alternative Entner–Doudoroff pathway is also present with the exception of one gene, 6-phosphogluconolactonase (PGL). It is likely that the pathway is complete, but the gene has not been sequenced or identified by the automatic annotation. The pentose phosphate pathway (PP) that generates pentoses from glucose and provides the cells with crucial metabolic precursors (pentoses for nucleic acid and erythrose for aromatic amino acids) is also present, with the exception, again of the 6-phosphogluconolactonase (PGL). All genes are present for the Citric Acid Cycle, a crucial pathway that supplies the cell with NADH and three precursor metabolites for amino acid synthesis.

Genes encoding enzymes for the utilization of other carbon sources, such as aminosugars (chitin and N-acetylglucosamine) di and oligosaccharides (sucrose, maltose, lactose and galactose) sugar alcohols (glycerol and inositol) are also present.

Energy metabolism/electron transport

Though Bergey's manual describes *Leptothrix* species as obligate aerobes (12), *Leptothrix discophora* SS1's draft genome revealed the genetic potential for the ability to perform fermentation and respiration using either oxygen or nitrate as terminal electron acceptors.

Fermentation

66 genes were attributed by RAST to the fermentation subsystem. Among these there were genes for the fermentation of lactate, butyrate, biosynthesis of butanol and mixed acid fermentation. Though *Leptothrix* was originally identified, described and grown as an obligate aerobe, in preliminary experiments we observed a tendency towards microaerophily: for example, colonies preferred to grow in cracks or holes of the agar plate. Moreover, we were able to grow *Leptothtrix* on plates overlaid with a layer of solid medium as well as in poured tubes (1% agar). In poured tubes *Leptothrix* preferentially grows as a thin layer located a few millimeters below the surface (Figure 4.1). Although we have not tested *Leptothri*x's ability to grow completely anaerobic, the genetic potential for fermentation supports our experimental observations that it can grow (and prefers to) as a microaerophile, potentially fermenting under these conditions.

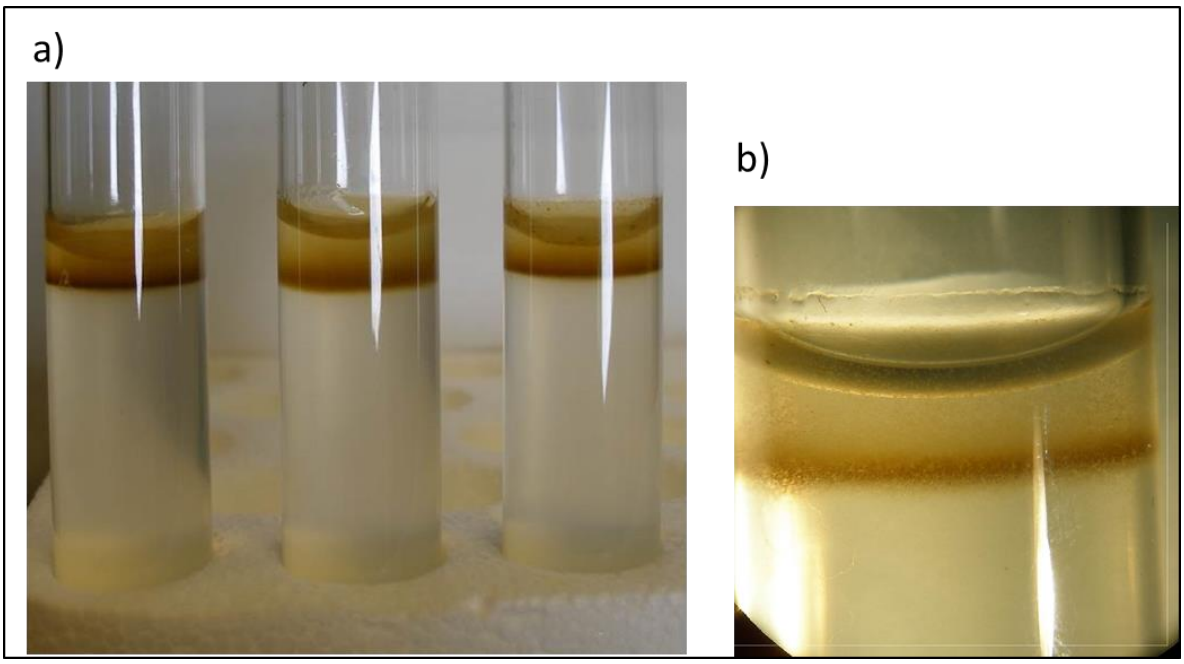


Figure 4.1: a) Growth of *L. discophora* in tubes shows the preferred area of growth under the surface b) close-up of a tube showing growth area in brown 3 to 4 millimeters under the surface

Aerobic respiration

The genes for a complete electron transport chain are present, including complexes I through IV. The presence of at least two kinds of terminal cytochrome oxidases (type aa3 and type cbb3) suggests the possibility of alternative pathways to enable the bacterium to adjust the efficiency of oxidative phosphorylation based on the amount of oxygen present: type aa3 cytochrome c oxidases are used under high oxygen tension, while type cbb3 cytochrome oxidases are used under low oxygen concentrations because they have higher affinity for oxygen.

Nitrate/Nitrite respiration

In oxygen limiting conditions nitrate and nitrite are the next preferred terminal electron acceptor after oxygen (13–15). Two dissimilar pathways of nitrate respiration, ammonification

and denitrification, involve the formation of a common intermediate, nitrite, but end in different products, ammonia and gaseous nitrogen oxides (NO or N_2O or N_2), respectively (16). The genome contains a set of genes that suggest the potential for the first steps in denitrification: an assimilatory nitrate reductase and four potential nitrate reductases (NirK, NirD, NirB, NirV) as well as transporters nitrate/nitrite ABC transporters, a signal transduction nitrate/nitrite sensor and response regulator couple as well as a regulatory NnrU family protein, required for expression of nitrite and nitric oxide reductases (Nir and Nor). No genes encoding nitric oxide reductases (*nor*), or nitrous oxide reductases (*nos*) for conversion of NO to N_2O or N_2O to N_2 respectively, were detected. Thus is seems that *Leptothrix* is genetically equipped to carry out the first two steps of denitrification and is expected to produce NO. The ability to perform only some of the steps involved in denitrification is not uncommon (17).

L. discophora and other organisms in this group often live at liquid air/interface environments, where concentration of dissolved oxygen can decrease dramatically within very small vertical distance gradients (millimeters). It seems reasonable that *L. discophora* would therefore benefit from alternative ways of generating energy (albeit small) through either fermentation or partial denitrification.

Autotrophy

Of the 12 enzymes required for the Calvin cycle, only sedoheptulose-1,7-bisphosphatase is missing from the current draft genome. However, this enzymatic activity in bacteria is often catalyzed by fructose-1,6-bisphosphatase which has dual sugar specificity and seems to replace sedoheptulose-1,7-bisphosphatase in other genomes (18). Moreover, this enzyme is not normally used for diagnostics of the Calvin cycle presence in a given organism. However, two

enzymes, ribulose bisphosphate carboxylase (RuBisCo) and phosphoribulokinase, which are both present in the genome, suggest a functional Calvin cycle.

Carboxysomes are often found in microorganisms that fix carbon, as a means of sequestering ribulose bisphosphate carboxylase/oxygenase from oxygen (the rate limiting step in the process of carbon fixation from CO_2). Since no genes coding for carboxyzomes are present, it is likely that if *L. discophora* fixes CO_2, it does so under high CO_2 pressure which allows normal function of RuBisCo without requiring a CO_2 concentrating/O_2 isolating mechanism (19).

Although *Leptothrix* was isolated and subsequently maintained as an organoheterotroph growing on organic carbon, CO_2 fixation could represent a convenient alternative for *Leptothrix* enabling it to cope with dynamic environments that occasionally offer a limited supply of simple organic carbon for metabolism.

Oxidation of sulfur compounds

Another possible adaptation of *Leptothrix* to its natural habitat is the potential for oxidation of reduced sulfur compounds, as these species are abundant at oxic/anoxic interfaces (20–22). The electrons derived from sulfur oxidation can be used by aerobic autotrophic bacteria during carbon dioxide reduction. The fact that *Leptothrix* possess both of these capacities suggests that it is capable of autolithotrophy. Genes predicted to code for the oxidation of sulfur compounds are present in two locations on the chromosome. The first location contains a sulfide dehydrogenase (cytochrome C subunit of flavocytochrome C), and soxF-a sulfide dehydrogenase flavoprotein chain precursor flanked by an integrase. The gene for a LysR-family regulator is located between this sulfur oxidation cluster and the CO_2 fixation gene cluster which is immediately upstream. The proximity of these clusters begs the question of coordinate regulation

given their role in autolithotrophy. The second cluster contains another set of Sox proteins required for oxidation of sulfur compounds (*sox HBXAZYDC*) together with a SoxR regulator and is also flanked upstream by an integrase. Upstream of the integrase there is region containing *tra* genes related to those of IncF conjugative plasmids. The presence of two clusters of genes for sulfur oxidation separated by a fragment of chromosome containing the *tra* genes between two integrases makes it tempting to speculate that the integration of the plasmid caused the splitting of the *sox* gene cluster. Finally there is a second SoxR homolog located about 100 genes upstream that is in close proximity to an ArsR regulator and two putative arsenate reductases.

***Tra* genes**

This region of the genome actually contains 17 *tra* genes similar to those of IncF conjugative plasmids(23). Conjugative plasmids encode the entire machinery required for their transmission to another bacterium. The number of genes encoding this process varies by plasmid type, however, *Leptothrix* appears to have a nearly complete complement, suggesting that it might be able to facilitate the transfer of non-self-transmissible plasmids. Although the *tra* genes are most often located on the plasmid rather than the chromosome, similar plasmids can integrate into the host's chromosome, as is the case with the F plasmid of *E. coli* . An autonomous plasmid has not yet been identified in *Leptothrix* and it would be interesting to know whether the *tra* genes integrated into the chromosome from a plasmid harbored by *Leptothrix* or by transfer from the genome of another bacterium. The latter option would also present the possibility of transfer of other genes during the process of plasmid transfer. Alternatively, the *tra* genes may be part of a integrative-conjugative-element (so called ICEs) that does not replicate like traditional plasmids, but resides almost exclusively in the chromosome (24).

Table 4. 2: The roles of some tra-gene encoded proteins during conjugation; genes in bold have been found in the *tra* cluster in the genome of *Leptothrix discophora.*

Pili Assembly and Production	*traA*, ***traB, traE, traC,*** ***traF, traG, traH, traK,*** ***traL***$_{2}$ *traQ*, ***traU***, *traV*, ***traW,***
Inner Membrane Proteins	***traB, traE, traG, traL***$_{2}$ *traP*
Periplasmic Proteins	***traC, traF, traH, traK, traU, traW***
DNA transfer	***traC, traD***$_{2}$ *traI, traM, traY*
Surface Exclusion Proteins	*traS, traT*
Mating Pair Stabilization	***traN, traG***

Manganese oxidation gene duplication and genomic context *of mofA* and *mofA2*

Initially identified from a clone library using antibodies raised against the manganese oxidizing fraction *of Leptothrix* supernatant, *mofA* was long thought to be the multicopper oxidase that is responsible for manganese oxidation in *Leptothrix.* However, as reported in chapter 3 of this dissertation, deletion of *mofA* did not eliminate manganese oxidation. Recent availability of the draft genome enabled the identification of another gene, with 85% (AA level) similarity to *mofA,* located approximately 1Mb downstream from it, Orf2977 that we named *mofA2* (Figure 4.2). Despite nearly 10x coverage of the genome, none of the manganese oxidization *deficient* mutants obtained from transposon mutagenesis of a Δ*mofA* strain contained insertions in *mofA2*. Rather, analysis of the sites of insertion of 23 manganese oxidation deficient mutants revealed that Mn oxidation was dependent on two sets of genes located in 2 regions on the chromosome, approximately 1Mb from each other. RAST automated annotation identified one of these genes (Orf 2665) to be a homolog of the *Bacillus sp. SG-1*

manganese oxidizing protein, MnxG; this gene was named *mnxG2*. MnxG2 shares 82% of their amino acids with Orf 1592, another putative multicopper oxidase which was named *mnxG1* (Figure 3.3). Although there is no direct evidence of the involvement of *mofA2* or *mnxG1* in manganese oxidation in *Leptothrix discophora SS1*, their high sequence similarity to genes involved in this process (*mofA* and *mnxG2*) as well as a pattern of duplication of these manganese oxidizing enzymes in other manganese oxidizing bacteria (25, 26) prompts us to include them in this analysis.

mofA/mofA2

Both *mofA* and *mofA2* are annotated as either multicopper oxidases (RAST) or as Fibronectin type III domain proteins, according to the J.Craig Venter Institute (JCVI) prokaryotic genome annotation pipeline. Multicopper oxidases (MCOs) are part of a large class of mixed function oxidases and oxygenases that oxidize their substrates using copper center active sites that transfer electrons to dioxygen and water (14, 27, 28). A motif search identified two copper center motifs in MofA (one type II, one type III) and three copper center motifs in MofA2 (one center of each type). Fibronectins (FN) were discovered and initially thought to occur exclusively in animals, but there is increasing evidence of their presence in bacteria (29–31). They are extracellular glycoproteins, whose major functional property is to create an extracellular matrix (ECM) and support cell adhesion. This is facilitated by the multiple binding sites identified along the fibronectin's fibril-like molecule, which allow for both self-association in aggregates as well as binding of several other molecules. Fibronectin type III repeats are predicted to occur in both MofA and MofA2, and some biochemical evidence suggests that MOF

is a glycoprotein (32). It is possible that MofA is a protein that encompasses both the functions of a multicopper oxidases and that of a fibronectin.

Upstream of *mofA* there is a thiol-disulfide isomerase, a multi-functional protein that catalyses the formation and isomerisation of disulfide bonds during protein folding and which could play a role in stabilizing the structure of MofA. Two proteins upstream of *mofA* there is a chaperone SecB-like protein, whose predicted role is to maintain proteins to be exported in an unfolded state; GspD of the general secretion pathway is located four ORFs downstream from *mofA*. The presence of these ORFs next to *mofA* as well as the signal peptide identified in the sequence of MofA (42) suggests that MofA is transported outside the cell which is consistent with the recovery of Mn oxidation activity in *Leptothrix* culture supernatants.

Downstream of *mofA* is the gene for a putative FKBP-type peptidyl-prolyl cis-trans isomerase FkpA precursor (EC 5.2.1.8) which is predicted to be a protein folding chaperon and which was originally called *mofB*. Immediately downstream of this is a hypothetical protein previously called *mofC*. These three genes (*mofA, mofB* and *mofC*) appear to be organized in an operon (ref) and *mofB* and *mofC* have been proposed to play a role in manganese oxidation, perhaps by assisting folding of MofA. Co expression of *mofB* with *mofA* in *E. coli* did not appear to impact *mofA* folding, while coexepression of all three genes did not recover manganese oxidation in *E. coli* (33). Despite the names, there is no direct biochemical evidence for the involvement of any of the three genes in manganese oxidation. Downstream of this is the gene for a cytochrome oxidase biogenesis protein Sco1/SenC/PrrC (a putative copper metallochaperone) and another hypothetical protein. Copper centers of MCOs require copper ions and the putative metalochaperone could be involved in providing this ion to MofA Cu centers. Further downstream there is an RNA polymerase sigma-54 factor RpoN next to a

transmembrane regulator protein PrtR. It important to note that a similar two component regulatory couple, containing a sigma-54 dependent response regulator (mnxR) and 2 histidine kinases (mnxS1/2) are required for Mn(II) oxidation in *Pseudomonas putida GB-1*.

The genomic context of *mofA2* is an area dense in two component regulatory systems, with three located immediately upstream and one downstream. Upstream of *mofA2* there is a signal transduction histidine kinase CheA (EC 2.7.3.-) followed by a chemotaxis response regulator CheY and a methyl-accepting chemotaxis protein. These types of proteins work together to detect changes in the concentration of signal molecules in the environment and transmit this information as chemoreceptor signals to the flagellum to adjust motility. Upstream of these there is another two component regulatory system, a Nitrate/nitrite sensor protein (EC 2.7.3.-) and a LuxR-type response regulator receiver; a third predicted two component regulatory system lies immediately upstream, with a signal transduction histidine kinase followed by a response regulator similar to UvrY. UvrY is a global regulator that has been shown to affect the transcript levels of more than 200 genes in *S. oneidensis* (34, 35). It can directly and indirectly regulate numerous processes including flagella biosynthesis, the type 2 quorum-sensing system and carbon metabolism. Immediately downstream of *mofA2* there is a hypothetical protein, followed by another regulatory group: a LuxR-family transcriptional regulator, a response regulator with sequence similarity to QseB, and a signal transduction histidine kinase similar to QseC. In *E. coli* QseB-QseC two-component system is part of the quorum-sensing regulatory cascade involved in the positive regulation of flagella and motility genes (36, 37) and it is possible that it plays the same role in *Leptothrix.* Although the draft genome does not specifically recognize genes of the quorum sensing in *Leptothrix*, there is evidence that many bacteria without a quorum sensing system *per se* do perceive quorum signals (38).

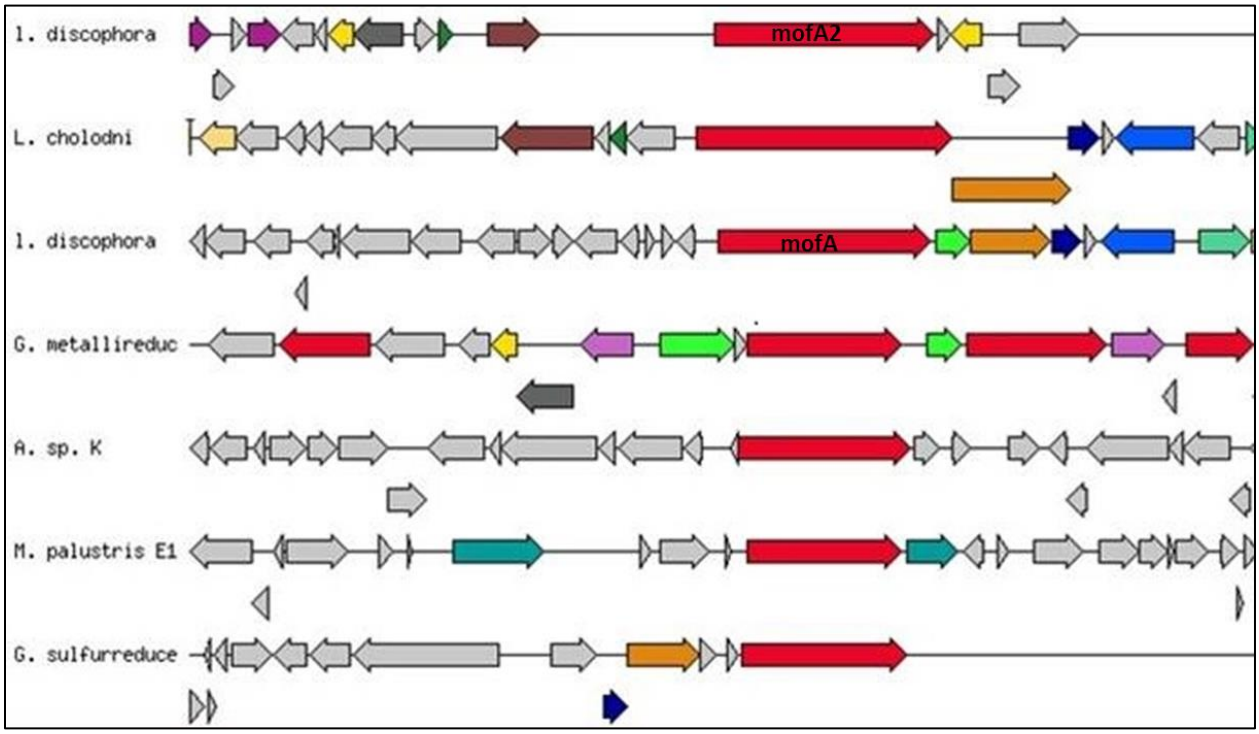


Figure 4.2 Schematic of the genomic context of *mofA* (line 3) in comparison with that of the *mofA* homolog in *L. discophora*, *mofA2* (line 1) as well as homologs of *mofA* in *L. cholodni (*line 2) and other bacteria (lines 4-7). While a *mofA* homolog seems to be present in the genome of other non- manganese oxidizers (red color arrow) the genomic context surrounding it is not conserved

Although MofA was long thought to catalyze manganese oxidation in *Leptothrix discophora* (32, 39, 40), its deletion did not affect the manganese oxidation ability of this bacterium and the role of MofA in manganese oxidation remains unclear. Likewise, it should be interesting to find out whether the high similarity between *mofA* and *mofA2* indicates a duplication in function, or if *mofA2* evolved to perform a different function (paralog of MofA). Initial evidence would suggest the latter, since no Δ*mofA* transposon mutants lacking manganese oxidation were isolated that had insertions in *mofA2*. Maintanence of manganese oxidation activity in a strain with a complete deletion of *mofA2* should confirm this hypothesis.

Alternatively, a more in depth analysis of the sequence of *mofA2* by comparison to the *mofA* sequence could reveal differences indicative of changes in the sequence of *mofA2* that altered its activity.

mnxG1/mnxG2

mnxG2 was the site of insertion in 9 of the 21 non-manganese oxidizing Δ*mofA* transposon mutants. Although no white mutants with insertions in *mnxG1* were found, its proximity to a putative operon where 14 insertions were found that resulted in non-manganese oxidizing mutants, and its 85% similarity to *mnxG2* prompted us to include it in this discussion. Both MnxG1 and MnxG2 have been annotated as either hypothetical proteins by RAST or as MnxG proteins by JCVI. Neither MnxG1 or MnxG2 have any homology to MofA or MofA2. Domain and motif searches identified MCOs domains, but no fibronectin-like domains, unlike MofA and MofA2. However, both *mnxG1* and *mnxG2* are accompanied by fibronectin type III domain proteins in their genomic neighborhoods; four genes upstream of *mnxG1* and 12 genes downstream of *mnxG2* (Figure 4.3). Only MnxG2 has a predicted signal peptide for export, while MnxG1 has a transmembrane domain that is predicted to occur in the N terminal.

mnxG2 is surrounded by either hypothetical proteins or proteins of unknown function (especially downstream). The sixth gene downstream is a copper-translocating P-type ATPase, while the 12th gene downstream is a fibronectin type III domain protein. Among other hypothetical proteins or proteins of unknown function there is a putative diguanylate cyclase and an acyltransferase 3.

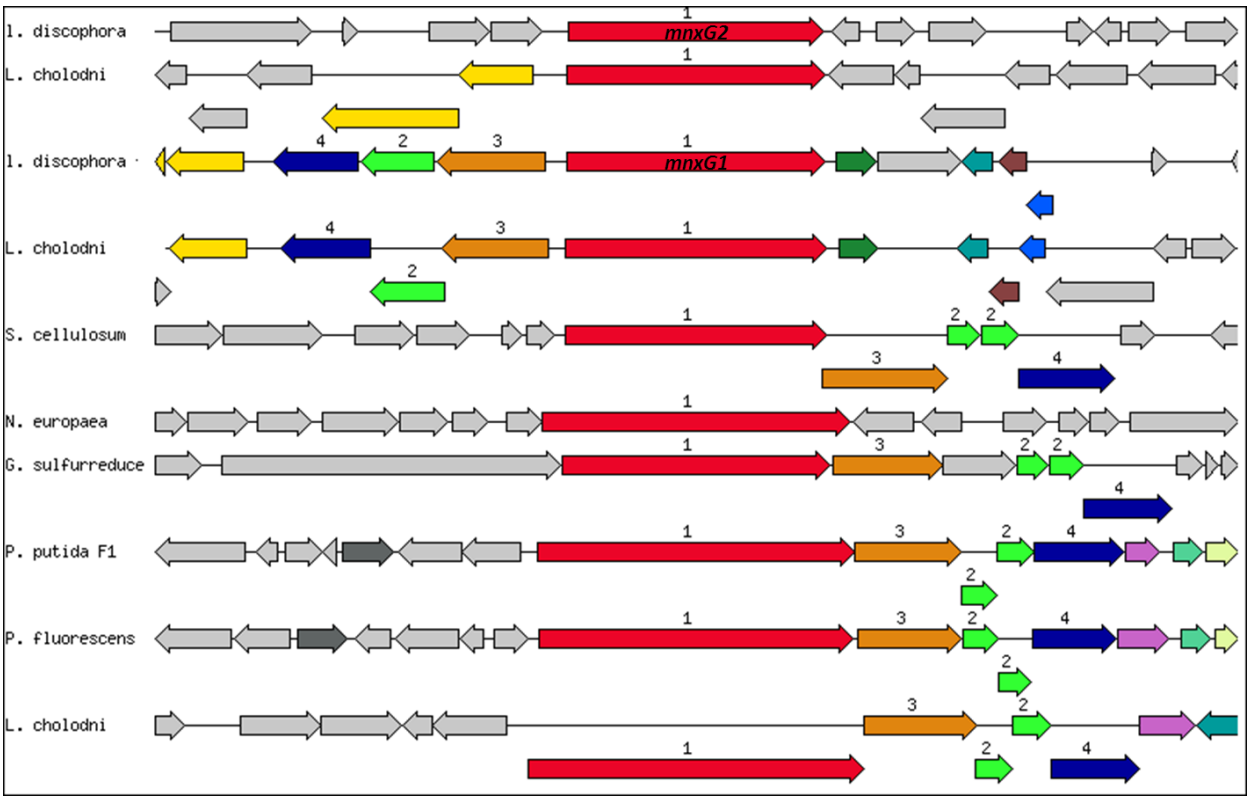


Figure 4.3 Genomic context of *mnxG2* and *mnxG1*. *mnxG2* (line 1) is surrounded by hypotehetical and proteins of unknown function, among which a copper-translocating P-type ATPase and a putative diguanylate cyclase. The genomic context of *mnxG1* (line 3) includes: (2) Sco1/SenC/PrrC, putative copper metallochaperone (3) surface antigen gene and (4) Putative cytochrome c family protein. This arrangement of genes is conserved *in L. cholodnii (line 4)*, which has two copies, as well as other non-manganese oxidizers.

4.5 CONCLUSION

A pattern of duplication of genes involved in manganese oxidation has been observed in two well characterized manganese oxidizers: *Pseudomonas putida GB-1,* encodes two MCOs, PputGB1_2447 and PputGB1_2665 that are independently capable of oxidizing both Mn(II) and Mn(III) and that are only weakly similar to each other (only 38% similarity over 20% of the query) (41). For *Aurantimonass sp.strain SI85-9A1,* preliminary experimental results, coupled with genomic mining suggest two variants of the putative Mn (II) oxidase, MoxA1 and MoxA2 (42). Unlike *Pseudomonas,* these putative manganese oxidizing proteins are 95% similar to each other, and the similarity extends over several genes in an area flanked by transposons, suggesting that an entire genetic fragment was duplicated during a transposition event (42, 43).

Preliminary experimental data for *Leptothrix* suggests that similar to *Pseudomonas putida GB-1,* two different putative MCOs, MofA and MnxG2 are involved in manganese oxidation. *In silico* analysis of the draft genome identified that both MofA and MnxG2 each have a homolog, MofA2 and with 82% and MnxG1 with 85% sequence similarity, respectively, as encountered in *Aurantimonas sp.strain SI85-9A1*.

Experimental evidence coupled with information provided by genome sequences establish a platform for investigation of the manganese oxidation process within and among the manganese oxidizing bacteria without precedent. For example, preliminary investigation of the genome of *Leptothrix* identified regulatory elements similar to *mnxS1, mnxS2* and *mnxR,* required for manganese oxidation in *Pseudomonas putida GB-1,* that are present in the genomic neighborhood of *mofA,* suggesting that this process could also be regulated in *Leptothrix.* Other genes shown to play a role in manganese oxidation in *Pseudomonas* and *Bacillus,* such as the

cytochrome C biogenesis pathway and genes from the general secretion pathway, have been found in the *Leptothrix* genome in the vicinity of genes encoding putative manganese oxidizing enzymes and should be investigated for their role in manganese oxidation in this bacterium.

A long standing hypothesis for the roles of manganese oxidation in the bacterial cells was that energy can be obtained from Mn(II)-derived electrons to the electron transport chain(7, 44). The preliminary genome analysis revealed the genetic potential for unexpected respiratory versatility and carbon metabolism in *Leptothrix discophora* that, in theory, could support this hypothesis. The recent availability of a genetic system for *Leptothrix* should facilitate experimental testing of a manganese oxidation derived chemoautothrophy.

4.6 REFERENCES

1. **Dondero NC**. 1975. The Sphaerotilus-Leptothrix Group. Annu. Rev. Microbiol. **29**:407–428.
2. **Schleifer K-H**. 2006. The Prokaryotes: A Handbook on the Biology of Bacteria. Springer.
3. **Ghiorse WC**. 1984. Biology of Iron-and Manganese-Depositing Bacteria. Annu. Rev. Microbiol. **38**:515–550.
4. **Ghiorse WC, Ehrlich HL**. 1992. Microbial biomineralization of iron and manganese. Catena Suppl. 75–99.
5. **Tebo BM, Johnson HA, McCarthy JK, Templeton AS**. 2005. Geomicrobiology of manganese(II) oxidation. Trends Microbiol. **13**:421–428.
6. **Ehrlich HL, Newman DK**. 2008. Geomicrobiology, Fifth Edition. CRC Press.
7. **Browers G-J**. 1999. Molecular Aspects of Microbial Manganese Oxidation. Thesis. Leiden University, Netherlands.
8. **Siering PL**. 1996. Application of molecular approaches to investigate the role of microorganisms in manganese cycling in wetland environments. Cornell University.
9. **Waasbergen LG van, Hoch JA, 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.
10. **Wilson K**. 2001. Preparation of Genomic DNA from BacteriaCurrent Protocols in Molecular Biology. John Wiley & Sons, Inc.
11. **Adams LF, Ghiorse WC**. 1987. Characterization of extracellular Mn2+-oxidizing activity and isolation of an Mn2+-oxidizing protein from Leptothrix discophora SS-1. J. Bacteriol. **169**:1279–1285.
12. **George Brenner Garrity, Noel R. Krieg, James T. Staley**. Bergey's Manual of Systematic Bacteriology.
13. **Bothe H, Ferguson S, Newton WE**. 2006. Biology of the Nitrogen Cycle. Elsevier.
14. **Nakamura K, GO N**. 2005. Function and molecular evolution of multicopper blue proteins. Cell. Mol. Life Sci. CMLS **62**:2050–2066.

15. **Prudêncio M, Eady RR, Sawers G**. 1999. The blue copper-containing nitrite reductase from Alcaligenes xylosoxidans: cloning of the nirA gene and characterization of the recombinant enzyme. J. Bacteriol. **181**:2323–2329.
16. **Kraft B, Strous M, Tegetmeyer HE**. 2011. Microbial nitrate respiration – Genes, enzymes and environmental distribution. J. Biotechnol. **155**:104–117.
17. **Zumft WG**. 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. MMBR **61**:533–616.
18. **Yagi JM, Sims D, Brettin T, Bruce D, Madsen EL**. 2009. The genome of Polaromonas naphthalenivorans strain CJ2, isolated from coal tar-contaminated sediment, reveals physiological and metabolic versatility and evolution through extensive horizontal gene transfer. Environ. Microbiol. **11**:2253–2270.
19. **Cannon GC, Bradburne CE, Aldrich HC, Baker SH, Heinhorst S, Shively JM**. 2001. Microcompartments in Prokaryotes: Carboxysomes and Related Polyhedra. Appl. Environ. Microbiol. **67**:5351–5361.
20. **Bueno E, Mesa S, Bedmar EJ, Richardson DJ, Delgado MJ**. 2012. Bacterial Adaptation of Respiration from Oxic to Microoxic and Anoxic Conditions: Redox Control. Antioxidants Redox Signal. **16**:819–852.
21. **Friedrich CG, Bardischewsky F, Rother D, Quentmeier A, Fischer J**. 2005. Prokaryotic sulfur oxidation. Curr. Opin. Microbiol. **8**:253–259.
22. **Friedrich CG, Rother D, Bardischewsky F, Quentmeier A, Fischer J**. 2001. Oxidation of Reduced Inorganic Sulfur Compounds by Bacteria: Emergence of a Common Mechanism? Appl. Environ. Microbiol. **67**:2873–2882.
23. **Arutyunov D, Frost LS**. 2013. F conjugation: Back to the beginning. Plasmid **70**:18–32.
24. **Wozniak RAF, Waldor MK**. 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat. Rev. Microbiol. **8**:552–563.
25. **Dick GJ, Podell S, Johnson HA, Rivera-Espinoza Y, Bernier-Latmani R, McCarthy JK, Torpey JW, Clement BG, Gaasterland T, Tebo BM**. 2008. Genomic Insights into Mn(II) Oxidation by the Marine Alphaproteobacterium Aurantimonas sp. Strain SI85-9A1. Appl. Environ. Microbiol. **74**:2646–2658.

26. **Geszvain K, McCarthy JK, Tebo BM**. 2013. Elimination of Manganese(II,III) Oxidation in Pseudomonas putida GB-1 by a Double Knockout of Two Putative Multicopper Oxidase Genes. Appl. Environ. Microbiol. **79**:357–366.
27. **Solomon EI, Sundaram UM, Machonkin TE**. 1996. Multicopper Oxidases and Oxygenases. Chem. Rev. **96**:2563–2606.
28. **Stoj CS, Kosman DJ**. 2006. Copper Proteins: OxidasesEncyclopedia of Inorganic Chemistry. John Wiley & Sons, Ltd.
29. **Mao Y, Schwarzbauer JE**. 2005. Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. Matrix Biol. J. Int. Soc. Matrix Biol. **24**:389–399.
30. **Hamburger ZA, Brown MS, Isberg RR, Bjorkman PJ**. 1999. Crystal structure of invasin: a bacterial integrin-binding protein. Science **286**:291–295.
31. **Pankov R, Yamada KM**. 2002. Fibronectin at a glance. J. Cell Sci. **115**:3861–3863.
32. **Adams LF**. 1986. Physiology, Cytology, and manganese oxidizing activityof Leptothrix discophora SS1. Cornell University.
33. **El Gheriany IA**. 2010. Manganese oxidation by Leptothrix discophora SS1. Cornell University.
34. **Binnenkade L, Lassak J, Thormann KM**. 2011. Analysis of the BarA/UvrY Two-Component System in Shewanella oneidensis MR-1. PLoS ONE **6**:e23440.
35. **Cruz-Garcia C, Murray AE, Klappenbach JA, Stewart V, Tiedje JM**. 2007. Respiratory Nitrate Ammonification by Shewanella oneidensis MR-1. J. Bacteriol. **189**:656–662.
36. **Krell T, Lacal J, Busch A, Silva-Jiménez H, Guazzaroni M-E, Ramos JL**. 2010. Bacterial sensor kinases: diversity in the recognition of environmental signals. Annu. Rev. Microbiol. **64**:539–559.
37. **Pereira M, Parente JA, Bataus LAM, Cardoso D das D de P, Soares RBA, Soares CM de A**. 2004. Chemotaxis and flagellar genes of Chromobacterium violaceum. Genet. Mol. Res. GMR **3**:92–101.
38. **Bassler BL**. 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. Curr. Opin. Microbiol. **2**:582–587.

39. **Corstjens PLAM, de Vrind JPM, Goosen T, Jong EW de V**. 1997. Identification and molecular analysis of the Leptothrix discophora SS-1 mofA gene, a gene putatively encoding a manganese-oxidizing protein with copper domains. Geomicrobiol. J. **14**:91–108.
40. **G. J. Brouwers, P. L. A. M. Corstjens, J. P. M. de Vrind, A. Verkamman, M. de Kuyper EW de VJ**. 2000. Stimulation of Mn2+ Oxidation in Leptothrix discophora SS-1 by Cu2+ and Sequence Analysis of the Region Flanking the Gene Encoding Putative Multicopper Oxidase MofA. Geomicrobiol. J. **17**:25–33.
41. **Geszvain K, McCarthy JK, Tebo BM**. 2013. Elimination of Manganese(II,III) Oxidation in Pseudomonas putida GB-1 by a Double Knockout of Two Putative Multicopper Oxidase Genes. Appl. Environ. Microbiol. **79**:357–366.
42. **Dick GJ, Podell S, Johnson HA, Rivera-Espinoza Y, Bernier-Latmani R, McCarthy JK, Torpey JW, Clement BG, Gaasterland T, Tebo BM**. 2008. Genomic Insights into Mn(II) Oxidation by the Marine Alphaproteobacterium Aurantimonas sp. Strain SI85-9A1. Appl. Environ. Microbiol. **74**:2646–2658.
43. **Anderson CR, Johnson HA, Caputo N, Davis RE, Torpey JW, Tebo BM**. 2009. Mn(II) Oxidation Is Catalyzed by Heme Peroxidases in "Aurantimonas manganoxydans" Strain SI85-9A1 and Erythrobacter sp. Strain SD-21. Appl. Environ. Microbiol. **75**:4130–4138.
44. **Nealson KH, Tebo BM, Rosson RA**. 1988. Occurrence and Mechanisms of Microbial Oxidation of Manganese, p. 279–318. *In* Allen I. Laskin (ed.), Advances in Applied Microbiology. Academic Press.

CHAPTER 5

SUMMARY AND RECCOMENDATIONS FOR FUTURE WORK

In this study I developed for the first time a genetic system for *Leptothrix discophora* SS1, a model organism for the study of manganese oxidation. The antibiotic sensitivity of *L. discophora* SS1 was characterized and a procedure for transformation with exogenous DNA via conjugation was developed and optimized, resulting in a maximum transfer frequency of $5.2*10^{-1}$ (transconjugant/donor). Genetic manipulation of *Leptothrix* was demonstrated by disrupting *pyrF* via chromosomal integration of a plasmid with an R6Kɣ ori through homologous recombination. This resulted in resistance to fluoroorotidine which was abolished by complementation with an ectopically expressed copy of *pyrF* cloned into pBBR1MCS-5. *pyrF* has potential as a selectable marker in *Leptothrix* and could be further developed into a more powerful tool for creating multiple deletions in the same strain. First a *Leptothrix* mutant with a clean *pyr*F deletion would be created and used together with a second pVIK165 derivative that contained a wild type copy of *pyrF* and the internal regions (or flanking regions, for deletions) of the gene of interest. This strategy would allow for selection of integrants into other chromosomal locations based on the restoration of *de novo* uracil biosynthesis and therefore growth on minimal medium. In theory, such a system could be used again and again to generate multiple markerless mutations in the same background (1).

In chapter two I was able to utilize some of the genetic tools I developed to investigate the role of MofA, a putative multicopper oxidase that has been suggested by others to encode the manganese oxidizing fraction of SS1 supernatant. *mofA* as well as four other genes candidates for encoding the manganese oxidizing activity in SS1 were interrupted by homologous

recombination and plasmid integration. All five interrupted mutants maintained the ability to oxidize manganese, suggesting that these genes may not play a role in manganese oxidation, as hypothesized. Deletion of *mofA* did not affect the ability of cells to oxidize manganese, however transposon mutagenesis in this Δ*mofA* mutant resulted in the isolation of white, non-manganese oxidizing mutants with transposon insertions in 4 genes located in 2 regions on the chromosome. One of the genes, named *mnxG2* is a putative multicopper oxidase similar to the manganese oxidizing enzyme in *Bacillus sp* SG-1. The other three genes with insertions encode a hypothetical protein, a putative cytochrome c next to a putative copper metallochaperone (Sco1/SenC/PrrC) involved in the biogenesis of cytochrome oxidase. To completely elucidate the role of *mofA* in this process as well as the role of *mnxG1,* the manganese oxidation activity of a Δ*mofA*Δ*mnxG1* mutant should first be investigated. If this mutant is indeed lacking manganese oxidation activity, then complementation of this mutant with a wild type copy of *mnxG1* should recover the manganese oxidation activity and should provide sufficient evidence for the requirement of MnxG1 for manganese oxidation. To completely elucidate the role of *mofA,* an SS1Δ*mnxG1* mutant should be assessed for the ability to oxidize manganese. If this mutant would still oxidize Mn, then a double knock-out mutant Δ*mnxG1*Δ*mofA* should be created and manganese oxidation assessed to investigate the role of *mofA.* Lack of manganese oxidation in this strain would suggest a role for *mofA* in the manganese oxidation process and restoration of this activity by complementation with a wild copy of *mofA* should solidify this argument. Additionally, transposon mutagenesis of the SS1Δ*mnxG1* (if this mutant would still oxidize manganese) can help identify other genes important in manganese oxidation as was the case with SS1Δ*mofA* transposon mutants. Next the role of the other three genes identified from the non-

manganese oxidizing transposon mutants (a hypothetical protein, a putative cytochrome c and a putative copper metallochaperone) should be elucidated.

In assessing the manganese oxidation activity of the mutants described we used a preliminary, qualitative assessment that only differentiates brown-manganese oxidizing from white-non oxidizing strains, but not between different shades of brown, which could be indicative of various levels of oxidation activity. A quantitative method however, such as Leuco Berbelin Blue (LBB) (2) which can quantify the amount of Mn(III) and Mn(IV), to measure the amount and the rate of manganese oxidizied by different mutants should be used since it could provide important additional information about the role of these genes in manganese oxidation. This quantitative assessment of manganese oxidation should be applied in particular to the remaining 31 off white SS1Δ*mofA* transposon mutants, not included initially in the pool of manganese oxidation deficient mutants, because they turned various shades of brown upon re-streaking. However, these mutants were derived from colonies that were initially off white or white with brown streaks, suggesting that the manganese activity was affected to some extent. Finally, quantitative analysis of the manganese oxidation activity of the 10 darker brown SS1Δ*mofA* transposon mutants as well as identifying the genes of transposon insertions can add knowledge about the manganese oxidation in *Leptothrix discophora* SS1.

Chapter three provides a short description of the draft genome of *Leptothrix discophora* SS1, which contains 4.2Mb with 3,791 identified protein coding sequences. In contrast to well established descriptions of *Leptothrix* as an obligate aerobic heterotroph, *in silico* analysis of the draft genome revealed the potential for much greater metabolic diversity than previously described, including fermentation, anaerobic respiration with nitrate and arsenate, as well as the potential for lithotrophy via sulfur oxidation and carbon fixation (3). Further work should

include a comparison between the draft genome of *L. discophora* SS1 and the completed genome of another manganese oxidizer, *L. cholodnii,* the closest phylogenetically related microorganism to *Leptothtrix* with an available genome. For example, initial comparison of these two genomes revealed that only *L. discophora SS1* contains the 17 tra genes encoding several proteins involved in conjugation. Conjugation experiments which would test the ability of SS1 to transfer DNA to other microorganisms can confirm whether these genes are functional or the mere result of horizontal gene transfer. The breadth of theoretical information about *Leptothrix's* metabolic diversity revealed by the draft genome should be confirmed in experiments designed to test the ability of SS1 to grown under anaerobic conditions with nitrate or arsenate as an electron acceptor or its ability to ferment. The possibility that manganese oxides could serve as terminal electron acceptor (4) can be tested by assessing the dissolution of manganese oxides under oxygen limiting conditions (5). Finally, to gain more insight about the role that manganese oxidation might play for *Leptothrix*, the metabolic and genetic information obtained theoretically or experimentally in the laboratory should be connected to physical and chemical information about the natural habitat of *Leptothrix*. In this respect, an analysis of the chemical species present in the water column of the Sapsucker woods wetlands as well as their vertical distribution and seasonal variation could represent important links for completing the manganese oxidation story in this bacterium.

5.1 REFERENCES

1. **Galvao TC, de Lorenzo V**. 2005. Adaptation of the Yeast URA3 Selection System to Gram-Negative Bacteria and Generation of a betCDE Pseudomonas putida Strain. Appl. Environ. Microbiol. **71**:883–892.
2. **El Gheriany IA, Bocioaga D, Hay AG, Ghiorse WC, Shuler ML, Lion LW**. 2009. Iron Requirement for Mn(II) Oxidation by Leptothrix discophora SS-1. Appl. Environ. Microbiol. **75**:1229–1235.
3. **Friedrich CG, Rother D, Bardischewsky F, Quentmeier A, Fischer J**. 2001. Oxidation of Reduced Inorganic Sulfur Compounds by Bacteria: Emergence of a Common Mechanism? Appl. Environ. Microbiol. **67**:2873–2882.
4. **Nealson KH, Myers CR**. 1992. Microbial reduction of manganese and iron: new approaches to carbon cycling. Appl. Environ. Microbiol. **58**:439–443.
5. **Mehta T, Coppi MV, Childers SE, Lovley DR**. 2005. Outer membrane c-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in Geobacter sulfurreducens. Appl. Environ. Microbiol. **71**:8634–8641.

APPENDIX A

PROTEINS IDENTIFIED BY LC MS/MS IN BANDS WITH MANGANESE OXIDIZING ACTIVITY FROM SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF SS1 CONCENTRATED CULTURE SUPERNATANT

SDS-PAGE of concentrated culture supernatant
Coomassie stain (left) and activity stain (right) of MOF

Leptothrix discophora SS1 culture supernatant was concentrated ~ 1000X and the protein mixture separated on a denaturing polyacrylamide gel electrophoresis. Brown bands of manganese oxides developed when the gel was soaked in a solution of $MnSO_4$ at approximately 100kDa marker and 55kDA marker. The brown band corresponding to the ~100kDa marker was excised, digested with trypsin and subjected to LC MS/MS (liquid chromatography tandem mass spectrometry). The library of peptides generated from the manganese oxidizing band was analyzed with MASCOT software used to search the NCBI protein database.

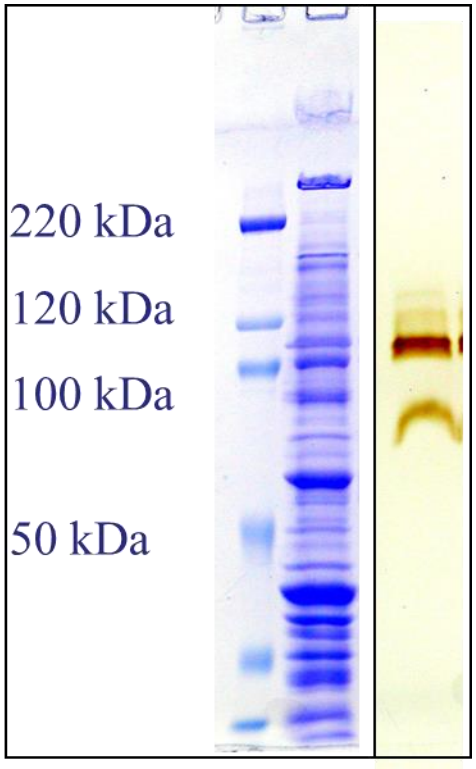


Figure 1: SDS PAGE of concentrated culture supernatant; left, coomassie stain ; right, manganese oxidizing activity

An initial initial investigation (2007) of the peptide library from the proteins contained in the active (brown, manganese oxidizing) fraction of SDS PAGE is shown in Table 1. Neither *L. cholodni*'s or *L.discohora's* genome were available at that time. This search identified 35 proteins, however *mofA* was not one of the proteins on this list.

Table 1: Proteins identified by MS/MS in SDS PAGE bands with manganese oxidizing activity from *Leptothrix discophora SS1* concentrated culture supernatant in a search against the NCBI protein database in 2007.

#		**Protein hit**	**Organism**
1	gi|110593603	chaperonin GroEL	Acidovorax sp. JS42
2	gi|84714563	S-adenosyl-L-homocysteine hydrolase	Polaromonas naphthalenivorans CJ2
3	gi|78780103	adenosylhomocysteinase	Prochlorococcus marinus str. MIT 9312
4	gi|118053932	adenosylhomocysteinase	Comamonas testosteroni KF-1
5	gi|87200019	adenosylhomocysteinase	Novosphingobium aromaticivorans DSM 12444
6	gi|84712483	2-oxo-acid dehydrogenase E1 component homodimeric type	Polaromonas naphthalenivorans CJ2
7	gi|149690	58-kDa common antigen	
8	gi|94263026	Adenosylhomocysteinase	delta proteobacterium MLMS-1
9	gi|110595193	2-oxo-acid dehydrogenase E1 component, homodimeric type	Acidovorax sp. JS42
10	gi|397462	excreted protein	Leptothrix discophora
11	gi|37958861	putative pyruvate dehyrogenase E1 complex	uncultured bacterium
12	gi|30248059	TCP-1 (Tailless complex polypeptide)/cpn60 chaparonin family	Nitrosomonas europaea ATCC 19718
13	gi|118602570	chaperonin GroEL	Candidatus Ruthia magnifica str. Cm (Calyptogena magnifica)
14	gi|84711720	TonB-dependent siderophore receptor	Polaromonas naphthalenivorans CJ2
15	gi|91786114	branched-chain amino acid aminotransferase	Polaromonas sp. JS666
16	gi|38606907	Clp protease	Lactobacillus johnsonii
17	gi|27378830	hypothetical protein blr3719	Bradyrhizobium japonicum USDA 110

18	gi|67938634	Chaperonin Cpn60/TCP-1	Chlorobium phaeobacteroides BS1
19	gi|46445664	probable 60 kDa chaperonin (GroEL)	Candidatus Protochlamydia amoebophila UWE25
20	gi|59711416	glutaminyl-tRNA synthetase	Vibrio fischeri ES114
21	gi|16264406	putative transcriptional regulator protein	Sinorhizobium meliloti 1021
22	gi|78044050	hypothetical protein CHY_1823	Carboxydothermus hydrogenoformans Z-2901
23	gi|18311423	ClpC adenosine triphosphatase	Clostridium perfringens str. 13
24	gi|50083807	hypothetical protein ACIAD0571	Acinetobacter sp. ADP1
25	gi|68544415	hypothetical protein SbalDRAFT_0589	Shewanella baltica OS155
26	gi|77362306	putative AMP-dependent synthetase and ligase	Pseudoalteromonas haloplanktis TAC125
27	gi|77459218	hypothetical protein Pfl_2996	Pseudomonas fluorescens PfO-1
28	gi|84703513	GDP-mannose 4,6 dehydratase	Parvularcula bermudensis HTCC2503
29	gi|88812264	Heavy metal efflux pump CzcA	Nitrococcus mobilis Nb-231
30	gi|89076449	hypothetical acyl-coenzyme A synthetase	Photobacterium sp. SKA34
31	gi|113474955	hypothetical protein Tery_1185	Trichodesmium erythraeum IMS101
32	gi|57167968	transcription-repair coupling factor	Campylobacter coli RM2228
33	gi|118729402	Extracellular ligand-binding receptor	Delftia acidovorans SPH-1
34	gi|37527242	hypothetical protein plu3368	Photorhabdus luminescens subsp. laumondii TTO1
35	gi|118587277	N-acetylgalactosamine transferase	Oenococcus oeni ATCC BAA-1163

A subsequent search of the same peptide library against the currently available *L. discophora* genome as well as the current protein database in NCBI (including the genome of *L. cholodni*) identified the proteins from both *L. discophora* and *L. cholodni* as shown in Table 2 and Table 3.

Table 2. Proteins identified by MS/MS in SDS PAGE bands with manganese oxidizing activity from *Leptothrix discophora SS1* concentrated culture supernatant in a search against the draft genome of *Leptothrix discophora SS1*.

	Protein ID	Protein score	Protein mass	Annotation
1	fig|6666666.41126.peg.1920	924	88455	Orf 1596 Glycoprotein gp2
2	fig|6666666.41126.peg.2804	835	33982	
3	fig|6666666.41126.peg.1926	789	169768	Orf 1592 *(mnxG1)* hypothetical protein
4	fig|6666666.41126.peg.2496	676	52774	
5	fig|6666666.41126.peg.3261	627	81605	
6	fig|6666666.41126.peg.1960	410	39672	
7	fig|6666666.41126.peg.1921	365	49107	Orf1597 Glycoprotein gp2
8	fig|6666666.41126.peg.1885	365	61917	
9	fig|6666666.41126.peg.2134	339	170217	
10	fig|6666666.41126.peg.3396	321	57440	
11	fig|6666666.41126.peg.2618	295	44474	
12	fig|6666666.41126.peg.2923	240	100730	
13	fig|6666666.41126.peg.301	170	44928	
14	fig|6666666.41126.peg.1384	150	33388	
15	fig|6666666.41126.peg.1386	113	7432	
16	fig|6666666.41126.peg.2305	111	71491	
17	fig|6666666.41126.peg.382	103	33089	
18	fig|6666666.41126.peg.3319	102	153069	
19	fig|6666666.41126.peg.916	90	106708	
20	fig|6666666.41126.peg.2011	86	51517	
21	fig|6666666.41126.peg.3249	77	85192	
22	fig|6666666.41126.peg.718	67	47226	
23	fig|6666666.41126.peg.1827	66	49067	
24	fig|6666666.41126.peg.3596	62	40074	
25	fig|6666666.41126.peg.1334	60	82760	
26	fig|6666666.41126.peg.1708	58	38117	
27	fig|6666666.41126.peg.1380	58	32706	
28	fig|6666666.41126.peg.2144	57	96910	
29	fig|6666666.41126.peg.54	53	46230	
30	fig|6666666.41126.peg.1714	52	80288	

31	fig|6666666.41126.peg.1539	51	37101	
32	fig|6666666.41126.peg.3248	50	46359	
33	fig|6666666.41126.peg.1413	46	54447	
34	fig|6666666.41126.peg.3164	44	45928	
35	fig|6666666.41126.peg.2141	44	82750	
36	fig|6666666.41126.peg.2881	39	68695	
37	fig|6666666.41126.peg.3075	38	51981	
38	fig|6666666.41126.peg.320	33	50706	
39	fig|6666666.41126.peg.3131	32	39615	
40	fig|6666666.41126.peg.550	31	43003	
41	fig|6666666.41126.peg.74	31	51157	
42	fig|6666666.41126.peg.534	29	14291	
43	fig|6666666.41126.peg.1280	28	30281	
44	fig|6666666.41126.peg.3122	28	108351	
45	fig|6666666.41126.peg.247	26	79289	
46	fig|6666666.41126.peg.826	26	29909	
47	fig|6666666.41126.peg.2995	25	45594	

Table 3: Proteins identified by MS/MS in SDS PAGE bands with manganese oxidizing activity from *Leptothrix discophora SS1* concentrated culture supernatant in a search against the NCBI protein database in 2013, which includes the genome of *L. cholodni.*

		Protein name	Protein score	Protein mass
1	gi|171057174	chaperonin GroEL [Leptothrix cholodnii SP-6]	493	57331
2	gi|124268470	S-adenosyl-L-homocysteine hydrolase [Methylibium petroleiphilum PM1]	424	52247
3	gi|171058329	pyruvate dehydrogenase subunit E1 [Leptothrix cholodnii SP-6]	400	100949
4	gi|121603402	S-adenosyl-L-homocysteine hydrolase [Polaromonas naphthalenivorans CJ2]	331	52275
5	gi|171059306	putative multicopper oxidase [Leptothrix cholodnii SP-6]	218	169523
6	gi|375106021	ABC-type branched-chain amino acid transport system, periplasmic component [Burkholderiales bacterium JOSHI_001]	199	40007
7	gi|171059608	serine hydroxymethyltransferase [Leptothrix cholodnii SP-6]	195	44940
8	gi|397462	excreted protein [Leptothrix discophora]	161	13385
9	gi|171058378	extracellular solute-binding protein [Leptothrix cholodnii SP-6]	148	44845
10	gi|239815711	extracellular ligand-binding receptor [Variovorax paradoxus S110]	113	41737
11	gi|154362103	RNA polymerase beta subunit [Comamonas kerstersii]	102	25502

12	gi|91788907	TonB-dependent siderophore receptor [Polaromonas sp. JS666]	99	79219
13	gi|91786114	branched-chain amino acid aminotransferase [Polaromonas sp. JS666]	87	34686
14	gi|171058572	glucose-1-phosphate adenylyltransferase [Leptothrix cholodnii SP-6]	64	47304
15	gi|254445776	Hsp20/alpha crystallin family [Verrucomicrobiae bacterium DG1235]	63	15301
16	gi|333984547	unnamed protein product [Methylomonas methanica MC09]	63	87203