Mn(II) oxidation by Leptothrix discophora SS-1

by Iman A Elgheriany

Mn(II) OXIDATION BY LEPTOTHRIX DISCOPHORA SS-1

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Iman A. Elgheriany

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Iman A. Elgheriany, Ph. D.

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The biological catalysis of Mn(II) oxidation is thought to be responsible for the formation of most naturally occurring insoluble Mn(III, IV) oxides. The physiological benefit and the regulatory mechanism of Mn(II) oxidation remains unknown. A better understanding of environmental conditions that favor or inhibit the production of the extracellular protein(s) responsible for Mn(II) oxidation is a prerequisite for the design of any successful technology that uses Mn(III,IV) oxides for biofiltration and biodegradation purposes. A controlled batch reactor system was developed to study the environmental conditions that regulate Mn(II) oxidation by the model Mn(II)-oxidizing bacterium *Leptothrix discophora* SS-1. Cells were grown in a chemically-defined medium at neutral pH. Mn(II)-oxidizing activity as well as *mofA* gene transcript levels were measured. A decline in Mn(II) oxidation occurred under iron-limited conditions, which was not accompanied by siderophore production and is therefore unlikely to be an artifact of siderophore complexation of Mn(III). The data are consistent with a direct requirement of Fe for Mn(II) oxidation. Additionally, Mn(II) oxidation is specifically stimulated by Cu(II) and not by toxic levels of Cd(II). These results indicate that Mn(II) oxidation is not a response to metal toxicity. Interestingly, the temporal variations in *mofA* transcript levels did not correlate with the observed decline and stimulation of Mn(II) oxidation. This result suggests that MofA may not be directly involved in Mn(II) oxidation. The role of *mofA* in Mn(II) oxidation was evaluated by cloning and expressing the three genes of the *mof* operon of *L. discophora* SS-1 in *Escherichia coli*. First, the signal sequences of the three

genes, *mofA mofB* and *mofC*, were screened using an assay that allows the discrimination between the major modes of transport across the inner bacterial membrane. All three proteins were shown to be transported across the inner cytoplasmic membrane by the twin-arginine translocation (Tat) pathway. Expression of full length and mature domains of MofA and MofB showed that the signal sequences enhance the stability of the two proteins in *E. coli*. Codon optimization was a prerequisite for the successful expression of MofC. MofB and MofC are shown to be extracellular proteins. In addition, MofC is shown to be needed for the folding of MofA and its highly probable subsequent translocation to the periplasm. Interaction of MofA and MofC appear to be responsible for the loss of the FLAG-tag sequence from the C-terminus of MofA which compromised detection of the MofA protein after it left the cytoplasm. Subsequent to cloning and coexpressing the three genes of the *mof* operon of *L. discophora* SS-1 in *E. coli*, no Mn(II)-oxidizing activity was detected in either the periplasm or the supernatant. These findings indicate that MofA is either not the Mn(II)-oxidizing protein of *L. discophora* SS-1 or that it needs other, as yet unknown accessory proteins for catalyzing Mn(II) oxidation. Further evaluation of the roles of MofA, MofB, and MofC and their link to Mn(II)-oxidation would be expedited by development of a reliable genetic system for *L. discophora* SS-1.

BIOGRAPHICAL SKETCH

Iman A. Elgheriany was born and raised in Alexandria, Egypt. She completed the German high school diploma (Abitur) in the German school (Alexandria) in 1996 and then attended the University of Alexandria, Egypt. In 2001, she received her Bachelor of Science in Chemical Engineering. She worked as a teaching assistant and received her Masters of Science in Chemical Engineering from the same University in 2004. She subsequently enrolled at Cornell University in August of 2004 and received her doctorate of philosophy in Chemical Engineering in August of 2010 under the guidance of Prof. Leonard W. Lion and Prof. Michael L. Shuler.

O Allah! We have no knowledge except what you have taught us,

you are the All- Knower and the All- Wise.

O Allah! Teach us what benefits us

and benefit us from what you've taught us

and enhance our knowledge.

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CHAPTER 1

INTRODUCTION TO THESIS RESEARCH

Mn(II) is the Earth's second most abundant transition metal next to iron (23). "Mn(III, IV) oxides" is a collective term that refers to the element's oxides, hydroxides and oxyhydroxides. In general, Mn(II) exists in a soluble form at low pH and oxygen concentration, while Mn(III) and Mn(IV) primarily form insoluble oxides and oxyhydroxides at high pH and oxygen concentration. Thus, oxidation of Mn(II) alters its transport and bioavailability in aqueous systems. Direct microbial oxidation of Mn(II) to biotic oxides is an extracellular enzymatic process. At circumneutral pH the rate of formation of biogenic Mn(III, IV) oxides is five orders of magnitude faster than that of abiotic oxides (16, 22); therefore at ambient pH values, Mn(II) oxidation kinetics is likely to be microbially mediated (23). The resulting biogenic Mn(III, IV) oxides are strong adsorbents of heavy metals (8, 9, 17) and participate in a wide range of redox reactions (20, 21). As a result, Mn(III, IV) oxides greatly affect the biogeochemistry and the fundamental cycling of many toxic and essential elements (11, 23).

The physiological importance of microbially mediated Mn(II) oxidation is not yet known. Scavenging trace metals required for growth and immobilizing toxic trace metals could be one advantage to organisms that catalyze this reaction (2). The ability of biogenic Mn(III, IV) oxides to oxidize biologically refractory organic matter-e.g., humic and fulvic acids- could enable cells to use the resulting low molecular weight organic compounds (e.g. pyruvate) as source of carbon and energy (21). It is also possible that the metal-encrusted surfaces of Mn(II)-oxidizing bacteria provide increased resistance to predation, UV-light or viral attacks (23).

Biogenic Mn(II) oxidation have been used for the removal of Mn(II), Fe(II) and As(III) from potable ground water (14, 15). This biological drinking water treatment

involves passing raw water through a filter colonized by Mn(II)-oxidizing bacteria. Despite the fact that Mn(II)-oxidizing bacteria e.g. *Leptothrix cholodnii* SP-6 (12, 15, 18) have been widely used in biological filters, the mechanism involved in Mn(II) oxidation are poorly understood (24).

Early work involving bacterial Mn(II)-oxidation was performed on the soil bacterium *Arthrobactor sp.* Strain B (6) and on *Pseudomonas* species (6, 13). The bacterial isolates that were subsequently studied include: *Bacillus* spores SG-1, *Pseudomonas putida* MB 1 and MG-1, *Leptothrix discophora* SS-1 and Leptothrix *cholodnii* SP-6, *α-Proteobacterium* SD-21, *Roseobactor* AzwK3b and *Pedomicrobium* sp. ACM 3067. Genome sequence information is available for the marine ά-*Proteobacterium Aurantimonas* sp. Strain SI85-9A1 and *Leptothrix cholodnii* SP-6.

Experimental evidence has revealed several common features of bacterial Mn(II) oxidation. The process is mainly extracellular. The Mn(II)-oxidizing activity is enzymatic since it is heat labile, sensitive to metalloprotein inhibitors like mercuric chloride and to redox enzyme inhibitors like azide and shows pH and temperature optima (1, 6, 10). Mn(II)-oxidizing factors appear to be multiprotein complexes (1, 10), since low molecular weight Mn(II)-oxidizing bands have been observed on SDS-PAGE gels. This result indicates that a single protein might be directly responsible for Mn(II) oxidation but needs to be present in multimeric form for optimal activity. No Mn(II)-oxidizing macromolecule has been purified sufficiently to allow biochemical and structural characterization.

Discovery of the genes responsible for Mn(II) oxidation relied either on producing mutants that did not oxidize Mn(II) e.g. in the case of *moxA* in *Pedomicrobium* (19) and *MnxG* in *Bacillus spores* SG-1(25, 26) and/or on the identification of the Mn(II)-oxidizing proteins by tandem mass spectrometry of Mn(II)-oxidizing bands visualized by native polyacrylamide gel electrophoresis in-gel

activity assays (4, 7). The primary amino acids of proteins linked to Mn(II) oxidation share sequence homology to either multicopper oxidases or heme peroxidases (4).

Leptothrix discophora SS-1 was isolated in 1979 from a metallic surface film and demonstrated the ability to oxidize Mn(II) (3). During early stages of cultivation this gram negative heterotrophic bacterium produced the Mn(II)-oxidizing protein(s) as part of an extracellular sheath material. After lab cultivation *L. discophora* stopped making the sheath. However it did not loose the ability to oxidize Mn(II). Regardless of the presence of Mn(II) in the growth medium, *L. discophora* SS-1 oxidizes Mn(II) to form brown Mn-oxide particles. The extracellular Mn(II)-oxidizing factor was separated using polyacrylamide electrophoresis and was associated with membrane blebs and polysaccharides. After purification it exhibited an apparent molecular weight of 110,000 (1). To date the Mn(II) oxidizing factor has not been isolated in sufficient quantities to allow further biochemical characterization. The Mn(II) oxidizing activity has optima at 28 ºC and pH 7.3 (1, 5).

The main objective of this research was to study the environmental factors that affect the production of the Mn(II)-oxidizing protein(s) produced by *L. discophora* SS-1. A controlled batch reactor system was developed in which I grew this model Mn(II)-oxidizing bacterium under defined growth conditions and assessed the effect of these conditions on the rate of Mn(II) oxidation as well as on the transcript level of *mofA*, the gene that is thought to encode the Mn(II)-oxidizing activity. Chapter 2 describes the reactor system used, while Chapters 3 and 4 present two important findings of this research. The first result is related to a decline in Mn(II) oxidation in iron-limited cultures, which implies that Mn(II) oxidation requires Fe. The second finding is related to the specific stimulation of Mn(II)-oxidation by Cu(II). My qRT-PCR data in both chapters showed poor correlation between changes in observed Mn(II) oxidation rates and transcript levels of *mofA*. While this result might be related

to the fact that Mn(II) oxidation could be regulated in a post transcriptional manner, I was interested in exploring the actual role of *mofA*. Chapter 5 describes my research effort in cloning and expressing the *mof* operon of *L. discophora* SS-1 in *Escherichia coli*.

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CHAPTER 2

DEVELOPMENT OF A BIOREACTOR SYSTEM FOR STUDYING Mn(II)-OXIDATION BY *LEPTOTHRIX DISCOPHORA* SS-1

Introduction

The objective of this research was to investigate the environmental conditions that regulate the production of the manganese oxidizing factor (MOF) produced by the model Mn(II)-oxidizing bacterium *Leptothrix discophora* SS-1. The first step was to set up a controlled bioreactor system, in which cells are grown in a defined medium with pH and oxygen control. The defined medium allows the control of the chemical composition and the speciation of metal ions e.g. Cu(II) and Fe(II). In addition to achieving stable growth in the defined minimal medium, establishing a reliable method to measure Mn(II)-oxidizing activity were the major challenges. The following chapter summarizes how I was able to set-up this controlled environment.

Materials and Method

Bioreactor. Experiments were performed in 2L magnetically stirred jacketed vessels shown in Figure 2.1. I used a stirred bar that does not rest at the bottom of the reactor to minimize shear on cells. The working volume of each reactor was 25% to insure that cells are not oxygen-limited. Glass autoclavable pH probes (Mettler Toledo 405-DPAS-SC-K8S/200) were installed in each reactor and connected to individual pH controllers (Jenco Electronics, LTD., pH/ORP controller 3676), in order to maintain pH at 7.0±0.1 by automatic addition of 0.01 N HNO_3 and 0.01N NaOH. Because of drifts in pH after autoclaving, the pH-probes were sterilized with a 70% ethanol mixture (pH=2) and installed aseptically after autoclaving the reactor with the autoclavable media components. Temperature was constant at 25-27ºC. The bioreactors were aerated with filtered air at constant flow rate of 0.8 SCFH.

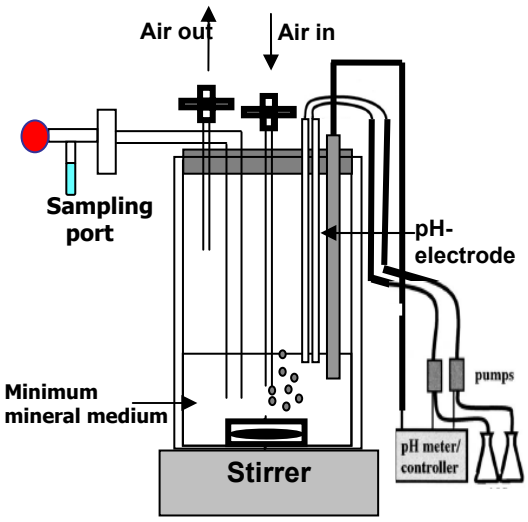

Figure 2.1. Bioreactor used for cell growth

Mn(II) oxidation. The LBB (Leuco Berbelin Blue) assay has frequently been used to measure the rate of Mn(II) oxidation. The color change to blue that occurs as a consequence of the oxidation of LBB by Mn(III,IV) was monitored by measuring absorbance at 628nm (A_{628}) using a Hewlett-Packard 852A Diode Array Spectrophotometer (Palo Alto, CA). A standard curve was constructed using serial dilutions of a 100 μM biogenic Mn-oxide suspension.

Pyruvate assay. Pyruvate was measured by an enzymatic assay using LDH (L-lactic dehydrogenase, Sigma, L-2500), β-NADH (Sigma, 340-110) and a pyruvic acid standard (Sigma: 726-4) according to the instructions of Sigma kit 726. The assay is based on the following reaction:

β-NADH + Pyruvate $\longrightarrow$ Lactate + β-NAD, that is catalyzed by LDH.

The extinction coefficient of β-NADH is 6.22 mM at 340nm. The concentration of pyruvate is determined by equation 1.

$$\text{mM Pyruvate in sample} = (\Delta A \text{ sample} - \Delta A \text{ blank})(3)(df)/(6.22) \quad (1)$$

Where:

3 = total volume of reaction mix

Df = dilution factor

6.22 = millimolar extinction coefficient of β-NADH at 340 nm

Results and Discussion

Stock cultures of *L. discophora* SS-1 (ATCC 43182) obtained from ATCC originally grew in the undefined medium PYG (Peptone-Yeast Extract-Glucose, containing 0.5g/L peptone, 0.5g/L yeast extract, 0.5g/L glucose, 0.6g/L $MgSO_4 \cdot 7H_2O$, 0.07g/L $CaCl_2 \cdot 2H_2O$ and 3.57g/L HEPES((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid))) and in the defined medium MSVP (Mineral Salt-Vitamin-Pyruvate, Table 2.1), but did not grow in MMS (Minimal Mineral Salt) medium, Table 2.1)(7). A series of shake flask experiments were used to determine the growth requirement for components of MSVP medium. Of the individual components of the vitamin solution, only 0.02μM biotin was essential for the growth of *L. discophora* in MSVP. In addition, cells did not grow unless MSVP contained 50μM of phosphate. Therefore MMS was supplemented with 0.02μM of biotin and 50μM of phosphate. I also evaluated the effect of the ionic strength of MMS on cell growth. The original MMS composition included the addition of 3.66g/L $NaNO_3$ to create an ionic strength of 50 mM. I observed that the 50mM ionic strength of MMS limited the growth of cells compared to cells growing in the absence of $NaNO_3$. A possible explanation could be the effect of ionic strength on the activity of the ferric ion and on the amount of ferric ion adsorbed to the cell surface. This effect has previously been reported in research related to the adsorption of heavy metals such as Pb and Cd to the surface of some model gram positive and negative bacteria (5). Therefore I omitted the addition of $NaNO_3$. The ionic strength of the medium without $NaNO_3$ is 11.7 mM. The modified MMS will be referred to as MMS-2 throughout this thesis.

Table 2.1. Compositions of media (*ddH_2O = Milli-Q deionized water)

Component	MMS Concentration in ddH_2O*, mg/L (µM)	MSVP Concentration in ddH_2O*, mg/L (µM)
$CaCl_2 \cdot 2H_2O$	30(200)	60(400)
$MgSO_4 \cdot 7H_2O$	35(140)	60(240)
$(NH_4)_2\ SO_4$	120(910)	240(1820)
KNO_3	15(150)	-----
$NaHCO_3$	0.84(10)	-----
KH_2PO_4	0.7(50)	20(142.86)
$FeSO_4 \cdot 7H_2O$	0.0278 (0.1)	2.78(10)
Na pyruvate	319(2900)	1000(9090)
Biotin	-----	**
Vitamin B_{12}	$2.0331 \cdot 10^{-3}$(0.0015)	**
HEPES	-----	30(10000)
Na_2HPO_4	-----	2383(211)
$NaNO_3$	3660(43 000)	-----

** 1mL of vitamin solution containing (20mg/L Biotin, 20mg/L Folic acid, 50mg/LThiamine . HCl, 50mg/L D-(+)-Calcium pantothenate, 1mg/L VitaminB12, 50mg/L Riboflavin, 50mg/L Nicotinic acid, 100mg/L Pyridoxine hydrochloride, 50mg/L p-Aminobenzoic acid) is added to 1000mL of MSVP

L. discophora SS-1 grows vegetatively in filaments during the logarithmic growth phase and then forms filamentous swarmer cells by the on-set of the stationary phase (2, 3). To insure that aggregation did not cause an error in the determination of cell concentration, cell growth was monitored using OD_{600} (optical density at 600 nm) and by cell dry weight during the logarithmic phase. Cell dry weights were determined by analyzing the total suspended solids in the broth (Standard Method 2540 D,(4)). A linear relationship was obtained between OD_{600} and dry weight of log-phase cells (Figure 2.2). Therefore I concluded that OD_{600} is a good measure of cell growth.

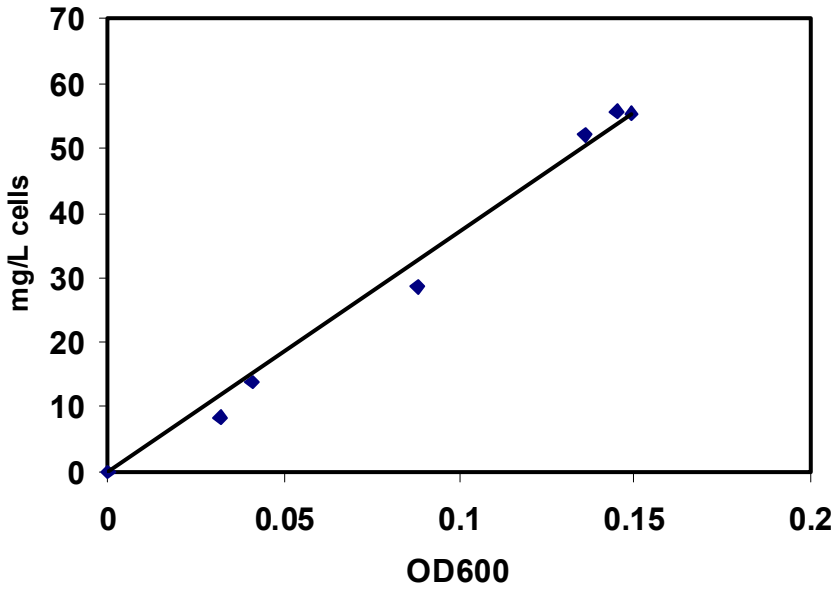

Figure 2.2. OD_{600} versus dry weight of cells in mg/L. The relationship between cell dry weight and OD_{600} is linear and has the slope of 370.72 (R^2=0.98)

Previous research has focused on characterizing Mn(III,IV) oxides and the kinetics of the Mn(II) oxidation process (8). In this research identifying the growth conditions that favor or inhibit the production of extracellular Mn(II) oxidases was of interest. Therefore, I examined cell growth conditions in modified MMS-2. Pyruvate consumption in presence of 2.9mM of added pyruvate was measured. The linear range of the assay is between 0-0.3mM pyruvate as shown in Figure 2.3.

To measure pyruvate consumption by cells; growth medium was separated from cells by filtration (0.2µm syringe filter) and stored at 4°C for two days. A 10 and 20 fold dilution was used when the initial pyruvate concentration in the medium was 3.2mM and 6.4mM, respectively. Pyruvate consumption in presence of 2.9mM of added pyruvate was measured as shown in Figure 2.4A.

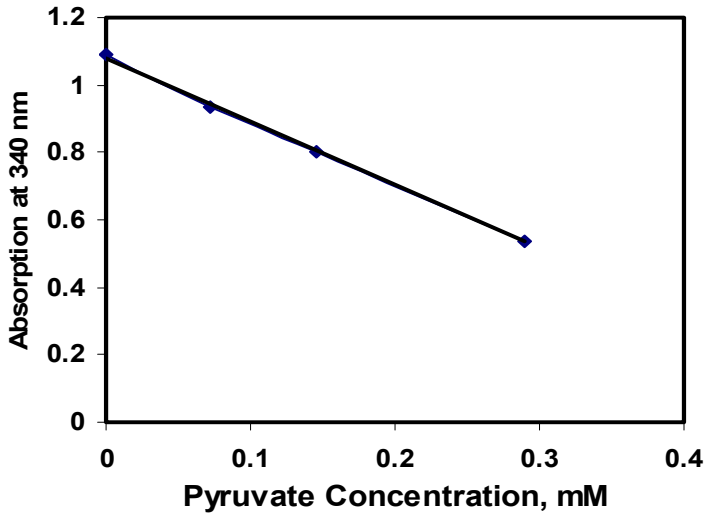

Figure 2.3. Standard curve of the pyruvate assay. The relationship between the absorbance at 340nm and pyruvate concentration is linear to 0.3mM and has the slope of -1.88 and an intercept of 1.08 (R^2= 0.99).

At this pyruvate concentration MMS-2 is carbon-limited. When the concentration of pyruvate was doubled, cell yield did not double. I then doubled the concentrations of phosphate, biotin, vitamin B12, iron ($FeSO_4$*$2H_2O$) and ammonium sulfate to determine the limiting nutrient. Only in the presence of double the concentration of iron, did the cell density double as shown in Figure 2.4B.

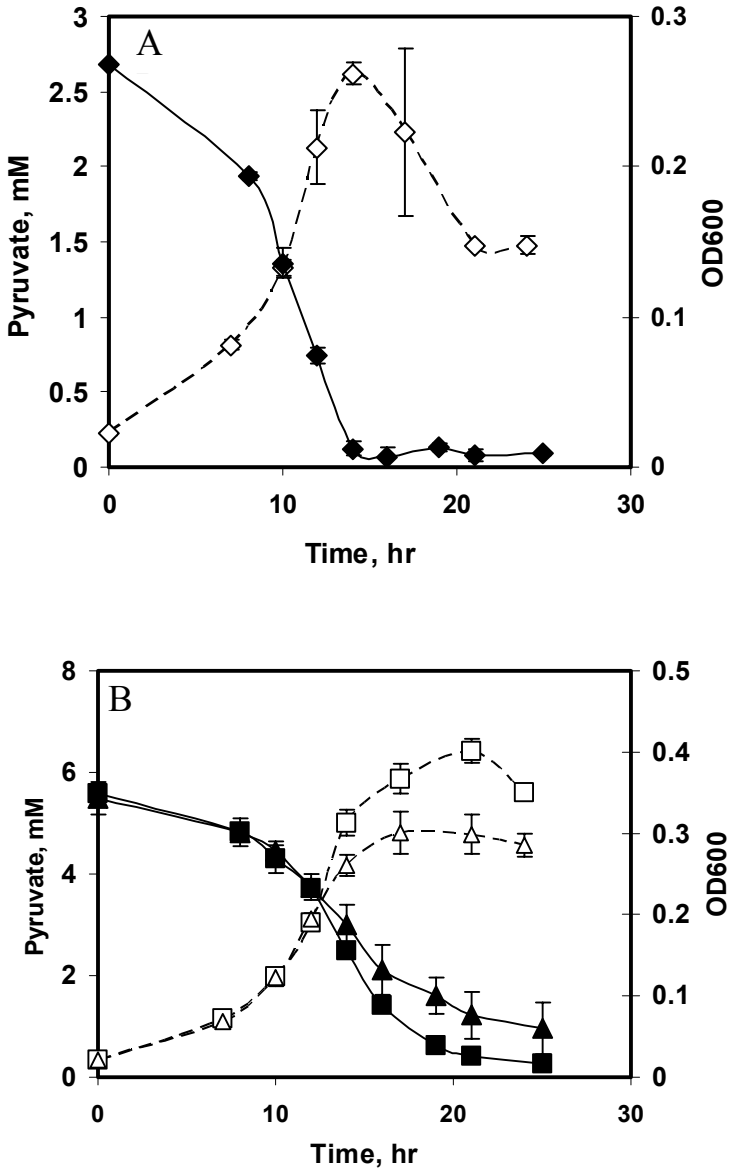

Figure 2.4. Growth (expressed as OD_{600}: open symbols) and pyruvate concentration (closed symbols) of *L .discophora* SS-1 in modified MMS containing A: 2.9mM pyruvate/0.1µM Fe (diamonds). B: 5.8mM pyruvate/0.1µM of $FeSO_4*2H_2O$ (triangles) and 5.8mM pyruvate/0.2µM $FeSO_4*2H_2O$ (squares).

Iron-sufficient and iron-deficient compositions were subsequently used to study the effect of iron limitation on Mn(II) oxidation, as will be described in chapter 3.

The LBB (Leuco Berbelin Blue) assay has been previously used to measure the rate of Mn(II) oxidation. 300µL of the Mn-oxide suspension were mixed with 900µL of LBB (0.04% of LBB in 45mM acetic acid). The color change to blue that occurred as a consequence of the oxidation of LBB by oxidized Mn(III,IV) was monitored by measuring absorbance at 628nm (A_{628}) using a Hewlett-Packard 852A Diode Array Spectrophotometer (Palo Alto, CA). Figure 2.5 shows that the absorbance is linear up to a concentration of 100 µM of MnO_2 and has a slope of 0.0168 (R^2= 0.99) at 628nm.

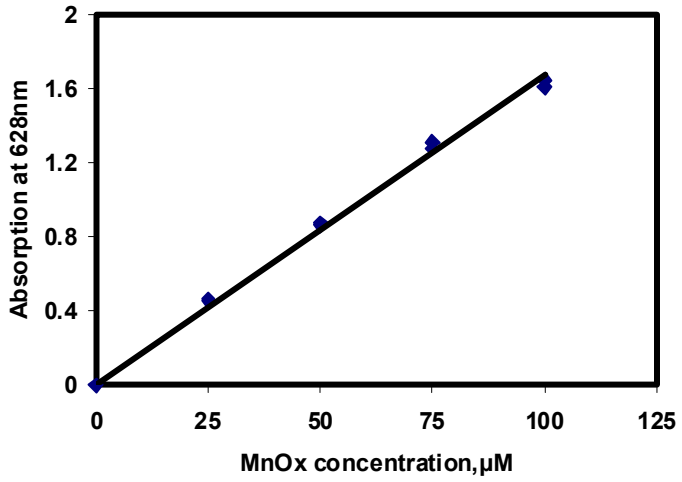

Figure 2.5. Standard curve for the LBB assay. The relationship between the absorbance at 628 nm and the biogenic MnOx concentration is linear to 100µM and has the slope of 0.0168 (R^2= 0.99).

The standard curve was constructed using serial dilutions of a 100 µM biogenic Mn-oxide suspension. In this study the LBB assay was used to measure the Mn(II)-oxidizing activity at different growth stages. Cells were grown in the *absence* of

Mn(II). Samples of the suspended cell culture were aseptically collected and analyzed for their ability to catalyze Mn(II) oxidation. Since Mn(II) can disappear through oxidation or by adsorption to the formed Mn(III,IV) oxides (6, 9), we wanted to quantify the amount adsorbed Mn(II) to ensure that it did not interfere with the assay. Adsorbed Mn(II) was evaluated by adding 4 mL of 10 mM $CuSO_4$ solution to an equal volume of the MnOx as described by Bromfield et al. (6). Cupric ion is expected to displace adsorbed Mn(II) from Mn (III/IV) oxide adsorption sites. In addition, 4 mL of a 10mM Na_2SO_4 solution were added to an equal volume of the suspension as a control. Then the mixtures were centrifuged at 3000xg for 10 min, and finally Mn(II) in the supernatant was measured by atomic absorption spectroscopy using an AAnalyst 100 instrument (Perkin-Elmer, Norwalk, Conn.) using the graphite furnace. Mn(II) in the control was 0.6 μM, while 6.6 μM of Mn(II) was adsorbed to the MnOx. Since adsorbed Mn(II) was less than 7% of the 100μM of added Mn(II), no correction was made for adsorbed Mn(II) in the assay.

As noted above the LBB assay was modified to measure the Mn(II)-oxidizing activity at the different growth stages. Mn(II)-oxidizing activity is reported as $\mu MMnO_x$ formed/(hr·(mL of cell suspension)). $\mu MMnO_x$ /hr is the initial rate (slope) of Mn(II) oxidation calculated from the time point measurements for each assay mixture. It is worth pointing out that the concentration of Mn(II) used in the assay (100μM) to initiate Mn(II) oxidation is high enough to render the rate zero order with respect to Mn(II) and directly proportional to enzyme concentration (2). The assay was validated by diluting a cell suspension and measuring the rate of Mn(II) oxidation by the LBB assay on the diluted and original samples. This was done to prove that the measured activity (initial oxidation rates) of the diluted samples could be correlated to the known dilution ratios of the cell suspension i.e. enzyme concentration as shown in Figure 2.6 and Table 2.2. In addition, the fact that the concentration of oxidized

Mn(III,IV) in each assay mixture increased linearly with time indicates that the Mn(II)-oxidizing factor is neither produced nor degraded during the assay.

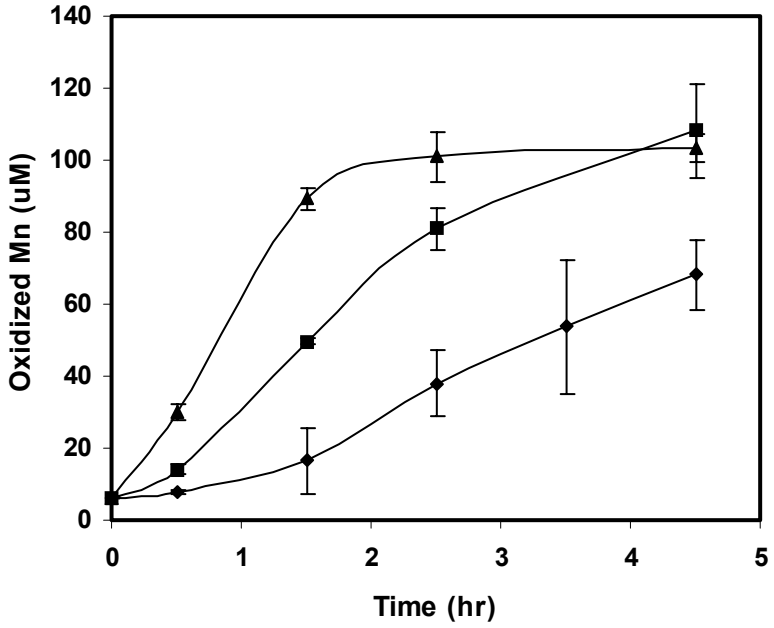

Figure 2.6. LBB assay using diluted cell suspensions at different OD_{600}values. (—▲—) OD_{600}=0.16, (—■—) OD_{600}=0.08 and (—♦—) OD_{600}=0.05.

Table 2.2. Comparison between actual dilution ratios and dilution ratios based on the initial rate of Mn(II) oxidation.

OD_{600}	Actual Dilution Ratio	Slope, μM MnOx/hr	Calculated ratio
0.16	1	56.0, R^2 = 0.99	1
0.08	1.9	31.0, R^2 = 0.99	1.80
0.05	3.0	19.0, R^2 = 0.97	2.94

An in-gel Mn(II)-oxidizing activity assay, as described by Adams et al. (1), was attempted to correlate variation in measured activity levels with changes in expression level. Cell-free supernatant collected from stationary phase cells had to be concentrated at least 700 to 900 fold using ultrafiltration to visualize the two Mn(II)-oxidizing bands. A 4 or 10 fold dilution of the concentrated supernatant renders the band not detectable as shown Figure 2.7. Therefore, I relied solely on the LBB assay to quantify Mn(II)-oxidizing activity. The low abundance of Mn(II)-oxidizing protein(s) is also reported by Dick et al. in a recent paper (7).

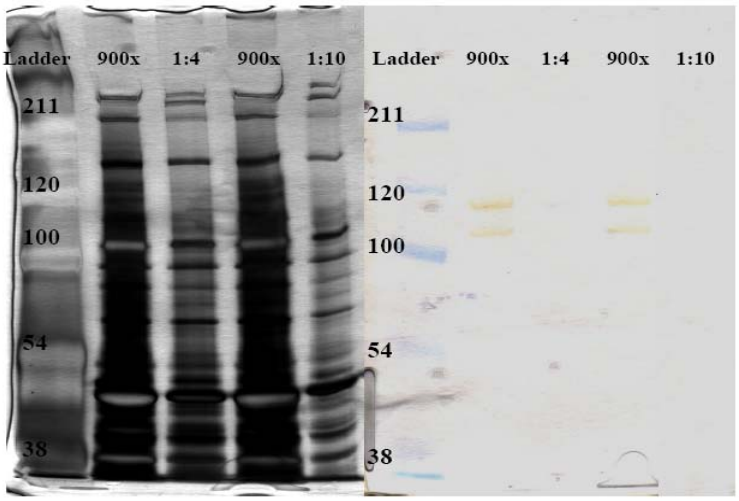

Figure 2.7. SDS-PAGE gel electrophoresis (7.5% gel) of 900-fold concentrated supernatant. The gel was stained with Silver staining (left) and an in-gel Mn(II) oxidation activity assay (right). The left lane is the ladder.

In summary, I have used the batch reactor system described above to grow *L. discophora* SS-1 under different defined growth conditions and evaluated how these conditions affect Mn(II) oxidation rates of the cell suspension.

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CHAPTER 3

IRON REQUIREMENT FOR Mn(II) OXIDATION BY *LEPTOTHRIX DISCOPHORA* SS-1*

Abstract

A common form of biocatalysis of Mn(II) oxidation results in the formation of biogenic Mn(III, IV) oxides and is a key reaction in the geochemical cycling of Mn. In this study, we grew the model Mn(II)-oxidizing bacterium *Leptothrix discophora* SS-1 in media with limited iron (0.1 μM iron/5.8 mM pyruvate) and sufficient iron (0.2 μM iron/5.8 mM pyruvate). The influence of iron on the rate of extracellular Mn(II) oxidation was evaluated. Cultures in which cell growth was limited by iron exhibited reduced abilities to oxidize Mn(II) compared to cultures in medium with sufficient iron. While the extracellular Mn(II)-oxidizing factor (MOF) is thought to be a putative multicopper oxidase, Mn(II) oxidation in the presence of zero added Cu(II) was detected and the decrease in the observed Mn(II) oxidation rate in iron-limited cultures was not relieved when the medium was supplemented with Cu(II). The decline of Mn(II) oxidation under iron-limited conditions was not accompanied by siderophore production and is unlikely to be an artifact of siderophore complex formation with Mn(III). The temporal variations in *mofA* gene transcript levels under conditions of limited and abundant iron were similar, indicating that iron limitation did not interfere with the transcription of the *mofA* gene. Our quantitative PCR results provide a step forward in understanding the regulation of Mn(II) oxidation. The mechanistic role of iron in Mn(II) oxidation is uncertain; the data are consistent with a direct requirement for iron as a component of the MOF or an indirect effect of iron resulting from the limitation of one of many cellular functions requiring iron.

*Adapted from El Gheriany I, Bocioaga D, Hay A, Ghiorse W, Shuler M, Lion L (2009) Iron Requirement for Mn(II) Oxidation by *Leptothrix discophora* SS-1. Appl Environ Microbiol 75:1229-1235.

Introduction

The biological catalysis of Mn(II) oxidation is thought to be responsible for the formation of most naturally occurring insoluble Mn(III, IV) oxides (40, 41) and consequently plays a key role in the biogeochemical cycling of Mn. The resulting biogenic Mn oxides have high adsorptive capacities for toxic metals (16, 17) and can oxidize both natural organic compounds (38) and organic contaminants (36). The binding of transition metals to biogenic Mn oxides can, in turn, greatly affect the phase distributions and residence times of these transition metals in many natural systems (16, 17). An understanding of environmental conditions that favor or inhibit the production of the extracellular enzyme responsible for Mn(II) oxidation will contribute to insight into this important ecological process and is also a prerequisite for the design of any successful technology that uses microorganisms for the production of Mn oxides.

A variety of phylogenetically distinct microorganisms are capable of the extracellular oxidation of Mn(II) (5). Based on the presence of conserved predicted amino acid motifs, the genes that encode putative Mn(II)-oxidizing enzymes (*mofA* in *Leptothrix discophora* (9), *cumA* in *Pseudomonas putida* GB-1 (7), and *moxA* in *Pedomicrobium* sp. strain ACM 3067 (33)) are all thought to produce multicopper oxidases. Recently, further support for the role of putative multicopper oxidases in Mn(II) oxidation has come from the recovery and sequencing of peptides excised from Mn(II)-oxidizing bands in polyacrylamide gel analyses of proteins from three Mn(II)-oxidizing *Bacillus* species (15). Specifically, Mn(II)-oxidizing bands from the exosporia of two of the three *Bacillus* species tested were shown by tandem mass spectrometric analyses to contain peptides with homology to the predicted C terminus of the putative multicopper Mn(II) oxidase MnxG.

Despite this growing body of evidence regarding the role of multicopper oxidases in Mn(II) oxidation, little is known about how the concentrations of different nutrients (e.g., iron, carbon, and nitrogen) or growth conditions such as pH and the oxygen concentration regulate the production of the enzyme(s) which oxidizes Mn(II). Nelson et al. (26) evaluated the minimal growth conditions needed for Mn(II) oxidation and found that the addition of 0.1 μM Fe(II) to the defined minimal mineral salt (MMS) medium used for growth was necessary for the complete oxidation of Mn(II) by *L. discophora* SS-1; however, adding 0.1 μM Fe(II) to stationary-phase cells did not allow complete Mn(II) oxidation.

In the present study, we grew *L. discophora* SS-1 in a controlled-reactor system and evaluated the time courses of Mn(II) oxidation and *mofA* transcript levels in batch cultures of cells with limited and sufficient iron. Parker et al. (30) observed that retarded Mn(IV) formation by iron-starved *P. putida* is a consequence of the binding of the Mn(III) intermediate (42) to the siderophore pyoverdine. Therefore, siderophore production was also evaluated as part of this research.

Materials and Methods

Medium compositions. Solutions and media were prepared with reagent-grade chemicals and Milli-Q deionized water. Glass reactors were silanated using a 5% solution of dimethyldichlorosilane in carbon tetrachloride. The trace iron concentration in the Milli-Q water was evaluated by inductively coupled plasma optical emission spectroscopy (ICP-OES) using quartz-distilled water to prepare standards and was found to be below the level of detection (≤10 ppb). Fe was added to the medium as $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich). We verified the iron concentrations in media containing 0.2 μM (mean ± standard deviation, 0.29 ± 0.03 μM) and 1 μM (0.91 ± 0.01 μM) Fe by ICP-OES. Table 3.1 shows the compositions of the two growth media used in this study. MMS-2 medium was modified from the MMS medium described by Nelson et

Table 3.1. Compositions of media[a]

Component	Concn (μmol/liter = μM) in ddH_2O[a]	
	MMS-2 medium	MSVP-2 medium
$CaCl_2 \cdot 2H_2O$	200	400
$MgSO_4 \cdot 7H_2O$	140	240
$(NH_4)_2 \cdot SO_4$	910	1,820
KNO_3	150	
$NaHCO_3$	10	
KH_2PO_4	50	142.86
$FeSO_4 \cdot 7H_2O$	0.1 or 0.2	10
Na pyruvate	2,900 or 5,800	9,090
Biotin	0.02	0.01
Vitamin B_{12}	0.0015	0.00075
HEPES	2,900	10,000
Na_2HPO_4		211

[a] ddH_2O, distilled deionized water

Table 3.2. Medium compositions and corresponding limitations

Composition	Carbon concn (mM)	Iron concn (μM)	Medium type
A	2.9	0.2	Carbon limited
B	5.8	0.2	Carbon limited
C	5.8	0.1	Iron limited
D	8.7	0.1	Iron limited

al. (26) by increasing the phosphate concentration from 5 to 50 μM and by adding 0.02 μM biotin. The minerals-salts-vitamins-pyruvate (MSVP) medium described by Adams et al. (3, 18) was modified by replacing the complex vitamin solution originally added to the medium with 0.75 nM vitamin B_{12} and 0.01 μM biotin to yield MSVP-2 medium.

Batch growth curves for which both optical densities at 600 nm (OD_{600}) and pyruvate concentrations were measured were generated using cultures in shake flasks with different iron/carbon ratios. Preliminary experiments revealed that in MMS-2 (Table 3.1.), carbon was the growth-limiting nutrient (i.e., the cell yield was limited by the carbon concentration) (24). When the iron/carbon ratio was lower than 0.1 μM Fe to 2.9 mM pyruvate, then iron became the growth-limiting nutrient. Adding 5.8 mM or 11.6 mM pyruvate/0.1 μM Fe did not lead to an increase in maximum cell density unless the $FeSO_4 \cdot 7H_2O$ concentration was increased. Increasing the concentrations of other medium components (e.g., phosphate and ammonium sulfate) did not relieve the growth limitation. In this study, carbon-limited (iron-replete) cultures were grown in media of compositions A and B and iron-limited cultures were grown in media of compositions C and D as defined in Table 3.2.

Bacterial strain and growth conditions. The model manganese-oxidizing organism used for this study was *L. discophora* SS-1 (ATCC 43182), which is a sheathless variant of the gram-negative bacterium *L. discophora* SP-6 (3). Consistent with the results of Emerson and Ghiorse (18), *L. discophora* SS-1 did not grow in MMS-2 medium unless iron was added. Stable growth (at least three transfers) of stock cultures of *L. discophora* SS-1, obtained from the ATCC, in MMS-2 medium could not be achieved. To obtain strains from these cultures that could grow in MMS-2 medium, five transfers in MMS-2 medium supplemented with decreasing concentrations of MSVP-2 medium were needed. Cells that grew in MMS-2 medium

were kept at –80°C in cryogenic preservation. Before every experiment, cells were removed from the cryopreservation and were allowed to grow for 5 days on MSVP-2-MMS-2 (1:1-ratio) agar plates containing 50 μM Mn(II) as an aid to visualize the colonies. Colonies from the MMS-2-MSVP-2 plates were inoculated into 50 ml of liquid medium (44 ml of MMS-2 medium, 6 ml of MSVP-2 medium) in 250-ml flasks. These cultures were allowed to grow at 26°C with rotary shaking at 150 rpm by an Innova 2000 instrument (New Brunswick Scientific, Edison, NJ) and were transferred into 50-ml volumes of MMS-2 medium (10% inoculum). These shake flask cultures were used as inocula for bioreactors. Each reactor contained 450 ml of sterilized MMS-2 medium and was inoculated with 50 ml of late-logarithmic-phase cells (OD_{600} = 0.17 ± 0.04 [mean ± standard deviation]). Experiments were performed in 2-liter magnetically stirred jacketed vessels at temperatures of 25 to 27°C (43). The working volume in each reactor was 25%. Glass autoclavable pH probes (designation 405-DPAS-SC-K8S/200; Mettler Toledo) were installed in each reactor and connected to individual pH controllers (pH-oxygen reduction potential controller model no. 3676; Jenco Electronics, Ltd.) to maintain the pH at 7.0 ± 0.1 by the automatic addition of 0.01 N HNO_3 and 0.01 N NaOH. The pH probes were sterilized with 70% ethanol (pH = 2) and installed aseptically after the reactor and its heat-stable components had been autoclaved. After autoclaving, filter-sterilized pyruvate, phosphate, vitamin B_{12}, and fresh $FeSO_4 \cdot 7H_2O$ were added to give the desired concentrations. The bioreactors were aerated at a constant flow rate of 0.8 standard cubic ft per h with air that had passed through a 0.2-μm-pore-size polytetrafluoroethane filter (Pall Life Sciences, MI). Autoclavable oxygen electrodes (model no. EW-05643-02; Cole-Parmer Instrument Co.) were used in this system in our preliminary experiments. The oxygen level was found to be stable at 98% saturation during the initial growth stage. The lowest oxygen level was 92% ± 4% saturation, measured during the late exponential growth phase.

L. discophora SS-1 was grown at pH 7.0 ± 0.1 by using 0.8 standard cubic ft of filtered air per h to provide sufficient oxygen so that pH and dissolved-oxygen effects on the production of the enzyme(s) responsible for Mn(II) oxidation were minimal.

Mn(II)-oxidizing activity assay. Cells were grown in the absence of Mn(II). Samples of the suspended cell culture were aseptically collected and analyzed for cell growth and for Mn(II) oxidation. The Mn(II)-oxidizing activity was evaluated using the cell suspension, since the use of the cell suspension was found to give a better estimate of total activity than the use of the cell-free supernatant. Enzymatic Mn(II)-oxidizing activity was quantified using the Leuco Berbelin blue (LBB) colorimetric assay (4). Activity was determined immediately after taking 1 ml from the reactor culture medium by adding 7.5 ml of sterilized MMS-2 medium (pH 7.2 ± 0.05; without Fe, pyruvate, phosphate, vitamin B_{12}, and biotin) and 1 ml of 150 mM HEPES buffer solution (pH 7.3). Mn(II) oxidation in the buffered solution was initiated by adding 0.5 ml of 2 mM $MnSO_4 \cdot H_2O$ solution. The pH of the final buffered solution was 7.2 ± 0.1, and the initial Mn(II) concentration was 100 μM. Samples of 300 μl of the assay mixture were collected every 15 to 30 min and were mixed with 900 μl of an LBB solution (0.04% LBB in 45 mM acetic acid). The color change to blue that occurred as a consequence of the oxidation of LBB by oxidized Mn(III) and Mn(IV) was monitored by measuring the absorbance at 628 nm (A_{628}) using a model 852A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). The relationship between A_{628} and the biogenic MnO_x concentration (where x is 2 or 3) is linear to 100 μM MnO_x, and the line has a slope of 0.017 ($R^2 = 0.99$). Using 100 μM Mn(II) in the assay makes the enzymatic oxidation reaction zero order with respect to the Mn(II) concentration and first order with respect to the concentration of the active Mn(II)-oxidizing factor (data not shown). Mn(II) oxidation is reported as the micromolar concentration of MnO_x formed per h·per milliliter of the cell suspension. The

micromolar concentration of MnO_x formed per h is the initial rate (slope) of Mn(II) oxidation calculated from the time point measurements for each assay mixture. The fact that the concentrations of oxidized Mn(III, IV) in each assay mixture increased linearly with time indicates that the Mn(II)-oxidizing factor was neither produced nor degraded during the assay. The activity was not normalized to the total extracellular protein concentration, since the protein concentration was below the detection limit (1 μg/ml) of the Bradford protein assay (microassay protocol) and the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA), even in supernatant that had been concentrated 200-fold (data not shown).

Consistent with previous results (27, 37), we found that pyruvate reduced the observed rate of Mn(II) oxidation, so experimental conditions were constrained to initial pyruvate concentrations at which the residual pyruvate (if present) had no more than a 10% effect on Mn(II) oxidation.

Growth-related iron effects were distinguished from the possibility that iron was limiting the activities of enzymes already present by measuring the rate of Mn(II) oxidation in culture fluids from stationary-phase cells. In these tests, different amounts of $FeSO_4 \cdot 7H_2O$ were allowed to equilibrate with the assay mixture for 1 h before the Mn(II) solution was added. The measured activity was compared to the activity of a control in which no $FeSO_4 \cdot 7H_2O$ was added to the assay mixture. To verify that Fe(II) did not interfere with the oxidation of LBB by Mn(III) and Mn(IV), Mn(II) oxidation was also assessed using a turbidity measurement (2, 23), which is a direct measurement of the formation of the fine suspension of yellow or light brown manganese oxides.

Siderophore assay. A universal chemical assay for siderophores (35) was applied to check for possible siderophore production by iron-limited cells. A shuttle solution was prepared by adding 5-sulfosalicylic acid (Sigma-Aldrich, St. Louis, MO) to a ternary-

complex [chromeazurol S-Fe(III)-hexadecyltrimethylammonium bromide] solution at a concentration of 4 mM to accelerate iron transfer. The presence of siderophores was determined by adding 0.5 ml of cell-free supernatant to 0.5 ml of the shuttle solution. After 2 h, the A_{630} was measured spectrophotometrically. Deferoxamine mesylate salt (Sigma) was used as a standard ($A_{630}/A_{\text{blank}} = -0.056x + 0.99$, where A_{blank} is the absorbance of a blank (without deferoxamine mesylate) and x represents the siderophore concentration [μM]; $R^2 = 0.99$). The detection limit for the deferoxamine mesylate siderophore by this method was found to be 0.8 μM, which is in agreement with previously reported results (22).

Nucleic acid extraction. At selected time intervals, 1-ml aliquots of cell suspensions were placed into centrifuge tubes and centrifuged at 21,000 x *g* and 4°C for 20 min. The supernatants were discarded, and the pellets were stored at –80°C and used for the extraction of RNA and DNA. DNA and RNA extractions were performed using the UltraClean microbial DNA isolation kit (Mo Bio Labs, Carlsberg, CA) and the RNeasy minikit (Qiagen, Valencia, CA). RNA samples were treated using the on-column RNase-free DNase I (Qiagen, Valencia, CA) digestion protocol to remove any contaminating DNA. The RNA concentration was quantified using the RNA 6000 Nano assay on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNA concentration in each sample was diluted to 25 ng/μl, and this concentration was used in subsequent steps.

Quantitative reverse transcriptase PCR (qRT-PCR). A second DNase treatment step was performed using RQ1 RNase-free DNase (Fisher Scientific, Rockville, MD) before cDNA synthesis. cDNA synthesis reactions were performed with random hexamer primers using the iScript cDNA synthesis kit according to the instructions of the manufacturer (Bio-Rad, Hercules, CA). Reactions were performed with 20-μl solutions containing 5.2 ng/μl of RNA. Quantitative PCR amplifications from

triplicate samples were performed using an iCycler real-time PCR machine (Bio-Rad, Hercules, CA). Copies of *mofA* (GenBank accession no. Z25774) were quantified by the amplification of 3 ng of cDNA with iQ SYBR green supermix (Bio-Rad, Hercules, CA) and 17.5 pmol of *mofA*-specific primers. The primer set was designed by the software package Beacon Designer 4 (Biosoft International) to amplify 127 bp (bp 2848 to 2974) of *mofA*. The sequences of the forward and reverse primers were 5'-TCA-CAC-CAT-CGG-CGT-CAC-3' and 5'-CGG-CAG-CAC-CTT-GTT-CAG-3', respectively. BLAST analysis and PCR amplification were used to confirm primer specificity. PCR amplifications were carried out with the following parameters: 2 min at 50°C, 3 min at 95°C, and 40 cycles of 1 min at 60°C and then 1 min at 95°C. Melting-curve analyses were performed after all runs to check the purity of the amplicon. Melting-curve analysis was also used to screen for primer dimers. cDNA target amplification was compared to DNA standards obtained by the serial dilution of genomic DNA. Original fluorescence data were analyzed by using the DART (data analysis for real-time PCR) method and adjusting for PCR efficiency differences (31, 34).

Results

Carbon-limited cultures. The time courses of extracellular Mn(II) oxidation by *L. discophora* SS-1 cells grown in two carbon-limited media are shown in Fig.3.1. The media contained the same initial $FeSO_4 \cdot 7H_2O$ concentration (0.2 μM) but different initial pyruvate concentrations (2.9 mM [composition A] and 5.8 mM [composition B]). Mn(II) oxidation was first detected during logarithmic growth phase (Fig. 3.1). The highest level of Mn(II) oxidation was measured after cells reached stationary phase. When cells were grown with the additional carbon available in composition B, the cell density increased by 85% while the maximum observed Mn(II) oxidation rate increased by 100% compared to those for cells grown in composition A.

Adding relatively higher amounts of $FeSO_4 \cdot 7H_2O$ (1 μM) did not affect cell growth but led to a small but statistically significant reduction in measured Mn(II) oxidation rates (Fig. 3.2). To determine if iron was causing growth-related effects on the observed rate of Mn(II) oxidation or merely interfering with the Mn(II) oxidation assay, the assay was performed with cultures to which iron had been added after growth cessation. Figure 3.3 demonstrates that the presence of Fe(II) in excess of 0.2 μM reduced the observed rate of Mn(II) oxidation, so the iron concentrations in all additional experiments were limited to no more than 0.2 μM.

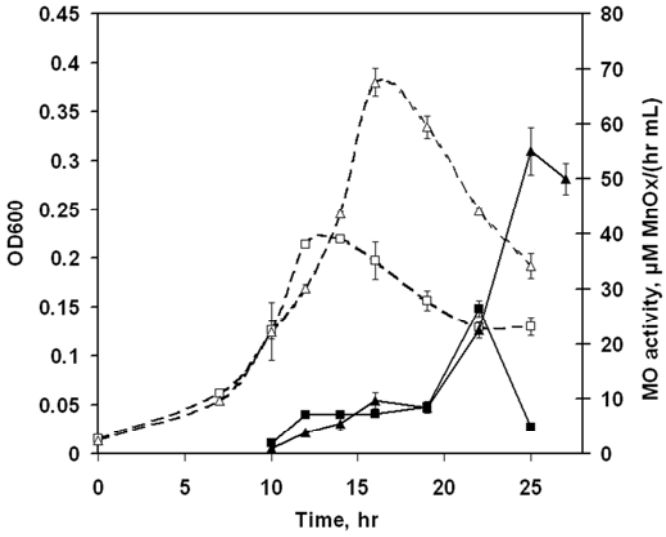

Figure 3.1. Growth (expressed as OD_{600}; open symbols) and extracellular Mn(II)-oxidizing (MO) activity (closed symbols) of *L. discophora* SS-1 cells in carbon-limited media. Media were as follows: composition A, 0.2 μM Fe-2.9 mM pyruvate (squares), and composition B, 0.2 μM Fe-5.8 mM pyruvate (triangles). Values for compositions A and B are means ± standard deviations for triplicate and duplicate cultures, respectively.

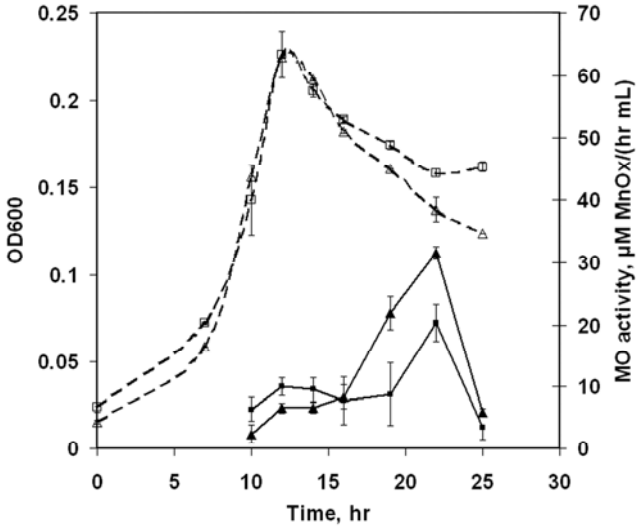

Figure 3.2. Growth (expressed as OD_{600}; open symbols) and extracellular Mn(II)-oxidizing (MO) activity (closed symbols) of *L. discophora* SS-1 cells in carbon-limited media. Media were as follows: 1 µM Fe/2.9 mM pyruvate (squares) and 0.1 µM Fe/2.9 mM pyruvate (control; triangles). The values are means ± standard deviations for duplicate cultures.

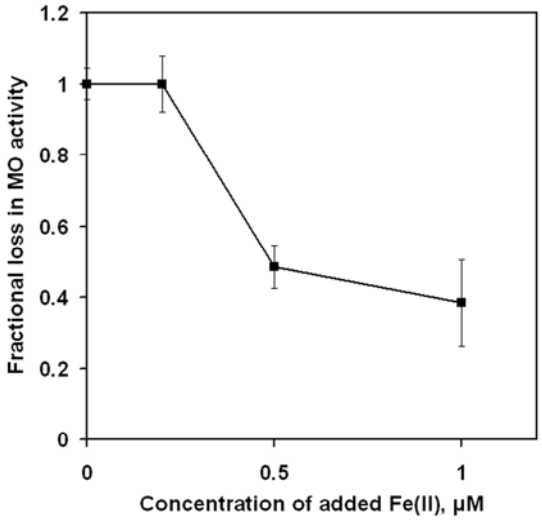

Figure 3.3. Effect of Fe (II) on the observed extracellular Mn(II)-oxidizing (MO) activity. Values shown are means ± standard deviations calculated for triplicate samples.

Iron-limited cultures. Reactor experiments were conducted using compositions C and D to study the effect of iron limitation on the time course of Mn(II)-oxidizing activity. Composition B was used as a control. The cell yields in compositions B, C, and D were similar, although the excess pyruvate in composition D did seem to forestall the onset of cell death (Fig.3.4). In composition D, the residual pyruvate concentration was 3.50 ± 0.11 mM at 17 h, confirming that Fe limitation affected cell yield. Iron-limited cells (those grown in compositions C and D) exhibited decreased abilities to oxidize Mn(II) compared to cells with sufficient iron (those grown in composition B), as shown in Fig.3.4. Reduced rates of Mn(II) oxidation in cell-free (filtered) supernatant were also measured. These results suggest that iron deficiency is the cause of either (i) a decline in the production of the enzyme(s) responsible for Mn(II) oxidation, (ii) reduced activity of the produced enzyme, or (iii) reduced ability

to detect Mn(II)-oxidizing activity because of the secretion of factors to counter iron limitation, such as siderophores. To address the third of these possibilities, the spent cell-free supernatants of cultures in compositions B, C, and D were tested for the presence of siderophores by using the chromeazurol S universal siderophore assay. No siderophores (≥0.8 μM) were detected in the supernatants at any time point.

To address the first possibility and determine if iron limitation was exerting transcriptional control over the production of the Mn(II)-oxidizing factor, we measured *mofA* mRNA levels. The time courses of *mofA* transcript levels in the batch cultures are shown in Fig.3.5. Transcript levels at 11 h were not significantly higher than those at 14 and 17 h ($P = 0.99$). A peak in *mofA* transcript abundance at 21 h correlates with the peak in observed Mn(II)-oxidizing activity .

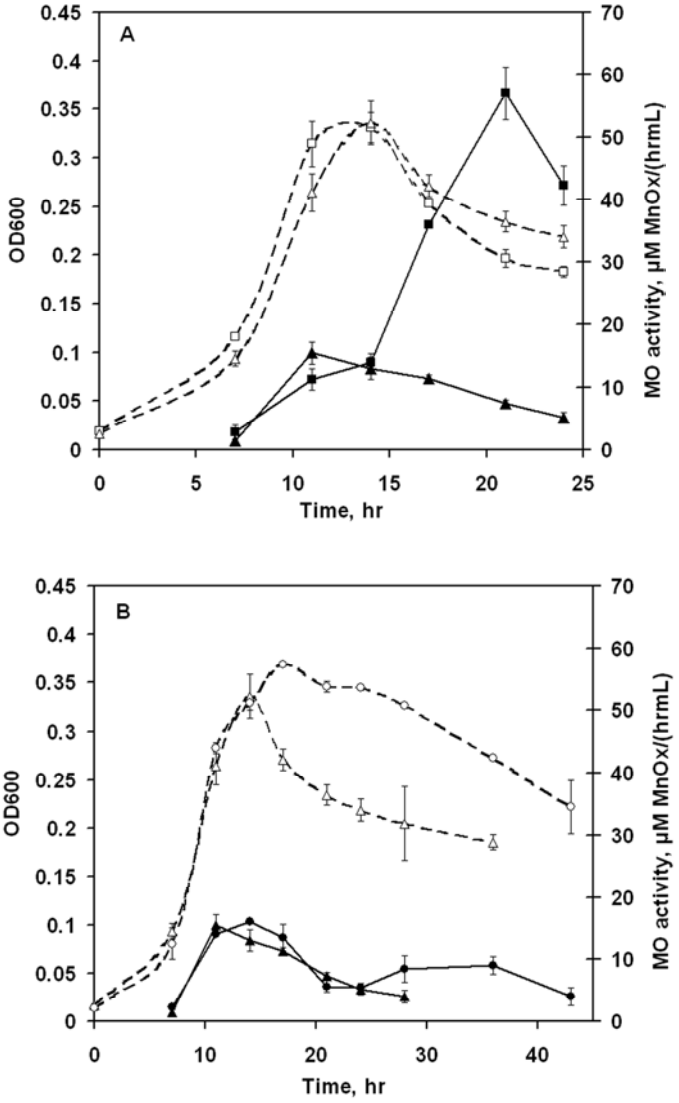

Figure 3.4. Growth (expressed as OD_{600}; open symbols) and extracellular Mn(II)-oxidizing (MO) activity (closed symbols) of *L. discophora* SS-1. (A) Cells were grown in iron-limited medium (composition C), with cells grown in composition B as a control. (B) Cells were grown in two iron-limited media (compositions C and D). Media were as follows: composition B, 0.2 µM Fe/5.8 mM pyruvate (squares); composition C, 0.1 µM Fe/5.8 mM pyruvate (triangles); and composition D, 0.1 µM

Fe/8.7 mM pyruvate (circles). The values for compositions B and D are means ± standard deviations for duplicate cultures. The values for composition C are means ± standard deviations for triplicate cultures.

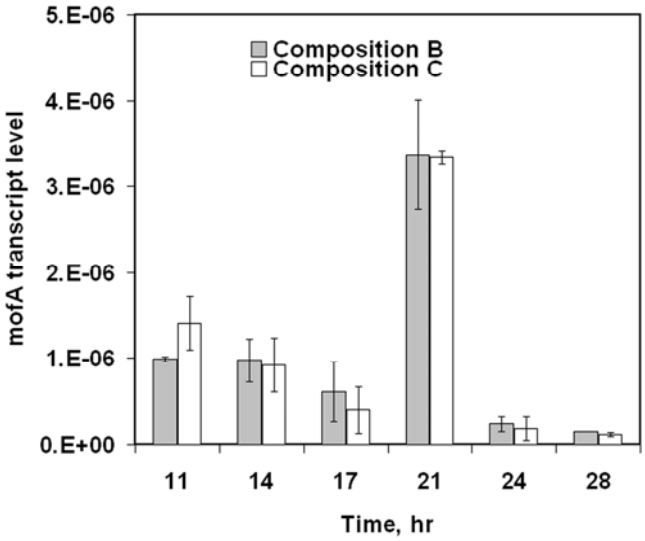

Figure 3.5. qRT-PCR results for RNA extracted from *L. discophora* SS-1 cells grown in the iron-limited (white bars) and replete (gray bars) cultures represented in Fig.3.4A. The values for compositions B (with sufficient iron) and C (with limited iron) are means ± standard deviations for duplicate and triplicate cultures, respectively. Transcript levels are expressed as numbers of transcripts per 104 ng of RNA.

While the extracellular Mn(II)-oxidizing factor is thought to be a putative multicopper oxidase, Mn(II) oxidation in the presence of zero added Cu(II) was observed. The decrease in the observed Mn(II) oxidation rate was not relieved when iron-limited cultures were supplemented with 0.02 or 0.04 μM Cu(II) (Fig.3.6). Consistent with previous results (6), we did observe the stimulation of Mn(II)

oxidation if these concentrations of Cu were added to cells with sufficient Fe (data not shown).

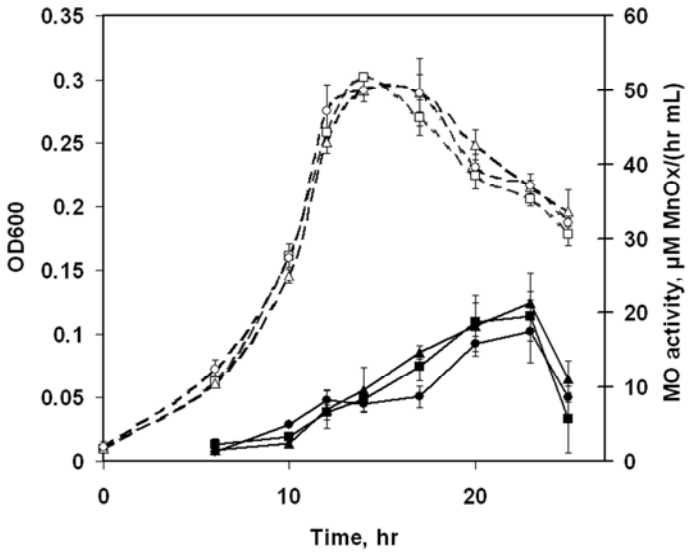

Figure 3.6. Growth (expressed as OD_{600}; open symbols) and extracellular Mn(II)-oxidizing factor (MOF) activity (closed symbols) of *L. discophora* SS-1 in iron-limited media supplemented with 0.02 and 0.04 µM Cu(II). Media were as follows: 0.1 µM Fe/5.8 mM pyruvate (composition C; squares), composition C with 0.02 µM Cu(II) (triangles), and composition C with 0.04 µM Cu(II) (circles). The values for composition C with 0.02 µM Cu(II) are means ± standard deviations for triplicate cultures. The values for composition C and composition C with 0.04 µM Cu(II) are means ± standard deviations for duplicate cultures.

Discussion

L. discophora SS-1 is a gram-negative heterotrophic bacterium that has the unique feature of producing two distinct extracellular macromolecules that catalyze the oxidation of Fe(II) and Mn(II) (10, 12). This bacterium is recalcitrant to genetic manipulation, and less is known about the molecular mechanism of Mn(II) oxidation in

this organism than about those in other Mn(II)-oxidizing bacteria such as *Pseudomonas*, *Pedomicrobium*, and *Bacillus* species (21, 40).

Our experimental results suggest that a threshold Fe-to-pyruvate ratio (approximately 0.1 μM $FeSO_4 \cdot 7H_2O$/2.9 mM pyruvate) is required for optimal Mn(II)-oxidizing activity and that the presence of more than 0.2 μM iron in the assay mixture leads to a decrease in observed Mn(II)-oxidizing activity. This result is consistent with prior reports of a decline in measurable Mn(II)-oxidizing activity in the presence of other transition metals such as Cu, Zn, Ni, and Co (1). The effect of excess Fe(II) on Mn(II) oxidation may result from a metal-catalyzed oxidative attack on active-site residues (28) or from competitive inhibition, given the similar sizes of Fe(II) (atomic mass, 55.8 U) and Mn(II) (atomic mass, 54.9 U). When the Fe concentration was less than the required threshold concentration, we observed a decline in Mn(II)-oxidizing activity. One previously reported reason for the reduction of Mn(II) oxidation under iron-limiting conditions is the presence of a Mn(III)-binding siderophore, such as pyoverdine, which has been shown to preferentially bind Mn(III) and retard Mn(IV) oxide formation (29, 30). This did not appear to explain our observations, however, as a common siderophore detection assay did not detect the production of any siderophore (indicating that the siderophore level was <0.8 μM) from *L. discophora* SS-1 under iron-limited conditions.

Another possible explanation for the reduced Mn(II)-oxidizing activity is that Fe deficiency interfered with processes requiring Cu. Diverse organisms use copper in high-affinity iron uptake systems induced during iron starvation (25, 39). Maldonado et al. (25) reported that iron-limited cells had a higher copper requirement than iron-replete cells, which may explain the connection between Fe limitation and Mn(II)-oxidizing activity since MofA is predicted to require Cu. However, we found decreased Mn(II)-oxidizing activity even when iron-starved cells were supplemented

with Cu(II) (Fig.3.6). Therefore, we conclude that the decrease of Mn(II)-oxidizing activity was specifically related to iron deficiency and that Fe is indispensable for optimal Mn(II) oxidation by *L. discophora* SS-1.

Diverse bacteria have been found to respond to iron starvation by repressing genes encoding iron-requiring products and inducing genes related to iron acquisition (11, 32). Therefore, we evaluated levels of transcripts of *mofA*, which is thought to encode the *Leptothrix* Mn(II)-oxidizing factor, in cells growing in compositions B and C (Fig.3.5). We found that the peak of Mn(II) oxidation coincided with the highest levels of *mofA* transcript accumulation but that *mofA* transcript levels were unaffected by iron limitation. This result suggests that Fe does not play a role in determining the *mofA* transcription level. Our RT-PCR results are consistent with the recent analysis (14) of the genome of a marine Mn(II)-oxidizing alphaproteobacterium. In silico analysis revealed that the regulation of the Mn(II) oxidase in that bacterium was unlikely to be metal dependent. However, the possibility that there may be either posttranscriptional or posttranslational control of MofA production or activity by iron cannot be excluded. Interestingly, the *mofA* transcript abundance did not reveal the same gradual increase that was observed in the Mn(II) oxidation activity. The reason behind this discrepancy is unclear and requires further investigation.

Performing an in-gel activity assay to provide more quantitative measures of the concentrations of the Mn(II)-oxidizing factor in iron-limited and iron-replete cultures was complicated by the low concentration of the Mn(II)-oxidizing enzyme(s). Cell-free supernatant collected at 22 h from cells grown in composition B needed to be concentrated at least 700-fold (via ultrafiltration with 10-kDa-molecular-mass-cutoff filters) in order to allow the detection of Mn(II)-oxidizing bands (1, 4). In fact, even a 4- or 10-fold dilution of that concentrated supernatant rendered the bands undetectable (data not shown) and precluded the routine use of in-gel assays for monitoring the

Mn(II)-oxidizing enzyme(s). *mofA* was originally identified by Corstjens et al. (9) after they screened an expression library of *L. discophora* SS-1 with antibodies raised against Mn(II)-oxidizing bands purified by sodium dedocyl sulfate-polyacrylamide gel electrophoresis followed by in-gel Mn(II) oxidation. However, the role of *mofA* in enzymatic Mn(II) oxidation in *L. discophora* SS-1 has never been unequivocally determined because of the recalcitrance of *Leptothrix* to genetic manipulation. Despite the fact that the involvement of multicopper oxidases seems to be a common feature of microbial manganese oxidation (7, 19, 33, 43), no bacterial Mn(II) oxidase has been purified in quantities sufficient for detailed biochemical study. In addition, no multicopper oxidase gene thought to encode a Mn(II) oxidase has ever conferred heterologous expression of an active Mn(II)-oxidizing enzyme (5, 40). The sequence of *mofA* contains regions that are common to multicopper oxidase genes and likely encodes at least part of the Mn(II)-oxidizing system in *L. discophora*.

Assuming that *mofA* codes for a component of the Mn(II)-oxidizing factor, it is also possible that Fe(II) influences the production of the active Mn(II)-oxidizing enzyme directly by serving as a cofactor of either MofA or an accessory protein, e.g., MofC, a protein with a predicted heme-binding motif specific for *c*-type cytochromes. The speculation of a role for MofC in Mn(II) oxidation by *L. discophora* SS-1 is due solely to the arrangement of *mofA* and *mofC* (6) in an operon. In *P. putida* (8, 13), active Mn(II) oxidation is inhibited by the disruption of the cytochrome *c* maturation operon. In *Aurantimonas* sp. strain SI85-9A1, *c*-type cytochrome-encoding genes were found in close proximity to both copies of the putative Mn(II) oxidase gene, suggesting that these genes may be functionally related (14). A requirement for accessory proteins is supported by the observation that *cumA*, the gene encoding a putative multicopper oxidase, is found in both Mn(II)-oxidizing and nonoxidizing

Pseudomonas strains (20). Thus, in *Pseudomonas* strains, CumA is obviously not the only protein needed for Mn(II) oxidation.

Despite the uncertainties regarding the exact nature of the Mn(II)-oxidizing factor in *L. discophora* SS-1, we have shown that Fe(II) levels influence the Mn(II)-oxidizing activity. The recalcitrance of *L. discophora* SS-1 to genetic manipulation constrains efforts to unequivocally define the mechanism it employs in Mn(II) oxidation. Thus, our ongoing research is directed toward developing genetic tools to study the molecular mechanism of Mn(II) oxidation by *L. discophora* SS-1.

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CHAPTER 4

AN UNCERTAIN ROLE FOR Cu(II) IN STIMULATING Mn(II) OXIDATION BY *LEPTOTHRIX DISCOPHORA* SS-1*

Abstract

In an effort to improve understanding of the role of Cu(II) in bacterial Mn(II) oxidation, a model Mn(II)-oxidizing bacterium, *Leptothrix discophora* SS-1, was grown in presence of toxic and non-toxic concentrations of Cu(II), Cd(II) and Mn(II). Enhanced Mn(II)-oxidizing activity was observed only when cells were grown in the presence of addedCu(II) (0.05μM to 0.18μM). Activity increased two fold at 0.18μM Cu(II). Comparison of transcript levels of the multicopper oxidase *mofA* gene in the presence and absence of added Cu(II) do not indicate a statistically significant change in *mofA* transcript levels in cultures supplemented with Cu(II). The lack of stimulation by toxic levels of Cd(II) challenges the hypothesis that Mn(II) oxidation is a response to metal toxicity. Stimulation by Cu(II) confirms the specific role of Cu(II) in Mn(II) oxidation. Thus, the exact role of Cu(II) in Mn(II) oxidation and its affect on *mof*A gene expression remain uncertain.

*The contents of this chapter have been submitted for publication with coauthors D. Bocioaga, A. Hay, W. Ghiorse, M. Shuler and L. Lion.

Introduction

Mn(III, IV) oxides greatly affect the biogeochemistry and the fundamental cycling process of many toxic and essential elements (Ghiorse 1984; Tebo et al. 2004). These highly reactive mineral phases are strong adsorbents of heavy metals and participate in a wide range of redox reactions (Stone 1987; Sunda and Kieber 1994; Nelson et al. 1999; Dong et al. 2000). At circumneutral pH values, the rate of formation of biologically formed Mn(III,IV) oxides is several orders of magnitude higher than the rate of chemical oxidation (Nealson et al. 1988; Tebo 1991; Wehrli et al. 1995). It is now widely accepted that Mn(II) oxidation kinetics are microbially mediated in aerobic natural waters (Tebo et al. 2004).

Bacterial Mn(II) oxidation was found to be catalyzed by proteins containing conserved copper-binding motifs that are characteristic of multicopper oxidases e.g. MoxA in *Pedomicrobium* sp. ACM 3067 (Ridge et al. 2007) and MnxG in *Bacillus* spores SG-1 (van Waasbergen et al. 1996; Francis et al. 2002; Dick et al. 2008b) and by heme-containing peroxidases (Anderson et al. 2009). In *Leptothrix discophora* SS-1, *mof*A is thought to encode for the Mn(II)-oxidizing factor. This gene was originally characterized by Crostjens et al. (1997) after screening an expression library of *L. discophora* SS-1 with antibodies raised against Mn(II)-oxidizing bands purified by SDS-PAGE electrophoresis. The role of the putative MofA protein in Mn(II) oxidation is uncertain, because the Mn(II)-oxidizing bands detected in activity assays (110 kDa and 116 kDa on SDS-PAGE gels (Adams and Ghiorse 1987; Corstjens et al. 1997)) are much smaller than the predicted amino acid sequence of *mofA* (180 kDa, (Corstjens et al. 1997)) and because no Mn(II) oxidation activity has ever been detected in heterologously expressed MofA (Corstjens et al. 1997).

Stimulation of Mn(II)-oxidizing activity was observed when Cu(II) was added to the growth medium of both *Pseudomonas putida* GB-1 (Brouwers et al. 1999) and *L.*

discophora SS-1 (Brouwers 2000; Zhang et al. 2002). In the case of *Pseudomonas putida* GB-1, the ability of metals to enhance Mn(II) oxidation was specific to Cu(II). The specificity of Mn(II) stimulation by Cu(II) in *L. discophora* SS-1 was not demonstrated, because the other metals tested (Ni(II) and Zn(II)) were added at cytotoxic concentrations (Brouwers, 1999, 2000). Furthermore, it has not been determined whether the reported increase in Mn(II) oxidation was a result of Cu(II)-enhanced folding of the multicopper oxidase (Wilson et al. 2004), the upregulation of the gene encoding for the Mn(II) oxidase, or some other effect. Thus, the exact role of Cu(II) in Mn(II) oxidation is uncertain.

The first aim of the research reported here was to determine whether the stimulation of Mn(II) oxidation by Cu(II) in *L. discophora* SS-1 is solely due to the presence of Cu(II) or whether it is an indirect response to Cu(II) toxicity. The underlying hypothesis for the latter possibility is that as biogenic Mn(III,IV) oxides are formed, they would adsorb the toxic metal and hence reduce its inhibiting effects on growth (Adams and Ghiorse 1985). In fact, this remains as one of the possible advantages of Mn(II) and Fe(II) oxidation by *L. discophora* SS-1 which does not appear to gain energy from these oxidation reactions. A second goal was to examine whether or not the increase in Mn(II)-oxidizing activity correlated with changes in abundance of the transcript of the gene purported to encode that activity (*mofA*).

Materials and Methods

We grew the Mn(II)-oxidizing bacterium *L. discophora* SS-1 in a series of parallel 2L continuously stirred batch reactors as described by El Gheriany et al. (2009) using the minimal mineral salt medium MMS-2 containing 0.2 μM of $FeSO_4 \cdot 7H_2O$ and 2.9 mM of pyruvate. The pH was maintained at 7.0±0.1 by the automatic addition of 0.01N HNO_3 and 0.01N NaOH. The bioreactors were aerated

with filtered air (0.2μm PTFE filter, Pall Life Sciences, MI) at constant flow rate of 0.8 SCFH (standard cubic foot per hour).

The toxicity of Mn(II), Cd(II) and Cu(II) in MMS-2 was evaluated using shake flask cultures at concentrations ranging from 0.05 to 5 μM. Cu(II), Cd(II) and Mn(II) were added as $CuSO_4 \cdot 5H_2O$; $CdSO_4$ and $MnSO_4 \cdot H_2O$ respectively. Inductively coupled plasma-atomic emission spectroscopy (ICP-OES) analysis of the standards prepared using quartz distilled water revealed the medium contained 0.16±0.06 μM Cu(II) after the nominal addition of 0.18 μM Cu(II).

Samples of the reactor cell culture were aseptically collected and the optical density at 600 nm (OD_{600}) was measured. Extracellular Mn(II)-oxidizing activity was assayed using the LBB (Leuco Berbelin Blue)(El Gheriany et al. 2009). The transcript level of *mofA* in cells with and without exogenously added Cu(II) was also measured using quantitative reverse transcriptase PCR (QRT-PCR) as described by El Gheriany et al. (2009).

The metal co-factor stripping and reconstitution procedure described previously by Larsen et al. (1999) for analysis of the Mn(II) oxidase of *Pedomicrobium* sp. was used to test the role of Cu(II) as a cofactor. Stationary phase cells were harvested and cells were separated by centrifugation; the supernatant was then filtered and concentrated using a 10 kDa centrifugal filter device (Amicon Ultra-15 10K, Millipore). The concentrated supernatant was incubated with 1, 10, 50, 100 and 500 mM of Diethyldithiocarbamic acid (DDC) and with 1 mM EDTA for 1 hr. The supernatant was then washed 3 times with 15mL of quartz distilled water using the Amicon Ultra-15 centrifugal filter units (10 kDa, cutoff). Mn(II)-oxidizing activity was assayed as above and compared to the activity of a control to which quartz distilled water was added instead of the metal chelator.

Results and Discussion

To test whether the previously reported enhancement of Mn(II) oxidation in *L. discophora* SS-1 was Cu(II)-specific, we needed to define toxic concentrations of Cu(II), Cd(II) and Mn(II). Mn (II) was the least toxic metal in the range of 0.05 to 5 μM, Mn(II) with concentrations of up to 0.5 μM Mn(II) permitting growth. No growth was observed in the presence of 0.2 μM of Cd(II) or Cu(II). At 0.1 μM Cd(II) cell yield decreased by 50%. In previous research (Brouwers 1999,2000) cells of *L. discophora* SS-1 were grown in a complex LD (Boogerd and de Vrind 1987) medium containing 0.5 g/L yeast extract and higher salt concentration. Since the concentration of metal-binding ligands is much lower in MMS-2, metal concentrations used in this study were several orders of magnitude lower than previously reported values.

Parallel cultures of *L. discophora* SS-1 were grown in presence of 0.05 μM of Cu(II), Cd(II) and Mn(II) and the cell suspensions were assayed for Mn(II) oxidation. Mn(II) oxidation was specifically stimulated by Cu(II) and was not affected by the presence of nontoxic concentrations of Mn(II) and Cd(II) as seen in Fig.4.1. Higher and more toxic concentrations of Cd(II) (Fig.4.2) and Mn(II) (data not shown) did not enhance Mn(II) oxidation. The fact that Mn(II) oxidation was not affected by the presence of toxic concentrations of Cd(II) and Mn(II) points away from the possibility that the production of the Mn(II)-oxidizing protein in *L. discophora* SS-1 is a direct response to the toxicity of heavy metals.

At 0.05 μM of Cu(II), the maximum Mn(II)-oxidizing activity increased by 40 % ±0.15 % and at 0.18 μM it almost doubled (Fig.4.3). Higher levels of Cu(II) inhibited cell growth. These observations are consistent with the involvement of one or multicopper oxidases in Mn(II) oxidation by *L. discophora* SS-1 as other members of the multi-copper oxidase family have also been shown to be stimulated by Cu(II)(Collins and Dobson 1997; Palmieri et al. 2000; Malhorta et al. 2004).

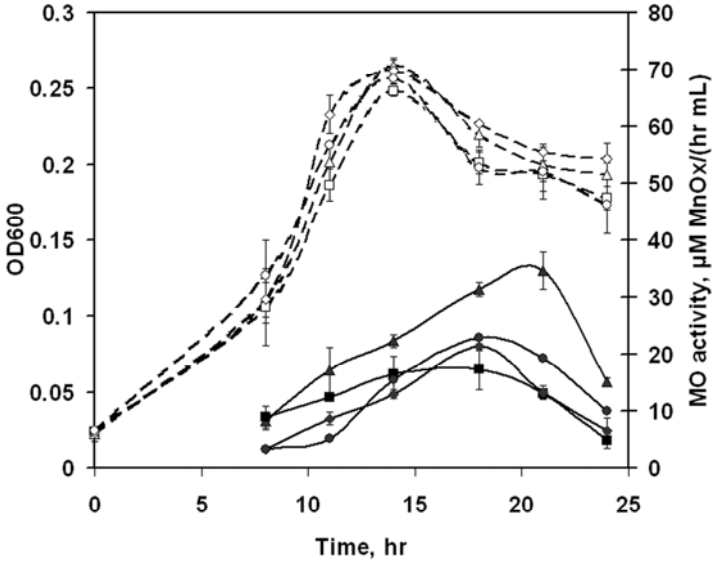

Figure 4.1. Stimulation of Mn(II)-oxidizing activity by the presence of Cu(II). Growth (OD_{600}) (open symbols) and extracellular Mn(II)-oxidizing (MO) activity (closed symbols) by *L. discophora* SS-1 supplemented with 0.05 µM Cu(II), 0.05 µM Mn(II) and 0.05 µM Cd(II). Control: (--○--)OD_{600} and (—●—) MO activity. 0.05µM of Mn(II):, (--◊--)OD_{600} and (—♦—) MO activity. 0.05µM of Cd(II):, (--□--)OD_{600} and (—■—) MO activity. 0.05µM Cu(II): (--Δ--)OD_{600} and (—▲—). The values are means ± standard deviations for duplicate cultures.

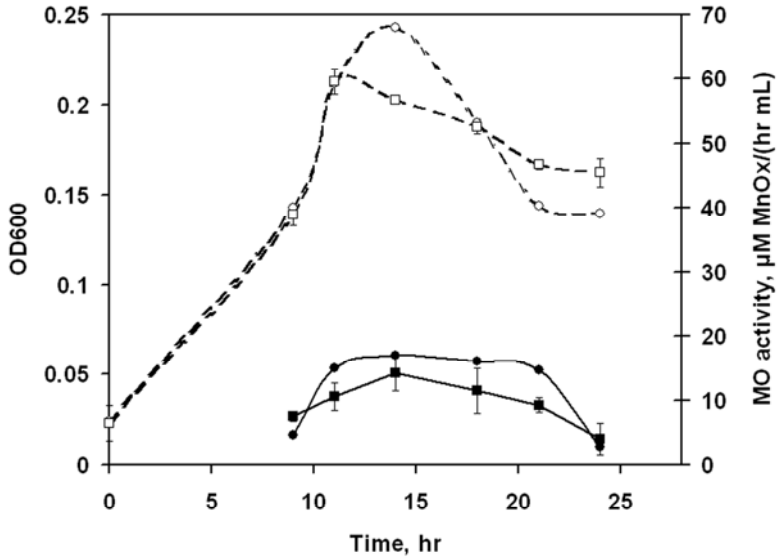

Figure 4.2. Growth in the presence of Cd(II) did not increase Mn(II)-oxidizing activity. Growth (OD_{600}) (open symbols) and extracellular Mn(II)-oxidizing (MO) activity (closed symbols) by *L. discophora* SS-1 supplemented with 0.08µM Cd(II). Control:(--○--)OD_{600} and (—●—) MO activity, 0.08µM of Cd(II):, (--□--)OD_{600} and (—■—) MO activity. The values are means ± standard deviations for duplicate cultures**.**

The recently completed genome of *Leptothrix cholodnii* SP-6, a close relative of SS-1, contains five putative multicopper oxidases in addition to a highly similar (GenBank accession no. CP001013.1; 78% aa identity) *mofA* homolog; so the exact role of *mofA* remains unclear. To gain further insight into the link of Mn(II)-oxidizing activity and *mofA* we asked whether Mn(II) oxidation was transcriptionally regulated by Cu(II). We measured transcript levels of *mofA* in cells grown in the absence and presence of Cu(II) as shown in Fig.4.4. At all Cu(II) concentrations used (data for 0.05

and 0.18 µM Cu(II) not shown), *mof*A transcript levels were not significantly ($p<0.05$) higher than the control with no added Cu(II).

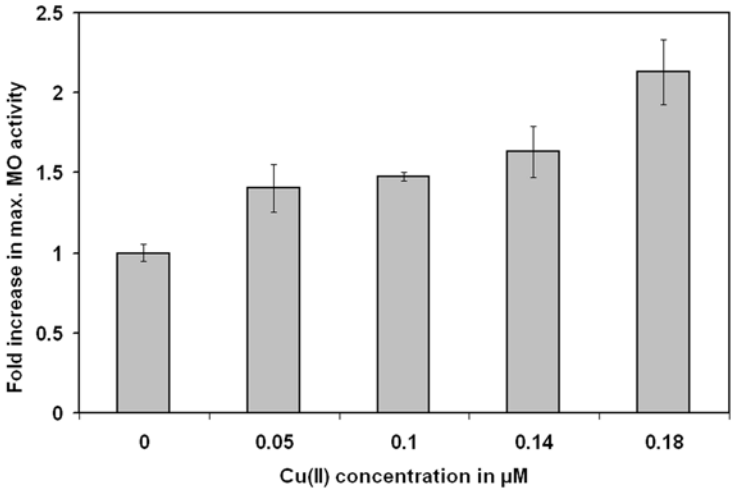

Figure 4.3. Effect of different added Cu(II) concentrations on the maximum Mn(II)-oxidizing activity by *L. discophora* SS-1. Values were normalized to the maximum of Mn(II)-oxidizing activity of a control with no added Cu(II). Mn(II) oxidation results were normalized to OD (optical density at 600 nm). Values shown are mean ± standard deviations for parallel duplicate cultures.

Our observation that Cu(II) mediated an increase in Mn(II)-oxidizing activity is consistent with previous reports (Brouwers 1999,2000), however, the lack of statistically significant evidence for a concomitant increase in *mofA* transcription leads one to consider alternative explanations for the observed increase in activity.

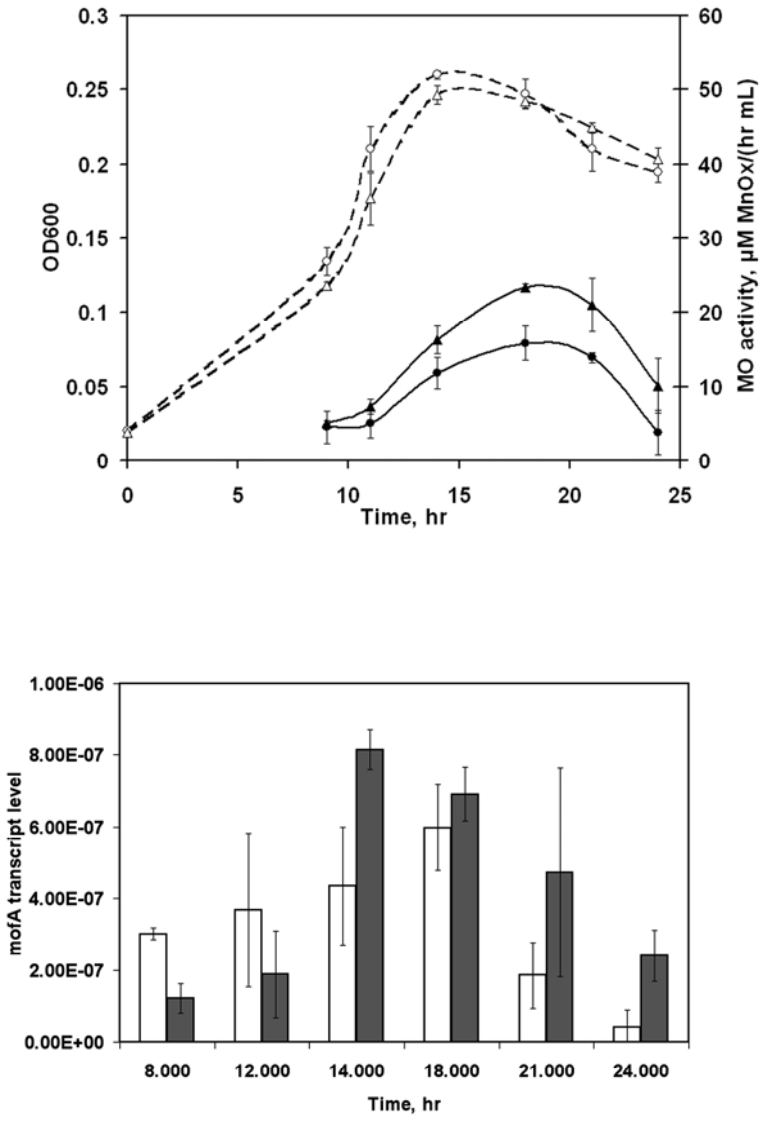

Figure 4.4. Q-RT-PCR revealed no difference in abundance of *mofA* transcripts in response to Cu(II). Growth (OD_{600}) (open symbols) and extracellular Mn(II)-oxidizing (MO) activity (closed symbols) by *L. discophora* SS-1 supplemented with 0.1 µM of Cu(II) and the corresponding Q-RTPCR results. (A) Control: (--○--)OD_{600} and (—●—) MO activity and 0.1 µM Cu(II): (--Δ--)OD_{600} and (—▲—) MO activity. (B) Q-RTPCR results from RNA extracted from *Leptothrix discophora* SS-1 grown with 0.1 µM

added Cu(II) (gray bars) and without added Cu(II) (white bars) shown in Figure 4.4A. Transcript levels are expressed as numbers of transcripts per 104 ng of RNA.

Given the QRT-PCR results reported in this study and our earlier work with Fe(II) (El Gheriany et al. 2009), it seems unlikely that the regulation of *Leptothrix*'s Mn(II) multicopper oxidase activity is metal-dependent, as has been suggested for the manganese oxidase of a recently sequenced Mn(II)-oxidizing α-proteobacterium (Dick et al. 2008a). However, it is possible that the Mn(II)-oxidizing activity is post-transcriptionally impacted by Cu(II).

Interestingly, we observe a decline in Mn(II)-oxidizing activity (Fig.4.5) when similar concentrations of Cu(II) are added to stationary phase cells grown in the absence of added Cu(II). This suggests that Cu(II) must be present during growth of *L. discophora* SS-1 for production of maximal Mn(II)-oxidizing activity. The decrease in observed rates of Mn(II) oxidation is similar to the decline in measured Mn(II)-oxidizing activity we observed in the presence of Fe(II) (El Gheriany et al. 2009) and may be due to oxidative attack on active site residues catalyzed by Fe(II) and Cu(II) (Park and Bauerle 1999). The Mn(II)-oxidizing activity seems more sensitive to Cu(II) than Fe(II), which may be due to the rapid oxidation of Fe(II) to Fe(III) under the aerobic conditions of the experiment (Park and Bauerle 1999).

The metal co-factor stripping and reconstitution experiment described previously by Larsen et al. (1999) was used to test the role of Cu(II) as a cofactor. None of the chelator concentrations used eliminated Mn(II)-oxidizing activity in the supernatant. This suggests that either the nature of the cofactor binding pocket is different in the Mn(II)-oxidase produced by *L. discophora* SS-1 from that produced by *Pedomicrobium* sp. or the active site is less exposed. The latter hypothesis seems

likely, since the Mn(II)-oxidizing protein of SS-1 has been reported to be bound to membrane aggregates (Adams and Ghiorse 1987).

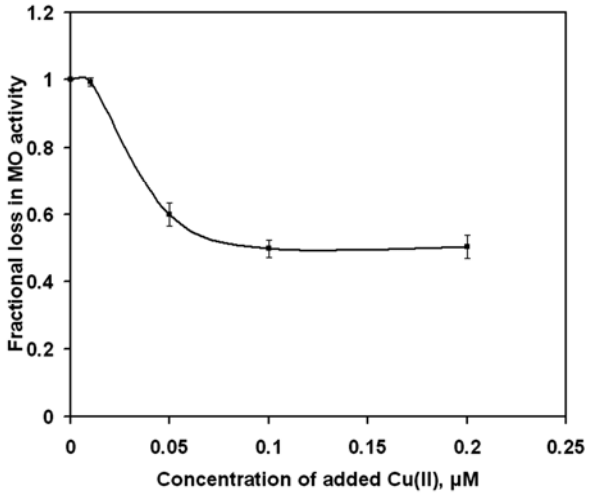

Figure 4.5. The effect of the addition of Cu(II) to stationary phase cells-grown in the absence of added Cu(II)-on the observed extracellular Mn(II)-oxidizing activity. Values shown are mean ± standard deviations calculated from triplicate samples.

As mentioned earlier, it is also possible that MofA is not the only protein and/or multicopper oxidase responsible for Mn(II) oxidation in *L. discophora*-SS1 (Adams and Ghiorse 1987; Boogerd and de Vrind 1987; Corstjens et al. 1992). Five genes encoding secreted proteins that contain sequence similarity to multicopper oxidase have been recently identified in the genome of *L. cholodnii* (YP_001789689.1, YP_001790298.1, YP_001790300.1, YP_001791655.1, YP_001792685.1). A homologue of MofA P_001792813) and a heme-peroxidase were also identified (YP_001791329.1).

In conclusion, the results obtained from this study show that the presence of Cu(II) stimulates Mn(II) oxidation by *L. discophora* SS-1. The presence of toxic and non-toxic concentrations of Cd(II) and Mn(II) had no effect on the Mn(II)-oxidizing activity. However, Cu(II) did not significantly enhance the transcription of *mofA*, the gene that is thought to encode the Mn(II)-oxidizing protein. The exact role of *mofA* in Mn(II) oxidation and/or copper resistance remains uncertain and awaits the development of a genetic system for SS-1.

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CHAPTER 5

CLONING AND EXPRESSION OF THE *MOF* OPERON OF *LEPTOTHRIX DISCOPHORA* SS-1 IN *ESCHERICHIA COLI*

Abstract

In this study we have cloned and expressed the *mof* operon of *Leptothrix discophora* SS-1 in *Escherichia coli* (*E. coli)*. The signal sequences of the three genes were screened using an assay that allows the discrimination between the major modes of transport across the inner bacterial membrane. We found that the three proteins are transported by the twin-arginine translocation (Tat) pathway. Expression of full length and mature domains of MofA and MofB showed that the signal sequences enhance the stability of the two proteins in *E. coli*. Codon optimization was a prerequisite for the expression of MofC. MofB and MofC are shown for the first time to be extracellular proteins. We have also shown that MofC is needed for the folding of MofA and its highly probable subsequent translocation to the periplasm. Interaction of MofA and MofC appear responsible for the loss of the FLAG-tag sequence from the C-terminus of MofA and compromised our ability to detect the protein after it left the cytoplasm. Coexpression of the three genes of the *mof* operon of *L. discophora* SS-1 in *E. coli*., no Mn(II)-oxidizing activity was detected in either the periplasm or the supernatant. Our findings indicate that MofA is either not the Mn(II)-oxidizing protein of *L. discophora* SS-1 or that it needs other accessory unknown proteins for catalyzing Mn(II) oxidation. Further evaluation of the roles of MofA, MofB, and MofC and their link to Mn(II)-oxidation would be expedited by development of a reliable genetic system for *L. discophora* SS-1.

Introduction

Although a variety of phylogenetically distinct Mn(II)-oxidizing bacteria have been isolated (42), the link between Mn(II) oxidation and the proteins catalyzing this reaction has only been established in a few of the bacterial systems studied to date. The multicopper oxidases, MnxG (19, 44) and MoxA (36) were found to be responsible for Mn(II) oxidation by the spores of several marine *Bacillus* species and by *Pedomicrobium* sp. ACM 3067, respectively. Heme peroxidases oxidize Mn(II) in *Aurantimonas manganoxydans* strain SI85-9A1 and *Erythrobacter* sp. strain SD-21 (2). The Mn(II) oxidase has not been identified in *Pseudomonas putida* GB-1 (25) and it remains uncertain whether *mofA* encodes the extracellular Mn(II)-oxidizing enzyme of *Leptothrix discophora* SS-1 (16).

MofA was discovered by Corstjens et al. (16) after screening an expression library of *L. discophora* SS-1 with antibodies raised against Mn(II)-oxidizing bands obtained from SDS-PAGE electrophoresis of a concentrated cell-free supernatant of this bacterium. Sequence analysis of the region downstream of *mofA* (9, 16) identified two genes belonging to the same regulon (*mofB* and *mofC*). Interestingly, the start and stop codons of *mofC* and *mofB* overlap. The predicted translation products of *mofA, mofB* and *mofC* contained a signal peptide sequence (9, 16). A signal peptide is a short peptide chain (18-30 amino acid residues) that functions as the targeting and recognition signal for proteins that are exported across the inner cytoplasmic bacterial membrane through the Sec-, SRP- (signal recognition particle) or Tat (twin arginine translocation)-dependent pathways (23).

Previously published efforts to subclone a 7262-bp *L. discophora* DNA fragment (GenBank accession no. Z25774.2) containing the complete *mofA* sequence and its putative promoter into the multicopy plasmid pUC21 failed (16). This fragment could only be cloned into a low-copy-broad-host vector and was expressed using

Escherichia coli (*E. coli)* S30 coupled transcription-translation system resulting in a ~180 kDa translation product that did not oxidize Mn(II). It is possible that the presence of the signal sequence of *mofA* may have complicated the cloning and expression of this gene (16). Sequence coding the mature protein of *mofA* was successfully cloned into a pET16B (Novagen) vector and overexpressed in *E. coli* BL21(DE3)(8). However, the recombinant protein could only be recovered in the insoluble cell fraction. Therefore, the exact relation between MofA (180kDa) and the extracellular 110 kDa Mn(II)-oxidizing factor remained unresolved.

The predicted translation product of *mofB* has a molecular weight of 24.2 kDa and shows homology to FKBP-type (FK506-binding protein) proteins (9). FKBP family members bind FK506 (an immunosuppressive drug) and show peptidyl-prolyl *cis/trans*-isomerase (PPIase) (24, 27, 32) and chaperone like activity (24, 29, 41). MofC (69.8 kDa) shows no overall homology with known proteins. It contains a consensus heme-binding motif, specific for *c*-cytochromes (9). Based on sequence analysis results, Browers, Corstjens and coworkers speculated that MofB might be needed for the folding and activation of MofA (9) and that MofC was involved in electron transport during Mn(II) oxidation (14, 17). However, to date, there has been no experimental evidence that MofA functions in concert with MofB and MofC or that any of these proteins is involved in Mn(II) oxidation.

In the present study, we report the first successful heterologous expression of the *mof* operon. First, we have used a novel experimental approach described by Marrichi et al. (34) to identify the export pathway of the three proteins coded by the *mof* operon. Then, the three genes of the *mof* operon of *L. discophora* SS-1 were successfully cloned and coexpressed in *E. coli*.

Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 5.1. The native signal sequences of the three genes found in the *mof* operon of *L. discophora* (*mofA, mofB and mofC*) were amplified by PCR from genomic DNA of *L. discophora* SS-1 using the primers (P1 through P6) shown in Table 5.2. The three native signal sequences were cloned into pMCS-ΔssPhoA (34) between NheI and XhaI sites resulting in the three plasmids pMCSMofAPhoA, pMCSMofBPhoA and pMCSMofCPhoA.

For the cloning of *mofA*, *mofB* and *mofC,* PCR reactions were performed according to the manufacturer's instructions with the long range PCR kit (Qiagen, CA) using the primer set P7 through P14 (Table.5.2). The reverse primers of *mofA*, *mofB* and *mofC* contained the DNA sequence of the FLAG tags c-Myc and HA (hemagglutinin), respectively, to allow detection of the expressed proteins by immunoblotting. The FLAG tag is an artificial 8-amino-acid residue-long peptide (28). The c-Myc (10 amino acid residues) and HA (9 amino acid residues) tags were derived from the human c-myc proto-oncogene and the HA of the influenza virus, respectively (22, 45). *mofA* with and without its native signal sequence (ss*mofA* and Δss*mofA*) was cloned into the pBAD-TOPO® (Invitrogen Life Technologies) expression vector. Full length and the mature domain of *MofB* were cloned between the KpnI/HindIII sites in the pZA31 vector (33). DNA sequence of a ribosomal binding site was added in the forward primer (P13) that was used to amplify ss*mofC*, which was cloned downsteam of ss*mofB* on the pZA31ssmofB using HindIII/BamHI restriction sites. All plasmids constructed in this study were confirmed by DNA sequencing.

Table 5.1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
Top10	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen life technologies
DR473	DHB4 Δ*trx*B *gor*552 Tn*10*Tet *ahp*C*Tn*10*Cm (*ara*C P_{ara}-*trx*B)	(18)
DRC	DR473 *tat*C::spec	(18)
Plasmids		
pMCS-ΔssPhoA	Δ(1-22)PhoA cloned in pBR322-Gm	(34)
pMCSMofAPhoA	PhoA with MofA signal sequence in pMCS-ΔssPhoA	This study
pMCSMofBPhoA	PhoA with MofB signal sequence in pMCS-ΔssPhoA	This study
pMCSMofCPhoA	PhoA with MofC signal sequence in pMCS-ΔssPhoA	This study
pBAD-TOPO® vector		Invitrogen life technologies
pBADssMofA	MofA with its signal sequence in pBAD-TOPO® vector	This study
pBADΔssMofA	MofA without its signal sequence in pBAD-TOPO® vector	This study
pZA31ΔssMofB	MofB without its signal sequence in pZA31	This study
pZA31ssMofB	MofB with its signal sequence in pZA31	This study
pZA31ssMofB ssMofC	MofB and MofC with their signal sequences in pZA31	

Table 5.2 List of oligonucleotides used in this study*

Primer	Sequence 5'---3'
P1: ssmofA-F	GCGATGGCTAGC**ATG**CGTAAAAGCACAGGTTTCCA
P2: ssmofA-R	GTAGCGCTCGAGAGACACCGGCCTGGGC
P3: ssmofB-F	GCGATGGCTAGC**ATG**AACTCGACTCTGCAAACCCT
P4: ssmofB-R	GTAGCGCTCGAGACGCCTCCGCCTGTGCCA
P5: ssmofC-F	GCGATGGCTAGC**ATG**AAGCGG
P6: ssmofC-R	GTAGCGCTCGAGCGCCGACACTCGTCGCG
P7: (1-33)mofA-F	GGCTTTGGCGCTGCGATCGA
P8: ssmofA-F	CGTAAAAGCACAGGTTTCCATCGCACGCCG
P9: mofA-Flag-R	ATGAAGCTT**CTA***TCCCTTGTCGTCATCGTCCTTGTA GTCTGCTCC*GGGCAGCGTGACGTC
P10: (1-33)mofB-F	GCGATGGGTACC**ATG**GCCGTCGGCAGCGC
P11: ssmofB-F	GCGATGGGTACCATGAACTCGACTCTGCAAACCCT
P12: mofB-cMyc-R	GTAGCGAAGCTT**CTA***CAGGTCTTCTTCAGAGATCA GTTTCTG TTC*TGA CGG CTT CTT GAT GTC GAG CAG CTC
P13: ssmofC-F	GCCATGAAGCTTAGGAGATATACATACCC**ATG** AAG CGG ATG CGG TGG TCG C
P14: mofC-HA-R	GTAGCGGGATCC**CTA***AGCGTAGTCCGGAACGTCGT ACGGGTA*CGGAATGAACCAGTCGGACACCACCTG

*The sequences of the primers are as follows (the epitope tag is written using italized spelling, restriction sites are underlined and the start or stop codons are in boldface). Sequences highlighted in grey in P2 and P4 were added to make the sequence in frame with *phoA*.

The three PhoA (alkaline phosphatase) fusion proteins were expressed in two *E. coli* mutant strains, DR473 and DRC as shown in Table 5.1. Strain DR473 carries

deletions in the genes *trxB* (thioredoxin reductase) and *gor* (glutaredoxin reductase) (7, 18) to allow protein oxidation in the-otherwise reducing-cytoplasm. In addition, these cells lack *phoA* and have a chromosomal copy of the *trxB* gene under control of an arabinose inducible promoter. This is of particular importance, since the folding of PhoA depends on disulfide bond formation (40) and requires an oxidizing environment. The use of these cells allows the folding of the signal peptide-PhoA fusions in the cytoplasm or periplasm by the addition of glucose or arabinose, respectively. DRC cells (18) are a DR473 derivative carrying an insertional deletion in the essential Tat translocase component TatC, which has been shown to be the primary recognition component for Tat signal peptides (1).

Export pathway prediction assay. PhoA activity of intact *E. coli* cells (DR473 and DRC) harboring the constructed plasmids (pMCSMofAPhoA, pMCSMofBPhoA and pMCSMofCPhoA) was monitored as described by Marrichi et al. (34) by streaking an overnight culture onto LB agar supplemented with appropriate antibiotics, 50 g/ml 5-bromo-4-chloro-3-indolylphosphate(BCIP)[3](Sigma), and 0.2% arabinose or 0.2% glucose. BCIP is a chromogenic substrate used to assay the cells for phosphate hydrolase activity. BCIP turns blue when the phosphate group is cleaved off. Plates were grown at 30 °C for 1 day. Cells that are capable of localizing PhoA fusions to the periplasm, inner and outer membranes appear blue. Cells that are export incompetent appear white/colorless (34).

Protein expression. An overnight culture of Top10 (Invitrogen Life Technologies) cells was subcultured in fresh LB at a starting optical density at 600 nm (OD_{600}) of 0.05 and then incubated at 37°C with shaking. At an OD_{600} of 0.5, L-arabinose was added to a final concentration of 0.002% to induce MofA synthesis. MofB and MofC were constitutively expressed from the Tet promoter on the pZA31 vector. After induction, cells were grown for 6 h at 37°C and overnight at 16°C or RT.

Subcellular fractionation. Cells were pelleted by centrifugation at 4,000x g for 15 min at 4 °C and then subjected to the ice-cold osmotic shock procedure as previously described (37) to obtain the periplasmic cell fraction. The cytoplasmic, soluble and insoluble fractions were obtained using the BugBuster reagent and r-Lysozyme solution (Novagen) according to the manufacturer's instructions. Protein was quantified by the Bio-Rad standard protein assay, with bovine serum albumin (BSA) as a standard.

Western blot analysis. Proteins were separated by SDS-PAGE. Unless otherwise stated, all lanes of SDS-polyacrylamide gels (Bio-Rad, Hercules,CA) were loaded with samples prepared from an equivalent number of cells harvested for each experiment. Samples were electrophoresed on a 12% (for MofB) or 7% (for MofA and MofC) Laemmli gel using the Bio-Rad (Hercules, CA) mini-protean cell. Samples were then electroblotted to Immobilon P (Millipore). The following primary antibodies were used: polyclonal rabbit anti-c-myc (Sigma) diluted 1:1,000, polyclonal rabbit anti-HA (Sigma) diluted 1:1000, monoclonal mouse anti-FLAG (Stratagene) diluted 1:3,000, and monoclonal anti-DnaK (Sigma) diluted 1:10,000. Secondary antibodies were either goat anti-mouse (Promega) or goat anti-rabbit (Promega) diluted 1:2,500. Detection was carried out using the Immun-Star horseradish peroxidasesubstrate kit (Bio-Rad) and visualized using x-ray film (Eastman Kodak Co.). The quality of all fractionations was determined by immunodetection of the cytoplasmic protein DnaK (13). Membranes were stripped in a solution consisting of 2.0% SDS, 7.0% mercaptoethanol, 0.03% NaCl, and 0.0025% Tris, reblocked, and probed with anti-DnaK antibody (Sigma).

Results

The three native signal peptides direct the export of PhoA in a Tat-dependent manner. The N-terminal signal sequences of the three proteins encoded by the *mof* operon of *L. discophora* SS-1 were predicted to be potential Tat signal peptides by TatP 1.0 (Tat signal peptide prediction server) (6). None of them, however, contained the Tat consensus motif ((S/T)RRxFLK). Expression of the three PhoA fusion peptides in DR473 resulted in blue phenotypes on glucose (oxidizing cytoplasm) containing plates and colorless/white phenotypes on arabinose (reducing cytoplasm) containing plates (Fig.5.1). Only cells that enable oxidative folding of PhoA in their cytoplasm can localize active PhoA to the periplasm. In a reduced-incorrectly folded state- PhoA is not translocated.

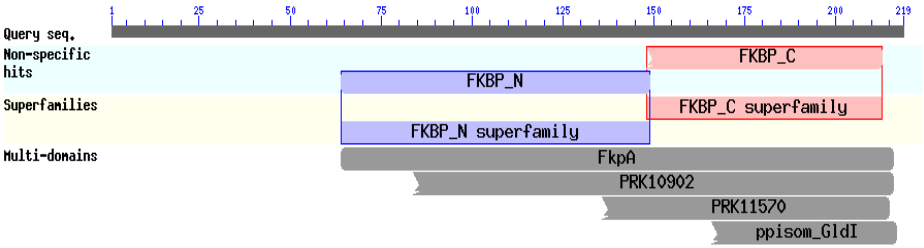
Figure 5.1. PhoA export monitored on BCIP indicator plates. Growth of DR473 expressing the three PhoA fusion proteins (A: ssMofAPhoA, B: ssMofBPhoA and C: ssMofCPhoA) after 24 h at 30ºC on agar plates supplemented with BCIP and either glucose (left) or arabinose (right).

Therefore, we conclude that the three N-terminal signals directed the export of PhoA via the Tat pathway, which only accepts proteins that have attained a native or nearly native (folded) structure in the cytoplasm (11, 18). To verify the Tat-dependent export conferred by the three N-terminal signals we expressed the three fusion proteins in DRC strain (Table 5.2). Cells grown on arabinose or glucose were colorless (data not shown). As expected, neither ssMofA-PhoA, ssMofB-PhoA nor ssMofC-PhoA was localized to the periplasm in *tatC*$^-$ cells.

***Mof* operon sequence update.** After cloning the genes of the *mof* operon, the plasmids were verified by sequencing. Point mutations in *mofA* were corrected by site directed mutagenesis. In addition, sequence discrepancies between the cloned genes and the GenBank sequence (GenBank accession no. Z25774.2) were identified. To confirm these differences, the PCR products of fragments containing the discrepancies were sequenced. The GenBank sequence of the *mof* operon was updated and has the accession number Z25774.3. A surprising finding was a 130 bp sequence we identified in the middle of *mofB*. The translation products of the previous and updated sequences of *mofB* were compared with sequences publicly available in databases using BLAST software at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Blastp results are shown in Figure 5.2. Interestingly, the predicted translation product of the updated *mofB* (28.4 kDa) allows a better alignment of the N- and C-termini of the protein with conserved domains of the FKBP-type family of proteins (COG0545 (FkpA), PRK10902, PRK11570). FkpA is one of the four known periplasmic cis-trans prolyl isomerases of *E. coli*. These proteins facilitate proper protein folding by increasing the rate of transition of proline residues between the cis and trans states (30). Ppisom_GIdI is a peptidyl-prolyl isomerase linked to a type of rapid surface gliding motility. This family is only found

in *Bacteroidetes* containing the suite of genes proposed to confer the gliding motility phenotype (35).

Previous GenBank version of MofB



Updated GenBank version of MofB

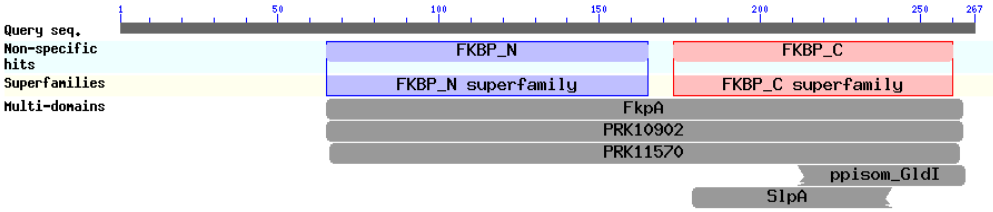


Figure 5.2. Blastp results of the previous and updated version of MofB. The updated version allows better alignment with the N- and C-termini of the protein with the periplasmic peptidyl-prolyl isomerase FkpA of *E. coli* and conserved domains of the FKBP-type family of proteins (PRK10902, PRK11570). Ppisom_GIdI is a peptidyl-prolyl isomerase linked to a type of rapid surface gliding motility. This family is only found in *Bacteroidetes* containing the suite of genes proposed to confer the gliding motility phenotype. SlpA is a 16 kDa peptidyl-prolyl isomerase of *E. coli*.

The presence of the native signal sequence increases the stability of MofA and MofB. Expression of *ssmofA* and Δ*ssmofA* resulted in a ~180 kDa product and revealed that the signal sequence increases the stability of MofA in the cytoplasm as shown in Figure 5.3. Similar to previous heterolgeous expression attempts, we recover ΔssMofA only in the insoluble cell fraction, when *E. coli* was grown at 37 ºC (data not shown). ΔssMofA is recovered in the soluble fraction only with growth at 16ºC, while ssMofA is recovered in the soluble fraction at room temperature (RT) and 16ºC. No Mn(II)-oxidizing activity could be detected in the soluble fraction in cells expressing *ssmofA*. The signal peptide increased the stability of MofB as well, as shown in Figure 5.4. The c-myc tagged protein was folded in the cytoplasm and exported to the periplasm at 37 ºC and at 16 ºC.

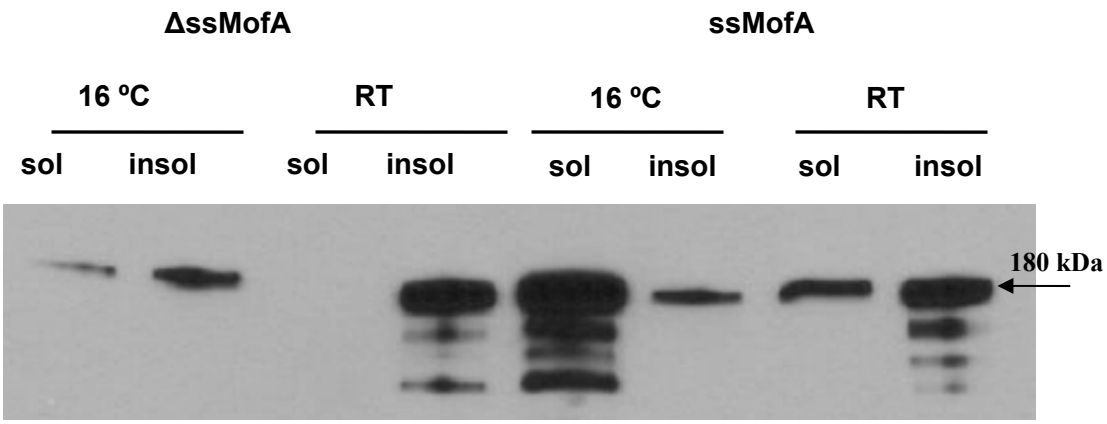


Figure 5.3. Solubility of truncated (ΔssMofA) and full-length (ssMofA) MofA. Soluble and insoluble fractions from cells expressing full length and the mature domain of MofA (devoid of its native signal sequence with an artificial start codon) were grown at 16ºC and at room temperature (RT). 10 µg of total protein was loaded in the soluble fraction and 5 µg of total protein was loaded in the insoluble fraction. All fractions

were immunoblotted with monoclonal anti-Flag. The expressed protein had the expected molecular weight of about 180kDa. The lower bands represent nonspecific degradation products.

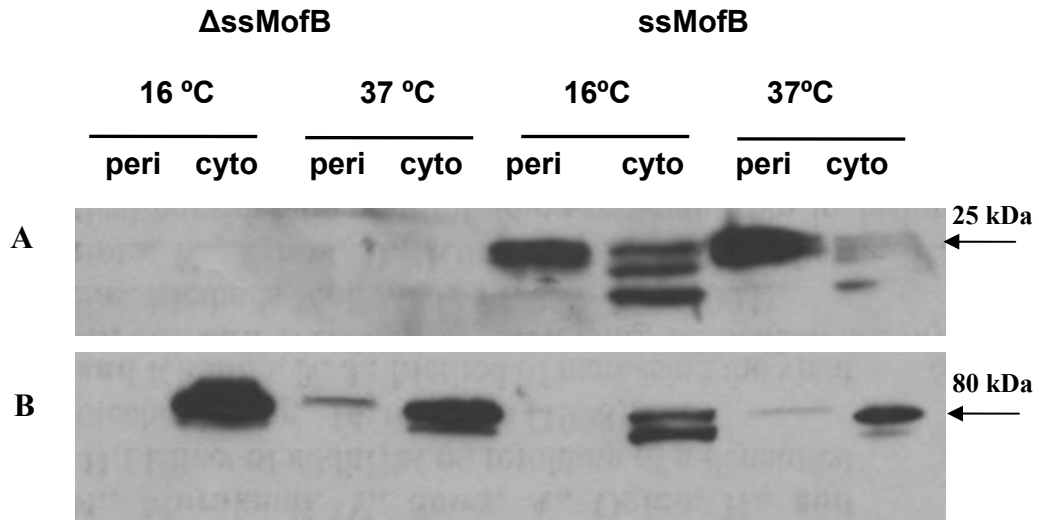


Figure 5.4. Subcellular distribution of full length and truncated MofB. Periplasmic (peri) and cytoplasmic (cyto) fractions from cells expressing ΔssMofB and ssMofB were immunoblotted with polyclonal anti-c-myc (A) and monoclonal anti-DnaK (B). Cells were grown at 37ºC and at 16ºC**.** The expressed protein had the expected molecular weight of about 25 kDa

MofC interacts with MofA. When *ssmofA* was expressed, MofA was not recovered in the periplasmic fraction i.e. it was not folded in the cytoplasm of *E. coli* (Figure 5.5). Coexpression of *ssmofB* did not lead to the export of MofA to the periplasm as shown in Figure 5.5. Therefore, we worked to also coexpress ssMofC. However, *mofC* was poorly expressed compared to *mofA* and *mofB* (Figure 5.5). Comparison of the codon usage of the three genes with that of *E. coli* by use of an online codon usage

database (http://www.faculty.ucr.edu/~mmaduro/codonusage/usage.htm) revealed that the three genes contain rare/low-usage codons. A low-usage codon is defined as a codon that is used rarely or infrequently in a genome and is decoded by a low-abundance t-RNA (rare tRNA) (31). The GC content of native *MofC* was 72%, while that of *E. coli* genes is 51% (38). Compared to *mofA* and *mofB*, *mofC* has a relatively higher number of the rare codons CGG (Arg) and UUG (Leu). A codon-optimized *mofC* gene was designed based on the *E. coli* O6:K15:H31 (strain 536 /UPEC) codon usage database (http://www.jcat.de/). Reconstitution of the gene by codon optimization resulted in a GC content of 58%. The optimized gene was synthesized and subcloned by GenScript Corporation (NJ). Codon optimized *mofC* was recovered in the cytoplasm and periplasm (data not shown). After coexpression of MofB and codon optimized MofC with MofA we recovered a reduced level of the recombinant 180 kDa protein in the cytoplasmic fraction (Figure 5.6).

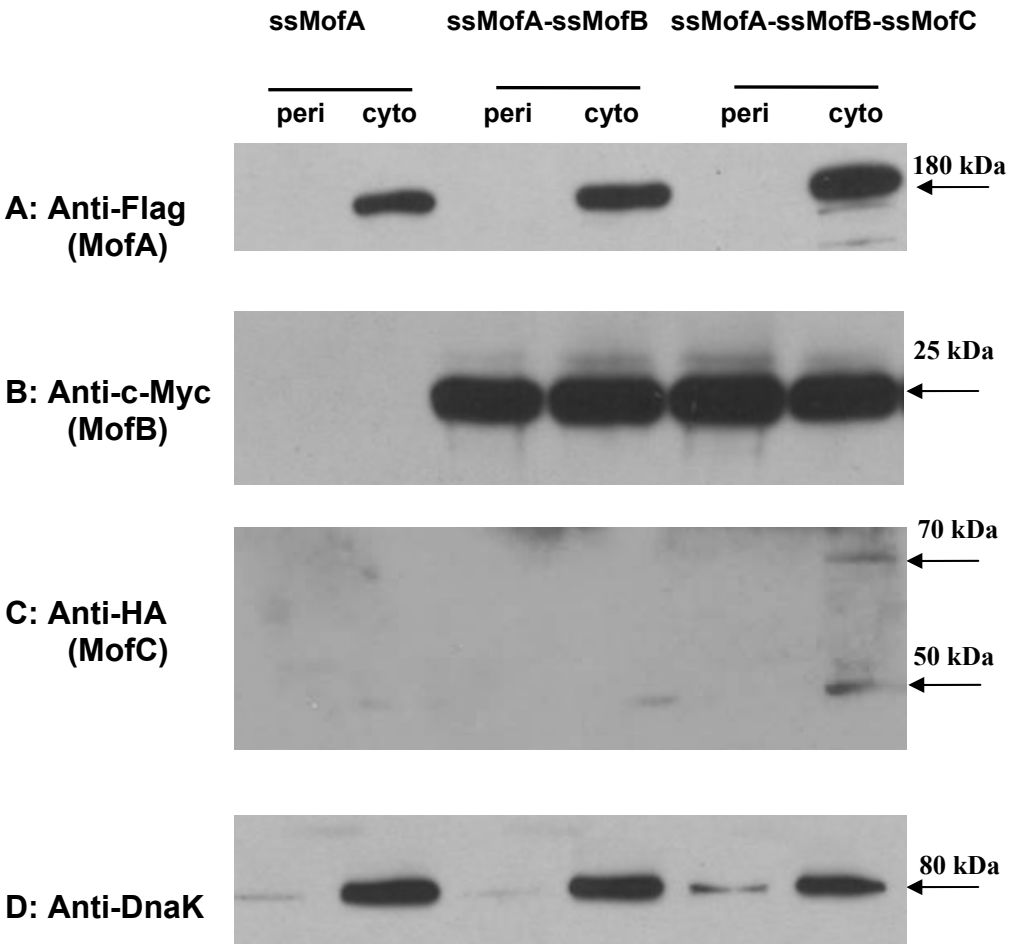


Figure 5.5. Subcellular fractionation of cells expressing ssMofA and ssMofA-ssMofB and ssMofA-ssMofB-ssMofC before codon optimizing *mofC*. Cells were grown at 16ºC. All fractions were immunoblotted with (A) monoclonal anti-Flag, (B) polyclonal anti-c-myc, (C) polyclonal anti-HA and (D) monoclonal anti-DnaK. MofC could be only detected in the cytoplasmic fraction using the SuperSignal West Femto Substrate (Thermo Scientific).

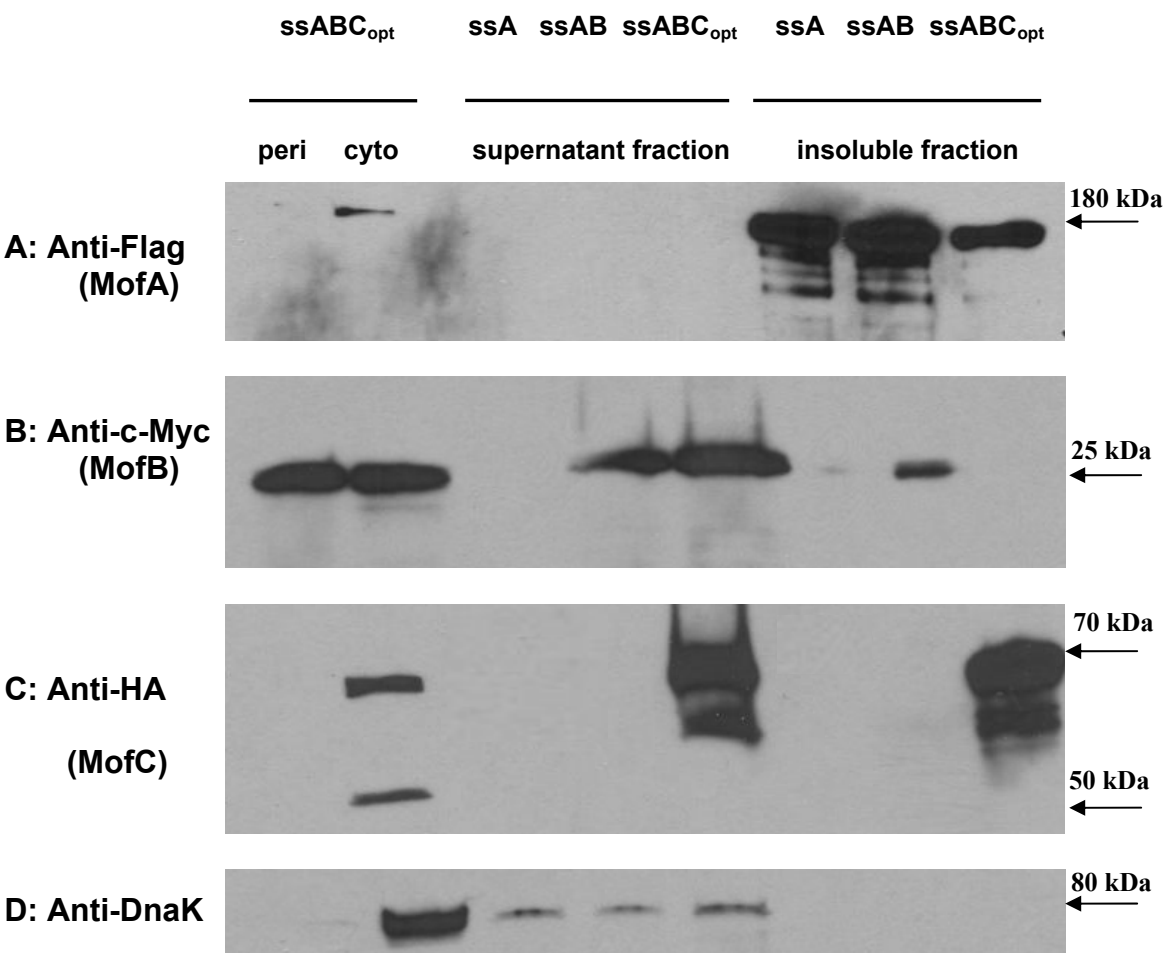


Figure 5.6. Subcellular fractionation of cells expressing ssMofA-ssMofB-ssMofC$_{opt}$ (ssAssBssC$_{opt}$), supernatant and insoluble fraction of cells expressing ssMofA (ssA), ssMofA-ssMofB (ssAssB) and ssMofA-ssMofB-ssMofC$_{opt}$ (ssAssBssC$_{opt}$). Cells were grown at 16ºC. All fractions were immunoblotted with (A) monoclonal anti-Flag, (B) polyclonal anti-c-myc, (C) polyclonal anti-HA and (D) monoclonal anti-DnaK.

Discussion

In an effort to improve understanding the role of the *mof* operon of *L. discophora* SS-1 and its connection to Mn(II) oxidation we have cloned and expressed *mofA*, *mofB* and *mofC* in *E. coli*. The signal sequence of *mofA*- the gene believed to encode the Mn(II)

oxidase- was thought by prior investigators to be toxic to *E. coli* (8, 16). Expression of *mofB* and *mofC* was not previously attempted. Therefore, initiating this study with the use of the experimental screen developed by Marrichi et al. (34) had a dual purpose. We wanted to verify whether any of the signal peptides was detrimental to *E. coli* and to reveal the export pathway of these proteins in *E. coli*. In agreement with the sequence-based prediction, the three proteins were confirmed to be Tat substrates. Each of the three native sequences exhibited cross-species compatibility with the secretion machinery of *E. coli* and successfully targeted PhoA across the inner cytoplasmic membrane. It is important to observe that the recently completed genome of *Leptothrix cholodnii* SP-6, a close relative of SS-1, contains homologues of components of the Tat translocon- tatA, tatB and tatC (YP_001790638.1, YP_001790639.1 and YP_001790640.1). As an added benefit, identification of the targeting specificity of the three signal peptides allowed us to exploit the quality control feature of the Tat pathway, that only exports proteins that have attained a native conformation in the cytoplasm (10, 18).

Because of the increasing indirect evidence that transportation of cofactor-containing folded proteins is a fundamental feature of the bacterial Tat apparatus (4) we were interested to test whether other known bacterial Mn(II) oxidases are targeted through the Tat pathway. The Mn(II) oxidase of *Pedomicrobium* sp. ACM 3067 MoxA (36) (GenBank accession no. CAJ19378.1) was predicted to be targeted through the Tat-translocation system by TatP 1.0 server (6). Unlike *Leptothrix*'s three signal peptides, MoxA signal contains the Tat consensus motif (RRSFM)(36). The N-terminal sequences of MnxG (GenBank accession no.ABP68899.1) and the Mn(II) peroxidase (GenBank accession no.ZP_01225898) were analyzed using the same online tool. Neither of these two sequences were identified as Tat substrates and the first 100 amino acids in these extracellular proteins were not predicted to be signal

peptides by SignalP 3.0 software either (21). The two proteins are likely to follow a signal peptide independent secretion pathway (5, 21). Indeed, the Mn(II) oxidase of the gram-negative bacterium *Aurantimonas manganoxydans* was predicted to be a non-classically secreted protein (SecP score = 0.87; SecP>0.5) by the online Secretome 2.0 software (5).

Expression of full length MofA and ΔssMofA showed that the yield of soluble protein increases with decreasing temperature (Fig.5.3). The same trend is also observed when we compare the yield of ssMofB and ΔssMofB at 37ºC and at 16ºC (Fig.5.4). Lowering the cultivation temperature was previously found to improve the yield and activity of overexpressed proteins in *E. coli* (15) and is considered as a traditional approach to improve the yield and solubility of heterologous proteins (3). Therefore, we selected 16ºC as the growth temperature for *mofA* expression in *E. coli*.

In addition, the higher stability of the full length protein in both cases might be due to the role played by the Tat leader sequence not only in the translocation of the substrate, but also in affecting the level of the Tat protein mRNA as recently suggested by Snyder et al. (39).

The export of MofB to the periplasm (Fig.5.4) indicates that MofB attained a folded state in the cytoplasm of *E. coli*. MofB belongs to the FKBP-type family of proteins. These facilitate the *cis-trans* isomerization of proline residues, which is known to be a rate limiting step in protein folding (24). Proline constitutes 7% of the amino acid composition of MofA. This proline content is higher than the average of *E. coli* proteins (4.29%, (31)).Therefore, we were encouraged to test the hypothesis that MofB was required for the folding of MofA and its subsequent translocation to the periplasm, since MofA-similar to Tat substrates- is expected be translocated to the periplasm only if attains a folded conformation in the cytoplasm (18).

Contrary to expectations, expression of MofB did not lead to the recovery of MofA in the periplasm. We then evaluated to possibility that MofC is needed in the processing of MofA. Coexpression of codon optimized $MofC_{opt}$ with the MofB and MofA proteins lead to a decline of the amount of MofA recovered in the cytoplasmic fraction (Fig.5.6). This observation suggests that MofC interacted with MofA in a manner that either led to the aggregation, folding or increased proteolysis of this Tat substrate. To test these hypotheses we analyzed the insoluble fraction for the presence of MofA. In addition, since MofA is present in the cell-free supernatant of *L. discophora* SS-1 and is therefore an extracellular protein, we harvested the supernatant fraction of cells expressing *ssmofA*, *ssmofA-ssmofB* and *ssmofA-ssmofB-ssmofC*$_{opt}$ and concentrated it 200 fold by TCA (2,2,2-Trichloroacetic acid) precipitation. Our results exclude the possibility that the interaction between MofA and MofC promotes the aggregation of MofA, since the amount of MofA recovered in the inclusion body in presence of MofB and MofC was less than the amount recovered when MofA is expressed alone or with MofB (Fig.5.6). The fact that less MofA was recovered in the cytoplasm in the presence of MofC indicates that MofA was likely to have been folded and then translocated to the periplasm and/or to the supernatant.

Despite the fact that MofC accumulated in the supernatant, it was occasionally not recovered (Fig.5.6) or recovered at a very low concentration in the periplasmic fraction (data not shown). Therefore, our inability to recover MofA in the periplasm does not necessarily mean that it was not translocated. It is probable that interaction between the 180 kDa MofA protein and MofC resulted in the loss of the tag and the formation of a protein with a lower molecular weight. This is supported by the fact that MofA was discovered using an antibody raised against a 110 kDa Mn(II)-oxidizing band and not a 180 kDa protein.

Surprisingly, we detected MofB in the supernatant by immunoblotting. Despite the fact that the two extracellular proteins-MofB and MofC-were expressed under the same promoter and using identical ribosomal binding sites, only MofB is abundant in the periplasm. The accumulation of MofB in the periplasm may be due to its interaction with other *E. coli* proteins. It is important to note that the four known *E. coli cis-trans* prolyl isomerases FkpA (a MofB homolog as seen in Fig.5.2), PpiA, PpiD, and SurA are periplasmic and were recently shown to be involved in pilus production and susceptibility to certain antibiotics (30). They are expected to have significant roles in survival in environmental and pathogenic niches.

Our inability to detect Mn(II)-oxidizing activity in all the cell fractions collected demonstrates for the first time, that expression of functional *mof* operon proteins in *E. coli* is not sufficient to confer Mn(II)-oxidizing activity and confirms previous results indicating that other accessory (possibly iron-containing) proteins are required (20). However, we cannot exclude that the FLAG tag- despite of its small size-has interfered with protein activity or folding of MofA (12, 26, 43).

In this study we have shown that MofC appears to play a role in the processing of MofA. The determination of the actual role of the *mof* operon proteins and understanding how they affect viability and growth of *L. discophora* SS-1 can best be achieved by developing a genetic system for this bacterium.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

In this study I have developed a batch bioreactor system in which control is maintained on the chemical composition of the medium, pH and the speciation of metal ions e.g. Cu(II). In addition, a method to monitor the time course of Mn(II)-oxidizing activity was established. I have used this reactor system to evaluate the influence of iron on the rate of extracellular Mn(II) oxidation by the Mn(II)-oxidizing bacterium, *Leptothrix discophora* SS-1. Cultures in which cell growth was limited by iron exhibited reduced ability to oxidize Mn(II) compared to cultures in medium with sufficient iron. The decline of Mn(II) oxidation under iron-limited conditions was not accompanied by siderophore production. Iron limitation did not interfere with the transcription of the *mofA* gene. These data are consistent with a direct requirement for iron as a component of the Mn(II)-oxidizing protein(s) or an indirect effect of iron resulting from the limitation of one of many cellular functions requiring iron.

The controlled reactor system was used to grow *Leptothrix discophora* SS-1, in presence of toxic and non-toxic concentrations of Cu(II), Cd(II) and Mn(II). Enhanced Mn(II)-oxidizing activity was observed only when cells were grown in the presence of Cu(II). The failure of toxic levels of Cd(II) to stimulate Mn(II) oxidation challenges the hypothesis that Mn(II) oxidation is a response to metal toxicity. Stimulation by Cu(II) confirms the specific role of Cu(II) in Mn(II) oxidation and the possible involvement of the multicopper oxidase family of enzymes. However, the addition of Cu(II) did not lead to a statistically significant increase in transcript levels of *mofA*.

The genes of *mof* operon of *L. discophora* were cloned and expressed in *E.* coli to evaluate their role in the oxidation of Mn(II). MofC was shown to require codon optimization for successful expression in *E.coli* and to interact with MofA. After

coexpression with MofB and the codon optimized MofC, the 180kDa MofA protein was not recovered in the supernatant, or periplasmic fractions. This result was not unexpected, since the antibody that lead to the discovery of *mofA* was developed against a 110 kDa protein (indicating that MofA gets truncated). It is recommended to rerun the samples on a 4-20% Tris-HCl gel to detect the cleaved fragment of MofA. Mn(II) oxidation was not detected. This result indicates the *mof* operon may not be involved in Mn(II) oxidation or that despite its expression, it was not functional. Future work must involve confirming the Tat-dependent translocation of the three full length proteins (ssMofA, ssMofB and ssMofC) by expressing the three genes in *tatC*⁻ deletion strains. Finally, it would be interesting to investigate how MofB and MofC are efficiently excreted to the medium by *E. coli* strain Top10 (K12 strain). Since proteins of type II secretion machinery are cryptic in K-12 strains, it is important to determine whether MofA serves as the outer membrane porin that allows the translocation of MofB and MofC across the outer membrane. This could be done by coexpressing MofB and MofC without MofA and check whether they will be recovered in the supernatant.

Future research directions in Mn(II) oxidation by *L. discophora* SS-1 should use the genome sequence of *Leptothrix cholodnii*, a close relative of SS-1, which was completed in 2008. It revealed the presence of seven other multicopper oxidases. Any of these could be responsible for Mn(II) oxidation. In addition, a heme peroxidase was identified. Interestingly, none of these Mn(II) oxidase candidates has a molecular weight of 110/116 kDa. The largest three multicopper oxidases (YP_001791621.1, YP_001792685.1, YP_001791655.1) have a predicted molecular weights of 98 kDa, 165 kDa and 167 kDa, respectively. The heme peroxidase (YP_001791329.1) is predicted to be 170kDa. Consequently, any of these possible Mn(II) oxidases cannot be responsible for Mn(II) oxidation unless it undergoes some processing-similar to

what we observe with MofA-to yield the observed 110 and or 116 kDa Mn(II)-oxidizing bands. Therefore, to identify the Mn(II)-oxidizing protein(s) in *L. discophora* these genes need to be cloned and expressed, or molecular methods for manipulation of *L. discophora* must be developed. Ideally plasmids would be constructed in a way that might allow simultaneous expression in *E. coli* and *L. discophora* SS-1. Pursuit of this objective has been inhibited because of the lack of information about regulatory sequences, e.g. promoter regions, which can be recognized by *L. discophora*. The availability of genome information and the appreciable progress achieved in the development of a genetic system for *L. discophora* SS-1 in the Hay Lab at Cornell University will hopefully make this possible.

Areas for future research also include the construction of a genome-scale metabolic model similar to that developed for *Geobacter metallireducens* (1). The model could then be used to generate a set of experimentally testable hypotheses and predictions related to the metabolic capabilities of *L. discophora* in general and related to Mn(II) and Fe(II) oxidation in particular. The reactor system and growth medium developed in this study could provide a useful tool for investigating hypotheses related to aerobic growth. The development of a similar reactor system for microaerophilic growth is another important aspect that merits attention in the future.

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