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Analysis of Genes Involved in Metal Resistance and Cytochrome C Maturation in Shewanella Oneidensis MR-1

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ANALYSIS OF GENES INVOLVED IN METAL RESISTANCE AND CYTOCHROME *C* MATURATION IN *SHEWANELLA ONEIDENSIS* MR-1

by

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ABSTRACT

ANALYSIS OF GENES INVOLVED IN METAL RESISTANCE AND CYTOCHROME *C* MATURATION IN *SHEWANELLA ONEIDENSIS* MR-1

by

Rini Banerjee

The University of Wisconsin-Milwaukee, 2019
Under the supervision of Professor Dr. Daad Saffarini

Metals play crucial roles in many cellular processes where they form active centers of metabolic enzymes or participate in electron transfer reactions during respiration. At high concentrations, metals can be toxic and result in the formation of reactive oxygen species and protein denaturation. Bacteria have evolved homeostasis systems to maintain intracellular concentrations of various metals and avoid their toxic effects. The aim of this project is to identify and characterize metal homeostasis systems in the metal reducer *Shewanella oneidensis* MR-1. This bacterium can use metals and radionuclides as electron acceptors during anaerobic respiration and is therefore a good candidate for bioremediation of metal-contaminated environments. Furthermore, this bacterium is able to maintain low internal levels of heavy metals through the use of multiple efflux pumps such as the P-type ATPase - CopA, and the HME RND efflux pump – CzcCBA. This study aims to understand the role of these efflux pumps and their regulators in metal resistance.

Shewanella oneidensis also expresses a large number of *c*-type cytochromes, many of which function as terminal reductases. All of these proteins contain the typical heme-binding motif CXXCH and require the Ccm proteins for maturation. SirA, the terminal sulfite reductase, also possesses an atypical heme binding site $CX_{15}CH$ which requires a specialized system for heme

attachment. *S. oneidensis* MR-1 encodes two cytochrome *c* synthetases (CcmF and SirE) and two apocytochrome *c* chaperones (CcmI and SirG). In this study we show that both apocytochrome *c* chaperones, CcmI and SirG, are required for the maturation of SirA and they each interact with the terminal sulfite reductase independently of each other, even in the absence of other components of the cytochrome *c* maturation system.

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CHAPTER I

1.1 INTRODUCTION

Members of the *Shewanella* genus are Gram negative facultative anaerobes and members of the γ-Proteobacteria. They are commonly found in diverse environments including freshwater and marine sediments and water columns, hydrothermal vents, activated sludge, marine fish, and oil pipelines (1–4). The majority of the *Shewanella* species are thought to be of marine origin. However, some species have been recovered from freshwater environments and their presence in these systems is thought to be recent (4, 5). *Shewanella* species, with the exception of *S. denitrificans*, are well known for their ability to transfer electrons extracellularly, and their genomes encode the genes required for this process (4).

Shewanella oneidensis MR-1 was isolated in 1988 from Oneida Lake in New York and was the first *Shewanella* species to have its genome sequenced (6). *S. oneidensis* was initially classified as *Shewanella putrefaciens* and later reclassified as a distinct species based on the analysis its of 16S rDNA and the DNA gyrase subunit B (*gyrB*) sequence (7). Analysis of *S. oneidensis* revealed that this bacterium can use Mn (IV) as an electron acceptor under anaerobic conditions and reduce it to Mn (II). *S. oneidensis* MR-1 has been extensively studied and is considered the model organism for the study of metal reduction. Other *Shewanella* species have since been isolated from various other aquatic environments including the Baltic Sea, the Black Sea and Lake Michigan (3). The genomes of many of these species have been sequenced and genes for metal reduction and other functions have been identified and analyzed.

S. oneidensis MR-1 has the ability to reduce a wide array of organic and inorganic compounds as electron acceptors during respiration. These include oxygen, dimethyl sulfoxide (DMSO), trimethylamine N-oxide (TMAO), nitrate, thiosulfate, sulfide, elemental sulfur, fumarate, iron and manganese oxides [Fe (III) and Mn (IV)], chromium as well as radioactive pollutants such as uranium and plutonium. (8–10). The versatility and ability of *S. oneidensis* MR-1 to use metals as electron acceptors makes it a model organism to study anaerobic respiration and metal reduction.

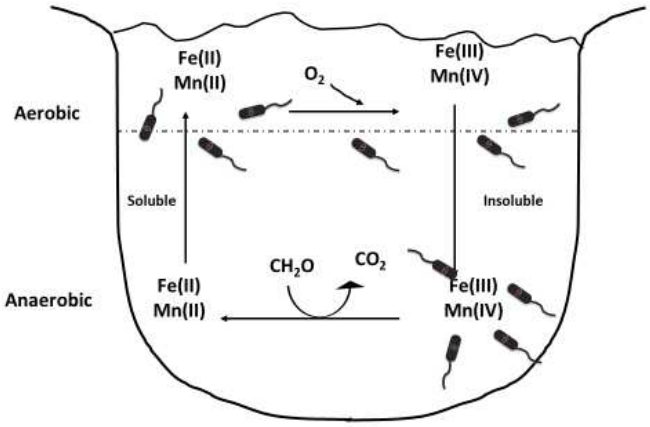


Figure 1.1 *Cycling of metals in an aquatic environment. Use of insoluble metal oxides by bacteria as electron acceptors during anaerobic respiration results in their reduction into soluble forms. Soluble metals are cycled into the aerobic zone where they are re-oxidized and settle down into the anoxic zone again* (11).

As mentioned above, *S. oneidensis* and other similar species are commonly found in aquatic environments. They are usually found at the interface between the aerobic and anaerobic zones and play an important role in cycling of metals in the environment (Fig. 1.1). Insoluble metal oxides such as Fe (III) and Mn (IV) that are found in the anoxic zone in aquatic environments are reduced by these bacteria. The resulting soluble metals diffuse into the aerobic zone where they become oxidized again, either chemically or through microbial activity, and settle down into the anoxic zone to be available for reduction by the bacteria, thus continuing the cycle. During oxidation of Fe (II), other metals can adsorb to the iron oxide particles that settle into the anaerobic zone. This cycle of oxidation and reduction results in the mobilization of adsorbed metals such as nickel, zinc and cadmium in between the aerobic and anaerobic zones (8, 12, 13).

1.2 Electron Transport in *S. oneidensis*

The genome of *S. oneidensis* MR-1 consists of a 4.97 Mb circular chromosome and a 161.6 kb plasmid. It encodes 42 *c*-type cytochromes that contribute to its ability to respire with a large number of electron acceptors. Some of these *c*-type cytochromes, such as CymA can function as intermediates during electron transport, while others are components of the respiratory terminal reductase complexes. Some of these cytochromes are present on the outer surface of the cell. This location allows this bacterium to reduce insoluble metal oxides on the outer surface of the cell.

The majority of the research on *S. oneidensis* has been focused on analyzing its electron transport chain and its ability to reduce metals as terminal electron acceptors. In *S. oneidensis*, electrons enter the Electron Transport Chain (ETC) via dehydrogenases, by the oxidation of carbon sources such as lactate. The electrons produced during this oxidation step are transferred through

the menaquinone pool to terminal reductases directly (such as SirA) or through intermediates such as CymA (Fig. 1.2). The terminal reductases reduce the available acceptors and the entire process results in the generation of energy. The presence of terminal electron acceptors associated with the inner membrane, periplasmic space and the outer membrane contribute to its ability to reduce a large number of electron acceptors.

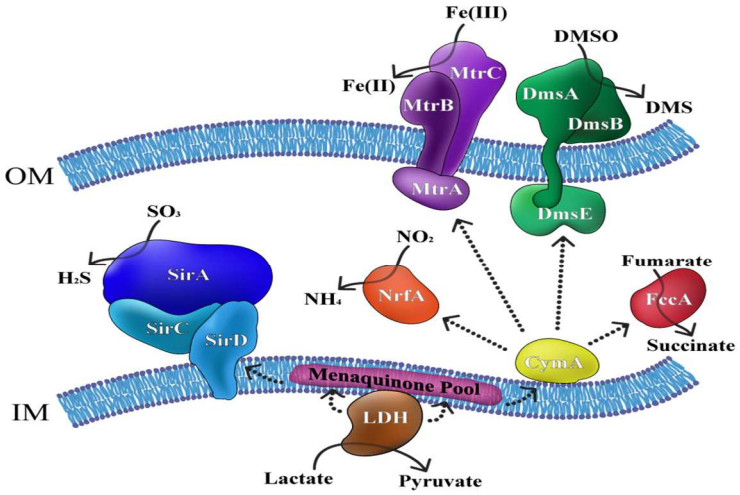


Figure 1.2. *Model of electron transfer during anaerobic respiration in S. oneidensis MR-1. Electrons generated by oxidation of a carbon source are transferred to terminal reductases where reduction of the terminal electron acceptor occurs, resulting in the generation of energy* (14).

1.3 Applications

Reduction of metals by bacteria during anaerobic respiration contributes significantly to the biogeochemical cycling of metals. Additionally, the ability of *S. oneidensis* MR-1 to reduce metals extracellularly can also be used in biotechnological applications such as generation of electricity as well as for the bioremediation of toxic metals.

1.3.1 Bioremediation of toxic environmental pollutants

Bioremediation is an important metabolic process that is associated with environmental bacteria. It involves the physical removal or detoxification of pollutants from the environment. The large number of *c*-type cytochromes in *S. oneidensis* and its respiratory versatility under both aerobic and anaerobic environments (15), coupled to its ability to reduce radionuclides and inorganic compounds to non-toxic forms, make this organism a suitable agent for bioremediation. For example, *S. oneidensis* reduces Uranium (VI) and Chromium (IV) to insoluble oxides U (V) and Cr (III) that can then be easily removed from the polluted environment. Similarly, *S. oneidensis* can use other compounds such as TMAO (trimethylamine-N-oxide), DMSO (dimethyl sulfoxide) and oxides of manganese and iron (Fe (III) and Mn (IV)) as electron acceptors during anaerobic respiration, converting them from their toxic forms to non-toxic states (3, 15).

1.3.2 Generation of electricity through microbial fuel cells

Generation of electricity using microbial fuel cells (MFCs) is another area of active research interest. MFCs are hybrid bio-electrical systems that generate current by using bacteria

as catalysts to oxidize organic and inorganic compounds (16, 17). These devices are generally operated as closed systems where the anode is maintained under anaerobic conditions, separated from the cathode by a membrane. MFCs are made such that the bacteria are associated with the anode where they oxidize organic or inorganic substrates and the electrons generated during this process flow to the cathode thus generating electric current (16). Since most terminal reductases in bacteria are associated either with the inner membrane or the periplasmic space, traditional MFCs required the use of mediators for the transfer of electrons from the bacterial cells to the anode. Most available mediators such as methyl vilogen and neutral red are expensive and toxic. *S. oneidensis*, on the other hand, contains multiple terminal reductases associated with the outer membrane, thus they can be used in MFCs without the need for mediators, making microbial fuel cells less toxic and safer to use (18, 19).

1.3.3 Hydrocarbon fuel generation

In addition to using *S. oneidensis* in mediator-free microbial fuel cells, this bacterium is being investigated as a source of hydrocarbon fuels. *S. oneidensis* has the ability to make long chain hydrocarbons as a product of certain metabolic processes. These hydrocarbons are synthesized by a mechanism called head-to-head fatty acid condensation. Attempts to modify hydrocarbons naturally produced by *S. oneidensis* into a fuel that resembles petroleum are underway (20). Additionally, scientists are trying to couple the production of hydrocarbons by *S. oneidensis* with the metabolism of certain photosynthetic bacteria thus potentially generating a renewable source of petroleum (20–22).

CHAPTER II

2.1 Metal Homeostasis/ Resistance

Heavy metals are defined as a group of elements that have a density over 5 g/cm^3. Most heavy metals fall under the category of transition elements and have incompletely filled d-orbitals, which gives the heavy metal cations the ability to form complex compounds with redox activity (23). These metals also serve as trace metals and play extremely important roles in various cellular processes. However, at higher concentrations, these metals become toxic for the cells by forming reactive oxygen species that cause the denaturation of cellular proteins. Most organisms thus show a biphasic response to a number of heavy metals – i.e. growth is stimulated at lower concentrations, but as the concentration of metal increases, growth is inhibited and finally stops. Thus, metal acquisition and transport to the correct location within the cell is tightly controlled (23, 24). Bacteria use two major systems to maintain metal homeostasis, also called as metallostasis, within cells; these are Metallochaperones and Heavy Metal Efflux Proteins.

2.2. Metallochaperones

Metallochaperones are a class of proteins that are essential for post-translational insertion of metal co-factors into their cognate metalloproteins. The primary function of metallochaperones is to bind to a specific metal, transport it through the cell and insert it into a specific apo-enzyme, thus facilitating its maturation (25). Since transport of metal co-factors by metallochaperones involves binding of metals by specific proteins for delivery, metallochaperones provide a dual function – they protect the cell from damage caused by free metal ions and transport and deliver the correct metals required for maturation of metalloproteins (26). Metallochaperones are found in

all domains of life and shuttle metals such as copper, iron, nickel, cobalt, zinc, etc. One of the most well studies examples of a metallochaperone found in enteric bacteria is CopZ – a copper chaperone that transfers Cu (I) to a CPx-type ATPase, CopA.

2.3 Heavy Metal Efflux proteins

One of the major means by which bacteria maintain metal homeostasis within the cells is by using efflux pumps that transport excess metals into the extracellular milieu. The heavy metal efflux RND Pumps, CPx-type ATPases and cation diffusion facilitators are the three heavy metal efflux protein systems found in bacteria.

2.3.1 Heavy Metal Efflux RND Pumps

Metal homeostasis can be maintained within cells by using various transport mechanisms. One mechanism involves the RND Family Proteins (resistance-nodulation-cell division protein family). This pump consists of a tripartite protein complex that can export a variety of toxic substrates from the cytoplasm or periplasm to the extracellular environment at the expense of Proton Motive Force (PMF). Members of the RND protein super-family can be involved in the transport of heavy metals, organic substrates as well as polypeptides and are found in all major kingdoms of life. The metal transporting RND proteins in bacteria and archaea pump out monovalent cations such as Cu (I) and Ag (I) as well as divalent cations such as Zn (II), Ni (II) and Co (II) (27, 28). The Heavy Metal Efflux RND efflux system forms complexes made up of 3 proteins - a central proton-substrate antiporter that belongs to the RND family, a Membrane Fusion Protein (MFP) and an Outer Membrane Factor (OMF). This complex can export substrates from

the cytoplasm, the cytoplasmic membrane or the periplasmic space directly across the outer membrane (29, 30). Two of the most well characterized metal transporting RND protein complexes are the CzcCBA complex from *Cupriavidus metallidurans* (*C. metallidurans*) and the CusCBA system from *Escherichia coli* (*E. coli*), where CzcA and CusA are the RND proteins, CzcB and CusB are the membrane fusion proteins and CzcC and CusC are the outer membrane factors. Some systems, such as the CusCBA system, require an additional periplasmic metal chaperone that is responsible for delivering the substrate to the membrane fusion protein. The genes encoding the respective proteins form polycistronic operons – *czcCBA* and *cusCFBA* (27, 31, 32).

The heavy metal efflux RND proteins, such as CzcA, are very large proteins that form trimers. Each protein monomer spans the inner membrane and has twelve transmembrane α-helixes in addition to a large hydrophilic periplasmic loop of about 300 amino acids (27, 33). Each of the RND protein monomers can undergo conformational changes and switch between three states – open access for binding of substrate, substrate-bound and extrusion of substrate to the outer membrane protein. These conformational changes are responsible for driving the proton coupled export by these systems (33–35).

The second component (CzcC) of RND efflux system is a trimeric outer membrane factor (OMF). This trimer forms a hollow tube spanning the outer membrane as a beta barrel, allowing substrates to pass through. The OMF connects the periplasmic domains of the RND protein trimer to the extracellular space. Multiple studies have shown that the OMF part of the Heavy Metal Efflux RND pump complex is not involved in substrate specificity, however an increase in their expression has been shown to increase resistance to certain toxic substrates (33, 36, 37). The third component of the HME RND protein complex is a periplasmic membrane fusion protein (CzcB).

The membrane fusion protein forms a ring around the RND proton-substrate antiporter and the outer membrane factor, thus stabilizing the interaction between these two trimeric proteins (33).

Two models have been proposed for transport of metals using RND driven efflux systems – trans-envelope efflux and periplasmic efflux. During trans-envelope efflux, metal ions may be transported through the entire transmembrane region of the RND protein and the outer membrane factor from the cytoplasm and released into the extracellular space, without release of the substrate into the periplasm. On the other hand, during periplasmic efflux, periplasmic substrates are bound by the RND protein and subsequently exported to the extracellular space through the Outer Membrane Factor (27).

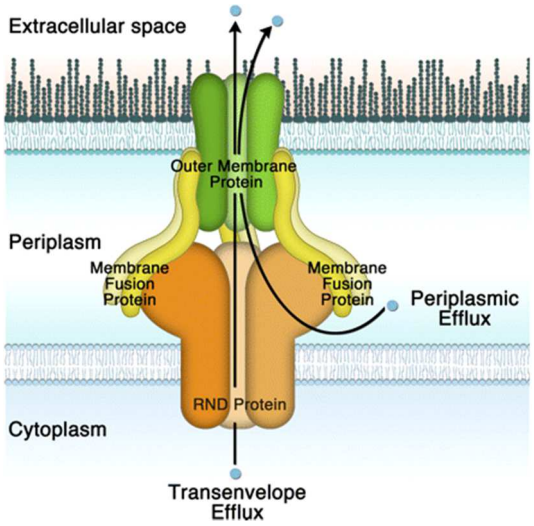


Figure 2.1. *Model depicting heavy metal efflux using Heavy Metal Efflux RND protein complex. The RND protein transports divalent or monovalent cations across the cytoplasmic membrane as a cation-proton antiporter. The membrane fusion protein stabilizes the contact between the RND protein and the outer membrane factor along with funneling the cations through the periplasmic space into the OMF which allows the cations to diffuse out into the extracellular space* (33).

2.3.2 CPx type ATPases

The second family of proteins involved in transport of heavy metals is the P-type ATPases. They constitute a superfamily of transport proteins that are driven by ATP hydrolysis. These proteins are found in all domains of life and are involved in the transport of various inorganic cations as well as small organic molecules against their concentration gradient. P-type ATPases can further be of two types – those involved in import of substrates from the extracellular environment or periplasm into the cytoplasm or those that export substrates from the cytoplasm to the periplasm or extracellular space. The exporting P-type ATPases play an important role in exporting heavy-metal cations by efflux (27, 38). Certain members of the P-type ATPase superfamily have a conserved cysteine-proline-x motif and are thus designated as CPx-type ATPases (39). Members of the CPx-type ATPase are only involved in the transport of heavy metals across the cytoplasmic membrane and their range of transported substrates includes Cu^{+}, Ag^{+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} and they are thus commonly called heavy-metal ATPases. CopA in *E. coli* is one of the most well characterized bacterial CPx-type ATPases and is involved in copper translocation. Although the CPx-type ATPases are a subgroup of P-type ATPases, they differ from other non-heavy metal P-type ATPases in several ways. They only have eight transmembrane helices compared to ten that are found in non-heavy metal ATPases. They also consist of one to six metal binding domains that contain a CXXC motif or a histidine-rich region close to the N terminus, along with a highly conserved CPx motif in the sixth transmembrane helix and a conserved Histidine Proline dipeptide (also called the HP locus) about 34 – 43 amino acids carboxy terminal to the CPx motif (39, 40).

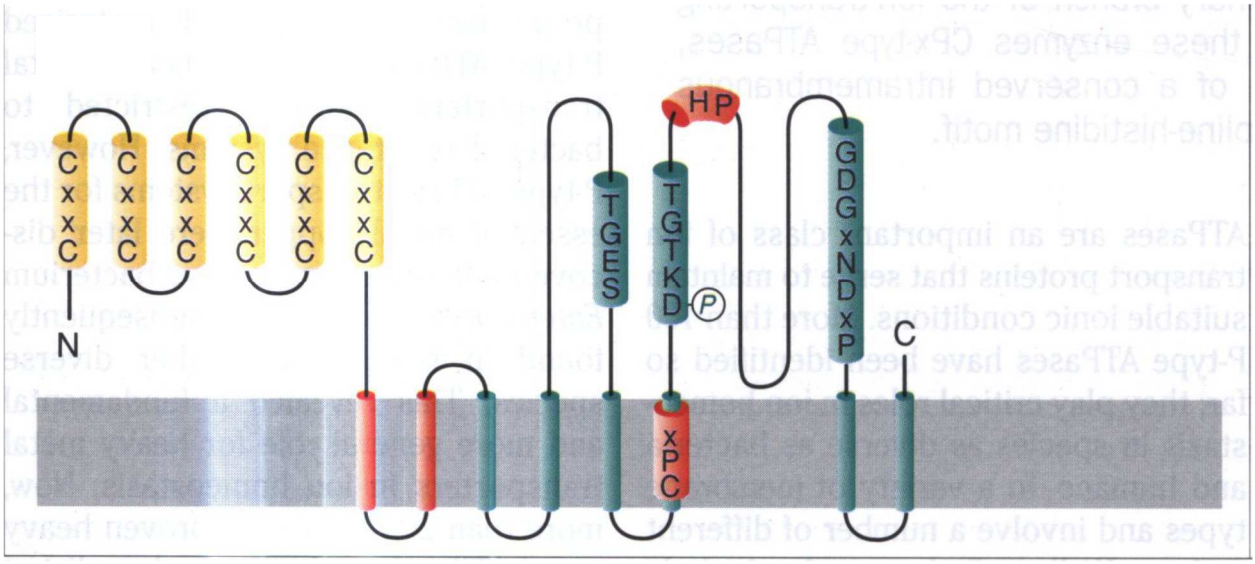


Figure 2.2. *Schematic representation of membrane topology of heavy metal or CPx-type ATPases. The TGES or phosphatase domain, DKTGT or Phosphorylation domain and GDGxNDxP or the ATP binding domain is common in all P-type ATPases. The conserved intramembranous CPx sequence, the HP or Histidine-Proline dipeptide locus and the N-terminus heavy-metal binding motifs CXXC are only characteristic of P-type ATPases involved in transport of heavy metals – also called CPx-type ATPases* (39).

2.3.3 Cation Diffusion Facilitator proteins

The Cation Diffusion Facilitators or CDFs are a family of membrane bound proteins that are considered as the third type of efflux pumps involved in the transport of heavy metal cations. These efflux pumps are common in all domains of life and are most commonly involved in the transport of divalent metal ions such as Zn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} and Fe^{2+}. The transport of metals using CDF proteins is driven by a concentration gradient, a chemiosmotic gradient or a pH gradient. CzcD from *C. metallidurans* was the first bacterial CDF Family protein to be characterized (27). Although most bacterial CDF Family proteins are involved in metal tolerance/resistance by exporting divalent metal ions, some bacterial CDFs have also been shown to be involved in other activities such as antibiotic resistance (CepA of *Klebsiella pneumoniae*) and magnetosome formation (MamB and MamF in *Magnetospirillum gryphiswaldense*) (41–43).

Current scientific data available on CDF transporters indicate that they function in an antiport mechanism to export divalent actions, using either a proton or potassium gradient. Most CDF proteins characterized in bacteria have a size of 300 – 400 amino acids and are predicted to function as dimers. Prokaryotic CFD proteins have six transmembrane helices. Residues that are predicted to function in metal binding are found in TM2 and TM5 while residues in TM3 form a hydrophobic gate that facilitates metal transport against a H^+/K^+ gradient (44). Additionally, some studies have reported the presence of a histidine-rich loop, known as intracellular loop 2 (IL2) which play a role in metal selectivity (45). A region of approximately 100 amino acids at the C-terminus of CFD proteins protrudes into the cytoplasm forming the C-terminal domain (CTD).

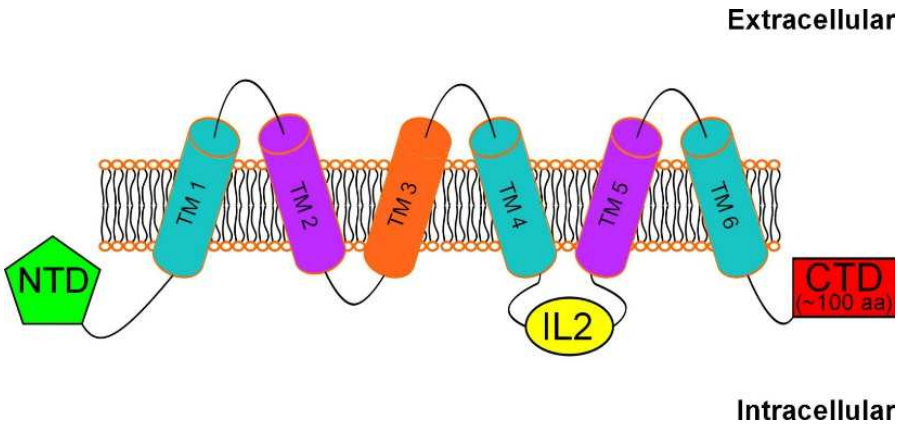


Figure 2.3. *Schematic representation of bacterial Cation Diffusion Facilitators (CDF). CDFs contain six transmembrane helixes (TM 1 -6). Metal binding sites are found in TM2 and TM5 or in the His-rich IL2 loop. Residues in TM3 are predicted to form a hydrophobic gate* (41).

CHAPTER III

ANALYSIS OF GENES INVOLVED IN METAL RESISTANCE IN *SHEWANELLA ONEIDENSIS* MR-1

1. INTRODUCTION

Transition metals have been known to play important roles in various physiological processes. They can act as cofactors that activate regulatory proteins as well as form cores of active centers of many enzymes. One of the best studied examples of a regulatory protein requiring metals for its activity is FNR of *E. coli*, which requires a Fe-S cluster for sensing O_2 and for activation. *S. oneidensis* also has many important metalloproteins including *c*-type cytochromes, which are heme containing proteins. In addition to the presence of essential metalloproteins within the cells, *S. oneidensis* also utilizes various metal oxides as electron acceptors during anaerobic respiration.

Although metals are essential for proper functioning of cells, they can also be extremely toxic when present in high concentrations due to their ability to form oxidative species that can easily denature cellular proteins. Therefore, the concentration of metals within the cell needs to be tightly regulated. Bacterial cells have evolved various mechanisms by which they can achieve metal homeostasis. They can regulate the uptake of metals depending on cellular requirement as well as express chaperones that bind free metal ions inside the cell, thus preventing them from denaturing other cellular components. When metal concentration within the cells are high, cells can use metal efflux pumps to rid themselves of excess metal. Bacterial cells have three different types of efflux pumps. These are the HME RND family of proteins, the CPx-type ATPases and Cation Diffusion Facilitator (CDF) family proteins.

CPx-type ATPases export metals out of the cytoplasm at the expense of ATP hydrolysis. The HME RND proteins, on the other hand, act in antiport fashion and transport metal ions at the expense of proton-motive force. The CDF family of metal transporters are usually involved in the transport of divalent metal ions and do so by using a concentration gradient, a chemiosmotic gradient or a pH gradient.

In *S. oneidensis* MR-1, two putative CPx-type ATPases encoded by SO_1689 (*copA*) and SO_2359 had been previously identified (46). Three potential RND efflux pumps have also been identified in the MR-1 genome. SO_0518-20 and SO_4596-98 are found on the chromosome while SO_A0153-55 are located on the megaplasmid. In addition to metal efflux pumps, predicted regulators of metal efflux pumps, CueR and ZntR, have also been identified in the MR-1 genome. The following work attempts to understand the role of these metal efflux pumps and their regulators in metal tolerance.

2. MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 3.1. LB (Lysogeny Broth) medium was used routinely during this study for aerobic growth of *Shewanella oneidensis* MR-1 and *E. coli* strains at 30°C and 37°C respectively. For anaerobic growth of *S. oneidensis* MR-1, LB medium was supplemented with 0.02% casamino acids (47) and 10mM Fumarate as an electron acceptor. Antibiotics chloramphenicol (25 μg/ml) and gentamycin (25 μg/ml) were added as needed.

2.2 Generation of chromosomal deletion mutants

To generate chromosomal deletions, the suicide vector pER21 was used (48). DNA fragments of approximately 1 kb in size flanking the gene(s) to be deleted were amplified by PCR using primers listed in Table 3.2 and Phusion polymerase (New England BioLabs, Ipswich, MA) using *S. oneidensis* MR-1 genomic DNA as template. Internal primers were designed to include restriction enzyme sites that would allow the ligation of the fragments obtained by PCR amplification. The ligated 2 kb fragments were then cloned into the *SmaI* site of the suicide vector pER21.

Recombinant plasmids were transferred into *E. coli* EC100 (Epicenter Technologies) cells by electroporation or by transformation of competent *E. coli* DH5α by heat shock and allowed to grow on LB plates containing gentamycin, bromo-chloro-indolyl-galactopyranoside (X-gal) and isopropyl β-D-1-thiogalactopyranisode (IPTG) for blue-white screening. Plasmids containing the insert of interest were confirmed by PCR using the outside flanking primers.

Plasmids with the appropriate inserts were used to transform *E. coli* β2155(49) by electroporation and were then introduced into *S. oneidensis* MR-1 wild type or mutant strains by conjugation. Transconjugants were selected by plating on LB agar containing gentamycin. To isolate mutants with the proper deletions, the primary integrants were allowed to undergo a second recombination and were then plated on LB (no salt) media containing 5% sucrose. Sucrose resistant colonies were screened by PCR to confirm deletion of the target gene (48).

2.3 Complementation of chromosomal deletion mutants

To complement deletion mutants, DNA fragments were amplified by PCR using Phusion Polymerase (New England Biolabs) and primers listed in Table 3.3. Amplified fragments were cloned into pJBC1 (50), followed by electroporation of *E. coli* EC100. Recombinant plasmids were screened for the presence of insert of interest and were then used to transform *E. coli* β2155. Plasmids were then transferred into *S. oneidensis* MR-1 mutant strains from *E. coli* β2155 via conjugation. Complemented strains were maintained on LB plates supplemented with chloramphenicol.

2.4 Growth assays

Cells were grown overnight in LB media. For aerobic growth assays, 6 µl of stationary phase cultures were used to inoculate 600 µl of LB with or without metals being tested, in 24-well polystyrene plates (Falcon) and grown at 30°C for up to 18 hours. OD_{600} was measured using the Infinite 200 Pro Microplate reader (Tecan). Concentration of metals tested are as follows: 1.5 mM

and 1.75 mM $CuSO_4$, 1.5 mM and 2 mM $ZnSO_4$, 0.25 mM and 0.5 mM $NiSO_4$, and 0.1 mM and 0.125 mM $CoSO_4$.

For anaerobic growth, 100 µl of overnight cultures were used to inoculate 10 ml LB supplemented with 0.02% casamino acids and 10 mM Fumarate as an electron acceptor. Cultures were grown in Hungate tubes at 30°C for up to 24 hours and OD_{600} was measured on a Genesys 20 spectrophotometer (Thermo Scientific) to determine growth. Metal concentrations used were as follows: 0.25 mM and 0.5 mM $CuSO_4$, 0.75 mM and 1.25 mM $ZnSO_4$, 1 mM and 1.25 mM $NiSO_4$, and 0.25 mM and 0.5 mM $CoSO_4$.

2.5 Promoter activity assays

Promoter activity assays were done using a constructed promoter-*lacZ* fusion plasmid. The promoter region directly upstream of *copA* (SO_1689) was amplified from *S. oneidensis* MR-1 genomic DNA by PCR using Phusion Polymerase and the primers listed in Table 3.3. Primers were designed to include HindIII and BamHI restriction sites on the forward and reverse primers respectively. The resulting 446 bp fragment was digested with HindIII and BamHI and cloned into pMC10 (51) digested with the same enzymes. Verified recombinant plasmid was transformed into *E. coli* β2155 followed by conjugation with *S. oneidensis* MR-1 wild type and mutant strains.

S. oneidensis cultures containing the promoter-*lacZ* fusion plasmid were assayed for β-galactosidase activity as previously described (52). Briefly, strains were grown overnight in LB with or without metals. Density of cultures were measured at 600 nm followed by dilution to an OD_{600} of approximately 0.2 using Z buffer (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.001 M $MgSO_4.7H_2O$, 0.05 M β-mercaptoethanol). Cells were lysed by adding 50 µl of

0.1% SDS and 100 µl of chloroform and vortexing for 5 – 10 seconds. 0.5 ml of 1 M ortho-nitrophenyl-β-galactoside (ONPG, Thermo Scientific), a lactose analog and an artificial substrate for β-galactosidase, was added and tubes were incubated at 30°C until a yellow colour was observed. To stop the reaction, 0.5 ml of 1 M Sodium Carbonate was added. OD was measured at 550 nm and at 420 nm. β-galactosidase activity was calculated using the following formula:

$$\text{Miller Units} = 1000 \text{ X } \frac{(OD_{420}\text{-}1.64 \text{ x } OD_{550})}{T \text{ x } V \text{ x } OD_{600}}$$

T = Incubation time (in minutes) with ONPG at 30°C

V = Amount of culture used/ml of Z Buffer

Table 3.1. Bacterial strains used in this study

Strain	Description	Source
Shewanella oneidensis		
MR-1	Oneida Lake *S. oneidensis* isolate	(53)
SR1492	MR-1 ΔSO_0518-20	This study
SR1536	MR-1 ΔSO_1687 (*cueR*)	This study
SR1493	MR-1 ΔSO_1689 (*copA*)	This study
SR1697	MR-1 ΔSO_0443 (*zntR*)	This study
SR1698	MR-1 ΔSO_0443-44	This study
SR1699	*ΔcueR ΔzntR*	This study
SR1700	ΔSO_0518-20 with p518-20C	This study
SR1701	*ΔcueR* with pCueRC	This study
SR1702	*ΔcopA* with pCopAC	This study
SR1703	*ΔzntR* with p443-44C	This study
SR1704	ΔSO_0443-44 with p443-44C	This study
SR1705	*ΔcueR ΔzntR* with p443-44C	This study
SR1706	*ΔcueR ΔzntR* with pCueRC	This study
SR1707	*ΔcueR* with pCopAP	This study
SR1708	*ΔzntR* with pCopAP	This study
SR1709	*ΔcueR ΔzntR* with pCopAP	This study

Strain	Description	Source
Escherichia coli		
EC100D+	*E. coli* EC100 derivative, pir^+	Epicenter Technologies
β2155	pir::RP4, Km^R	(49)

Table 3.2. Plasmids used in this study

Plasmid	Description	Source
pER21	R6K *ori*, Gm^R, SacB, *lacZ* α - fragment	(54)
pJBC1	Cloning and sequencing vector, Cm^R	(50)
pMC10	Promoter probe vector, *lacZ*, Cm^R	(51)
Plasmids for Chromosomal deletions		
p518-20	ΔSO_0518-20 in pER21	This study
pCueR	ΔSO_1687 in pER21	This study
pCopA	ΔSO_1689 in pER21	This study
pZntR	ΔSO_0443 in pER21	This study
p443-44	ΔSO_0443-44 in pER21	This study
Plasmids for Complementation		
p518-20c	SO_0518-20 fragment in pJBC1	This study
pCueRC	SO_1687 fragment in pJBC1	This study
pCopAC	SO_1689 fragment in pJBC1	This study
p443-44C	SO_0443-44 fragment in pJBC1	This study

Plasmid	Description	Source
Plasmid for promoter expression		
pCopAP	SO_1689 promoter in pMC10	This study

Table 3.3. Primers used in this study

Name	Sequence	Description
Chromosomal Deletion Primers		
518F	GATCGATTGGGTGGGTAATGCGGTGCG	ΔSO_0518-20 Upstream DNA Fragment
518R	GATCGGATCCCGTGGCATTGGCAGTCGAGGC	
518F1	GATCGGATCCGCGTCTATCACCCAATGGCG	ΔSO_0518-20 Downstream DNA Fragment
518R1	GATCCAGCTATCCCAAGCGTGCC	
1687F	GTACCGAACTTGTGTATGCGCCAG	ΔSO_1687 Upstream DNA Fragment
1687R	GATCGGATCCCTAACTCAGTTTCTAATCCCGCAGCC	
1687F1	GATCGGATCCCCCTGTGTGCTTTGCCACTTCGCC	ΔSO_1687 Downstream DNA Fragment
1687R1	CCATGCAGTGCTGGTACCTAACACG	
1689F	GATCGATGGGTCGATGCGAATAAGCTGG	ΔSO_1689 Upstream DNA Fragment
1689R	GATCGGATCCCGGCCTCGTTTGCGCAGAAC	
1689F1	GATCGGATCCCGGCCTCGTTTGCGCAGAAC	ΔSO_1689 Downstream DNA Fragment
1689R1	GATCGCGCCTGTGGTGACGTCACTC	
443UpF	GGTGCGTGGGAACTTTGAGCC	ΔSO_0443 Upstream DNA Fragment
443UpR	CGTAAGGATCCCGGCTAACTCACCAATTCGGTAC	

443DnF	CGCTTGGATCCCAGCAGATAAATCAGGGG	ΔSO_0443 Downstream DNA Fragment
443DnR	GCAACCTGCGAGCTTGTGCTGC	
444DnF	CGCTTGGATCCCCGCAGCGATTGTTGCTC	ΔSO_0443-44 Downstream DNA Fragment
444DnR	CCAGATTCGCCTCACCCC	

Complementation Primers

C518F	GATCAAGCTTATCGCTGGCTGCGCTTCGTAAGTGC	SO_0518-20 Complementation DNA Fragment
C520R	CATGGATCCCGAAACAGCGTACATTGCCCC	
1687compF	GGCGAAGTGGCAAAGCACACAGGG	SO_1687 Complementation DNA Fragment
1687compR	GGCTGCGGGATTAGAAACTGAGTTAG	
1689compF	ATGTCTCAAATAAAACTCTACGTCGCC	SO_1689-90 Complementation DNA Fragment
1689compR	CGGACAAACACGGAAGATAACCTCC	
443compF	GGCGCGCGTATTCTACCG	SO_0443-44 Complementation DNA Fragment
444compR	GCAAGTTAGCGATTCCCTGTGAGC	

Primers for Promoter expression

1689PF	GCGTAAGCTTCGCCTATTTTCATCTTGACCTCGG	SO_1689 Promoter DNA Fragment
1689PR	CCTAAGGATCCGAGACATAGAAGATCCCTCACTTGC	

3. RESULTS

3.1 Effect of metal sulfates on aerobic and anaerobic growth of *S. oneidensis* MR-1

To determine the sensitivity of *S. oneidensis* MR-1 to various metals, we grew wild-type cells under aerobic and anaerobic conditions in LB supplemented with two different concentrations for each metal tested, under both aerobic and anaerobic conditions.

Our results indicated that wild type MR-1 cells grown in LB aerobically with 1.5mM $ZnSO_4$, 0.25 mM $NiSO_4$ and 0.1 mM $CoSO_4$, show a slow growth phenotype with a 4 – 5-hour lag phase. When grown with 2mM $ZnSO_4$, MR-1 cells were unable grow. Growth with 0.5 mM $NiSO_4$ and 0.125 mM $CoSO_4$ was also slow, with 7 – 8-hour lag phase. Growth with 1.5mM and 1.75mM $CuSO_4$ also resulted in slower growth. The final OD_{600} of all cultures tested, except 2 mM $ZnSO_4$, were comparable to cells grown without any addition metals in the media. (Fig. 3.1).

To test the response of *S. oneidensis* to metals under anaerobic conditions, cells were grown in LB supplemented with 0.02% casamino acids and 10 mM Fumarate, with or without metals. *S. oneidensis* MR-1 showed growth deficiencies with all the metals that were tested as compared to its growth without any additional metals, with higher concentration of metals resulting in a more prominent growth defect as compared to lower concentration of metal tested (Fig. 3.2).

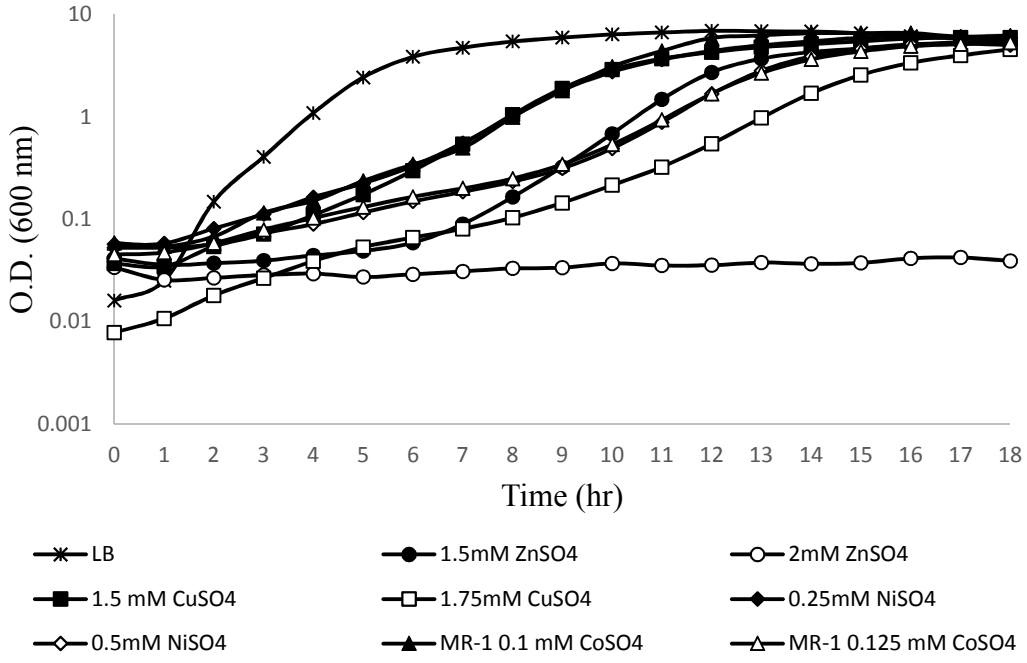


Figure 3.1. *Effect of metal sulfates on aerobic growth of S. oneidensis MR-1 wild-type cells. The bacterium showed growth with long lag phases when grown with both concentrations of $CuSO_4$, $NiSO_4$ and $CoSO_4$ and with 1.5 mM $ZnSO_4$ and was unable to grow with 2 mM $ZnSO_4$.*

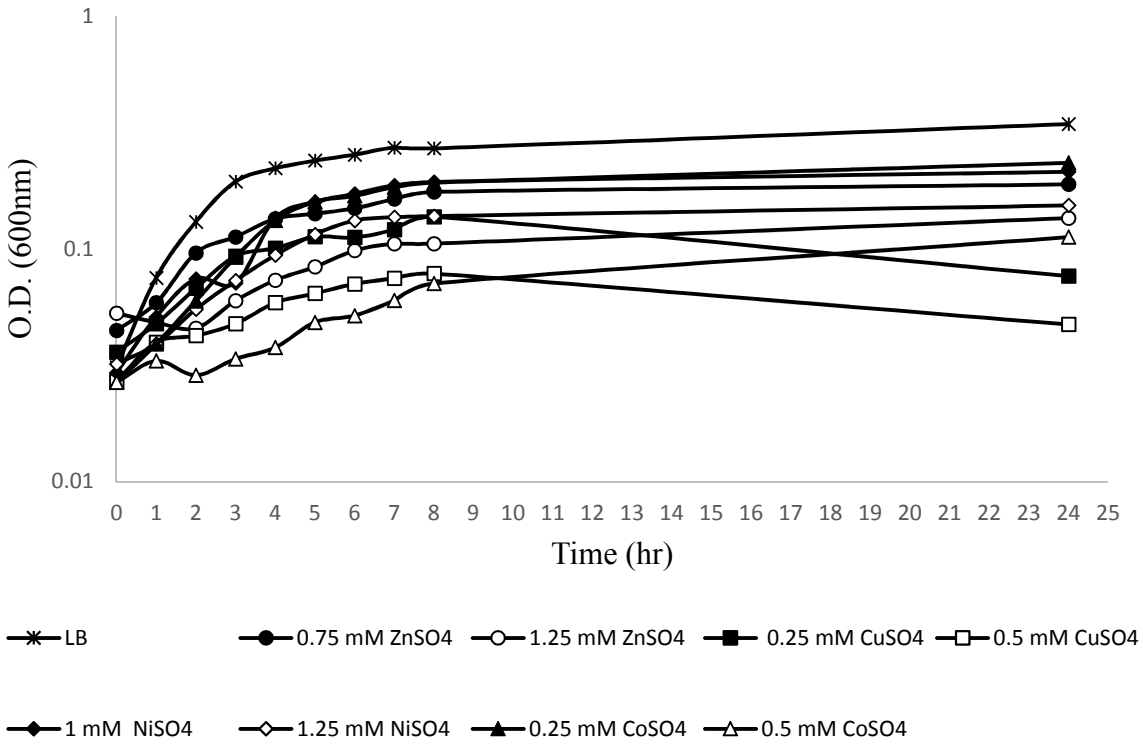


Figure 3.2. *Effect of metal sulfates on anaerobic growth of S. oneidensis MR-1 wild-type cells. Growth deficiency was observed on growth with all metals tested.*

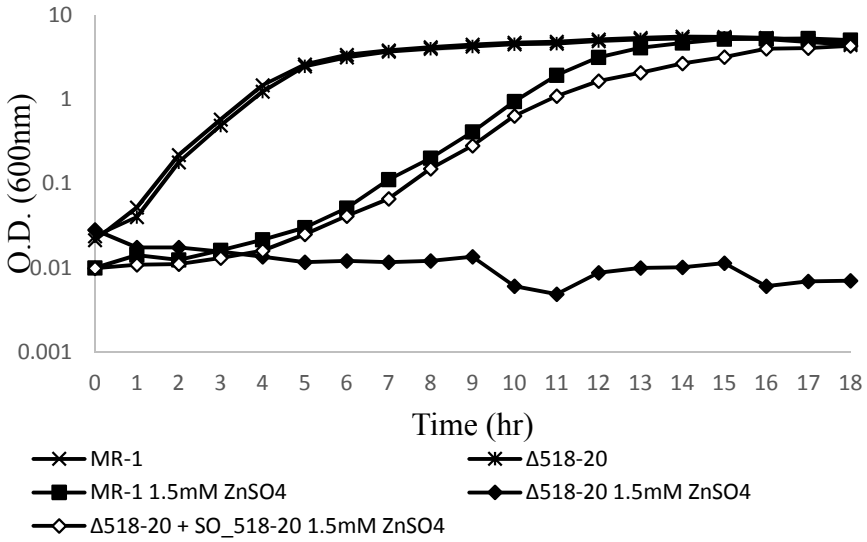
3.2. SO_518-20 encodes an HME RND pump that exports zinc under aerobic conditions and nickel under anaerobic conditions

Previous studies with *S. oneidensis* showed the presence of genes predicted to encode Heavy Metal Efflux RND pumps that are thought to be involved in transport of Cobalt, Zinc and Cadmium (Czc type) or Copper (Cus-like proteins). To determine the role of one of these predicted HME RND efflux pumps in transport of metals in *S. oneidensis* MR-1, we generated a deletion mutant that lacks all components of the efflux pump [SO_0518 *(czcC)*, SO_0519 (*czcB*) and SO_0520 (*czcA*)] using the chromosomal deletion method described above. The deletion mutant was tested for growth in the presence or absence of various metals as described above, both aerobically and anaerobically.

When grown aerobically without addition of metals in the media, the ΔSO_0518-20 mutant grew similar to wild-type MR-1, indicating that the deletion did not affect growth in the absence of any metal stress. However, when the strains were grown in the presence of 1.5 mM zinc, the mutant cells did not grow. Introduction of a plasmid that carries SO_0518-20 fragment into the deletion mutant resulted in complementation and restoration of resistance to zinc to wild-type levels (Fig. 3.3 A).

Under anaerobic conditions, the deletion mutant showed a similar growth phenotype as the wild-type cells when grown with 0.75 mM zinc. In the presence of 1.25 mM zinc, the mutant cells were unable to grow, and this growth defect was restored to wild-type level growth upon complementation with a plasmid carrying SO_0518-20 (Fig. 3.3 B).

A.



B.

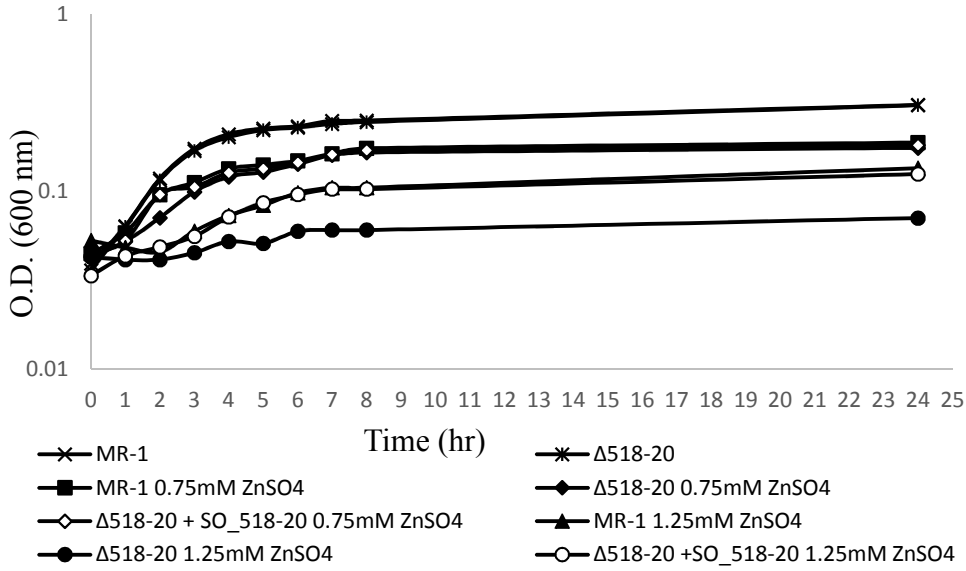


Figure 3.3. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1, ΔSO_0518-20 mutant and ΔSO_0518-20 complemented with plasmid containing SO_0518-20 fragment from MR-1 in the presence of* $ZnSO_4$. ***A.)*** *Mutants lacking the predicted HME RND pump did not grow in the presence of zinc. Introduction of plasmid containing SO_0518-20 genes into the mutant restored resistance against zinc to wild-type levels.* ***B.)*** *Growth deficiency observed in mutant cells when grown with 1.25mM zinc, which was restored upon complementation with plasmid containing SO_0518-20 genes.*

In addition to testing the growth of the ΔSO_0518-20 mutant strain with zinc, its response to the presence of other metals such as nickel, copper and cobalt was also tested. When grown with 1 mM Nickel under anaerobic conditions, the mutant grew slower than the wild-type and complementation of the mutant with plasmid containing SO_0518-20 fragment restored growth to wild-type levels. Under aerobic conditions also, the mutant strain showed severe growth defect in the presence of 0.25 mM and 0.5 mM $NiSO_4$. Complementation of this mutant restored the wild-type growth phenotype (Fig. 3.4). Testing of the ΔSO_0518-20 mutant strain with other metals did not reveal any differences in growth phenotype when compared to wild-type MR-1 – either aerobically or anaerobically (Fig. 3.5 – 3.6). These results suggest that SO_0518-20 encodes an efflux pump complex that appears to transport zinc and nickel under both aerobic and anaerobic conditions.

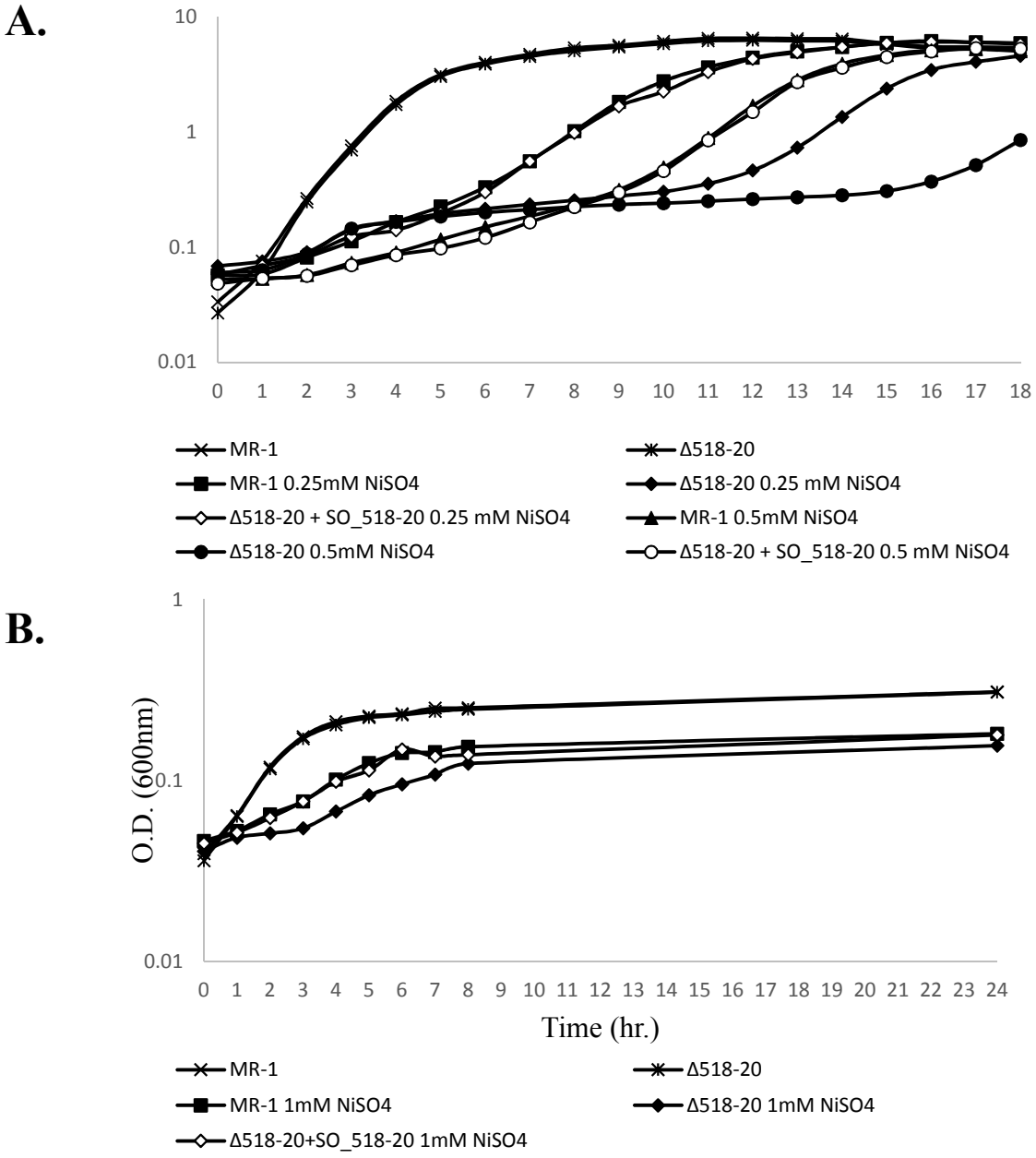
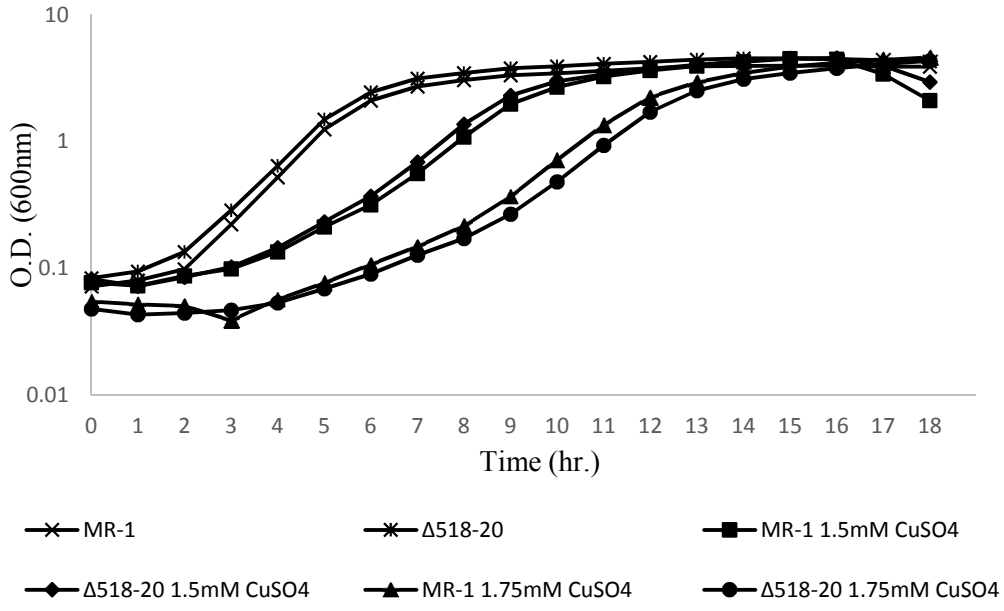


Figure 3.4. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_0518-20 mutant in the presence of $NiSO_4$.* ***A.)*** *ΔSO_0518-20 grew slower than wild-type MR-1 cells in the presence of 0.25 mM and 0.5mM $NiSO_4$.* ***B.)*** *Under anaerobic conditions, mutant ΔSO_0518-20 grew slower as compared to MR-1. Growth was restored to wild type levels on introduction of plasmid containing SO_518-20.*

A.



B.

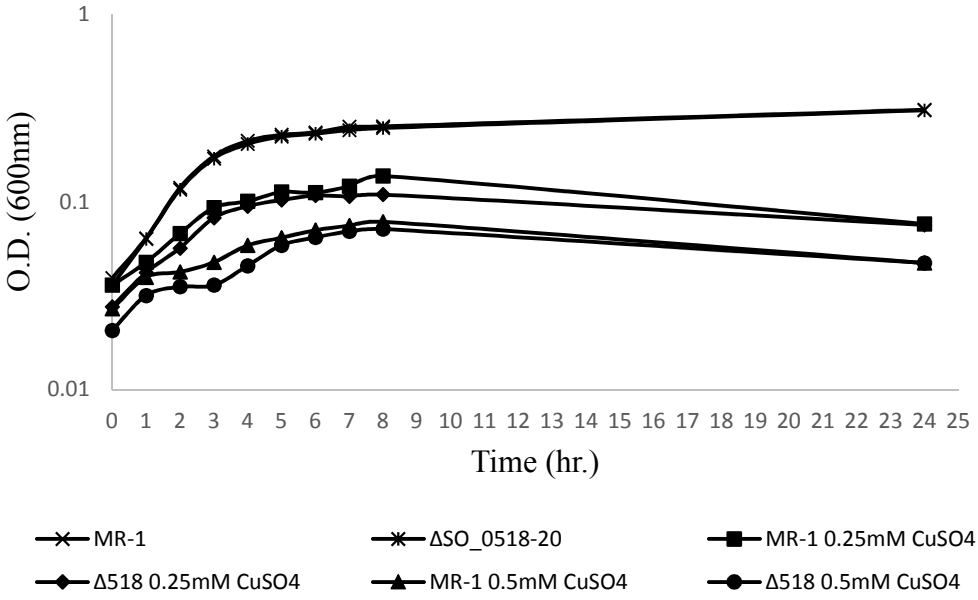
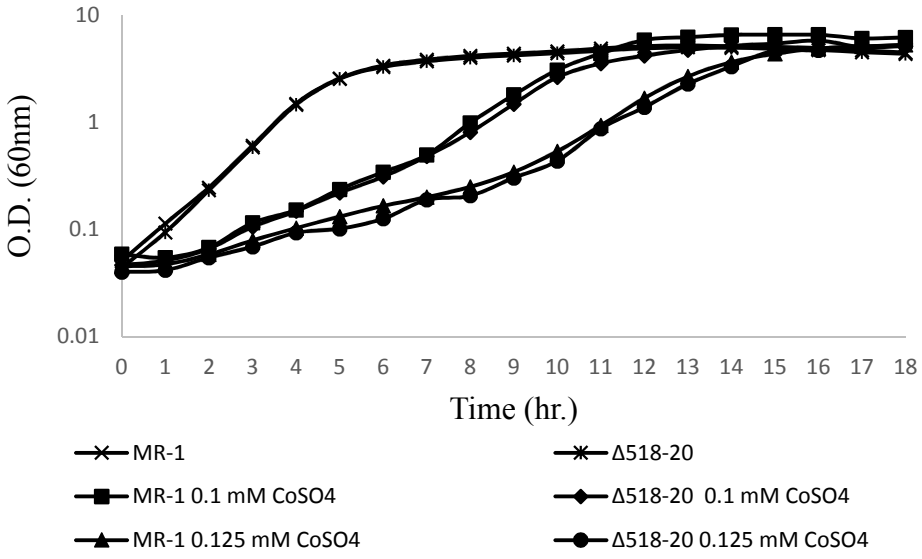


Figure 3.5. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_0518-20 mutant in the presence of $CuSO_4$. Under both aerobic and anaerobic conditions, the mutant and wild-type cells showed a similar growth phenotype.*

A.



B.

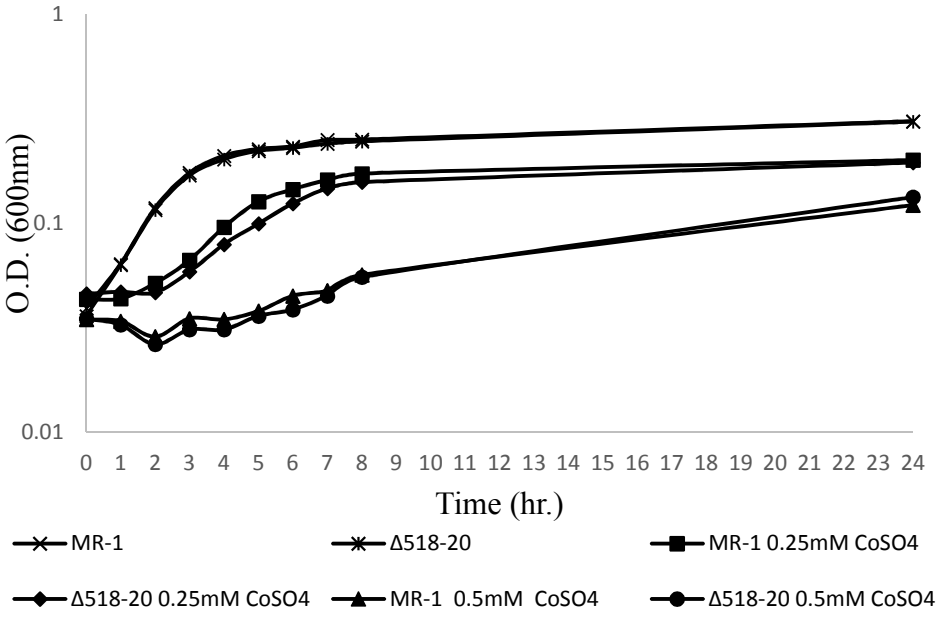


Figure 3.6. *Aerobic* ***(A)*** *and anaerobic* ***(B)*** *growth of wild-type S. oneidensis MR-1 and ΔSO_0518-20 mutant with $CoSO_4$. Under both conditions tested, the mutant and wild-type cells grew slower than when grown without additional metals, however no difference in growth was observed between the mutant and wild type strains.*

3.3. CopA, a CPx-type ATPase is only involved in transport of copper in *S. oneidensis*

Genome analysis of *S. oneidensis* revealed the presence of two CPx-type ATPases encoded by SO_1689 and SO_2539, that are predicted to be involved in copper transport. SO_1689, which has been annotated as *copA*, encodes a protein that has sequence similarity to *E. coli* CopA. Previous studies in *S. oneidensis* have shown that the induction of *copA* expression occurs under anaerobic conditions in presence of low concentration of copper (46). To test if this predicted CopA homolog is involved in the export of Cu in *S. oneidensis*, we generated a deletion mutant and tested its growth compared to wild-type MR-1 using the same growth conditions as described in the previous section.

Deletion of *copA* did not cause any growth defects in the mutant when grown in the absence of additional metals in the media. When tested for growth under aerobic conditions, the only defect in the growth of the *ΔcopA* mutant was observed when it was grown in the presence of 1.75 mM $CuSO_4$. The growth phenotype was restored to wild-type levels upon complementation of the mutant strain with a plasmid containing SO_1689. When both wild type and mutant strains were grown under anaerobic conditions, the mutant cells grew slower than the wild-type when grown with 0.25 mM copper, while it was unable to grow in the presence of 0.5 mM copper (Fig. 3.7). Growth was restored to wild-type levels by complementation with plasmid containing SO_1689. When growth with other metals such as zinc, nickel and cobalt was tested, no significant difference was observed in the growth of wild type and *ΔcopA* mutant (Fig. 3.8 – 3.10).

Although previous studies have shown that *copA* is only induced to high levels under anaerobic conditions (46), our results suggest that this efflux pump is involved copper resistance under both aerobic and anaerobic growth conditions. Additionally, this CPx-type ATPase seems to show substrate specificity to copper and is not involved in the transport of any other metals tested in this study.

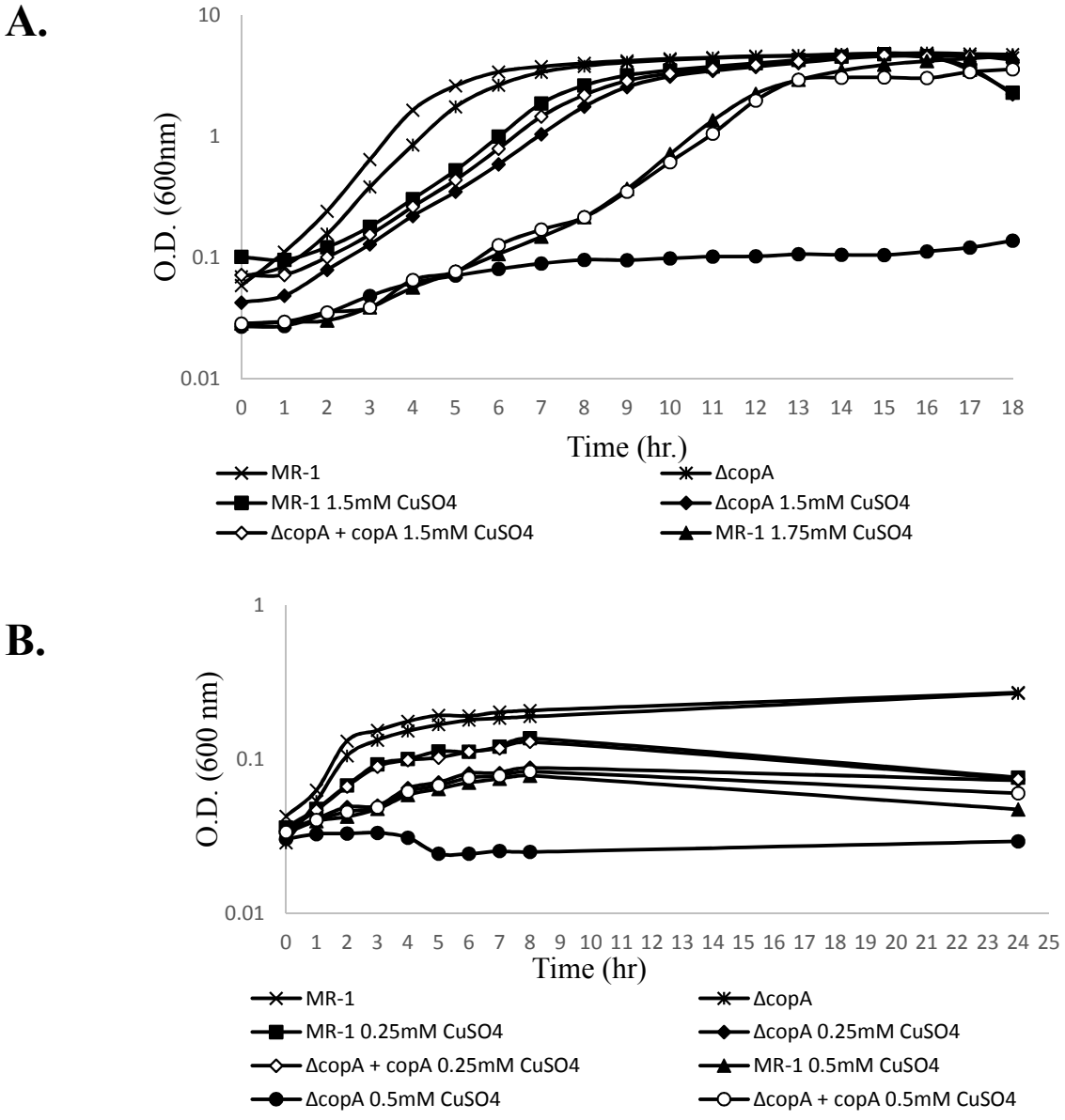
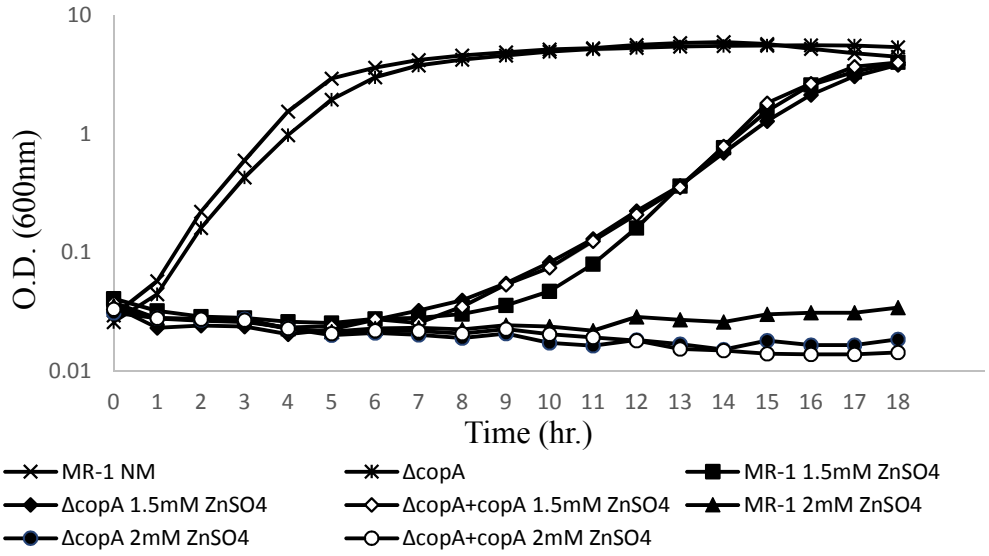


Figure 3.7. *Aerobic and anaerobic growth of wild-type S. oneidensis MR-1 and ΔSO_1689 (copA) mutant in the presence of $CuSO_4$.* ***A.)*** *Upon deletion of copA, cells are unable to grow aerobically in the presence of 1.75mM Cu and complementation of mutant strain results in restoration of wild-type growth phenotype.* ***B.)*** *When grown anaerobically, a growth deficiency is observed in the mutant strain in the presence of 0.25 mM CuSO4, while it is unable to grow with 0.5 mM CuSO4. Complementation of mutant restores growth to wild-type levels.*

A.



B.

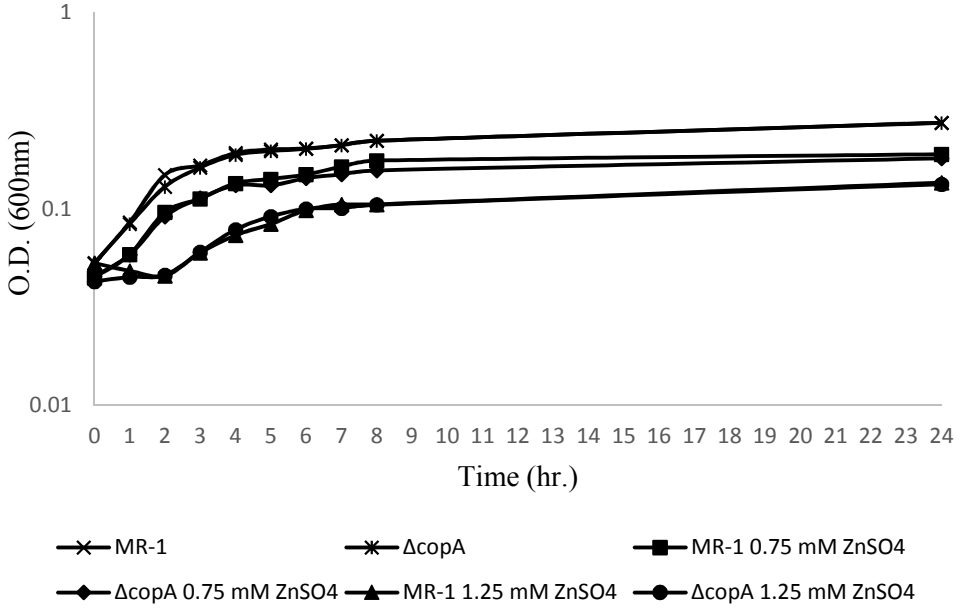
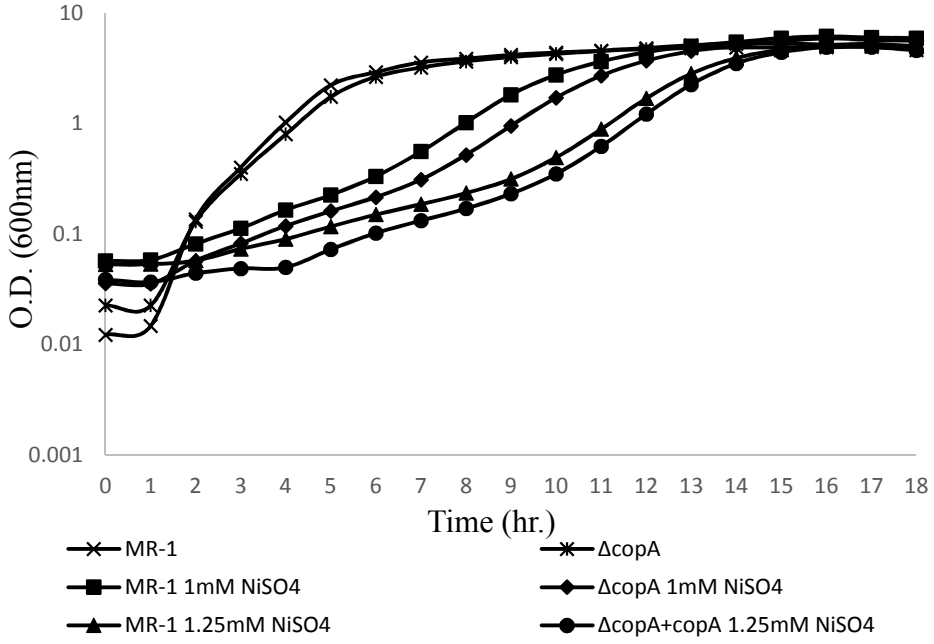


Figure 3.8. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_1689 (copA) mutant in the presence of* $ZnSO_4$*. Under both conditions tested, the mutant and wild-type cells grew slower than when grown without additional metals, however no difference in growth was observed between the mutant and wild type strains.*

A.



B.

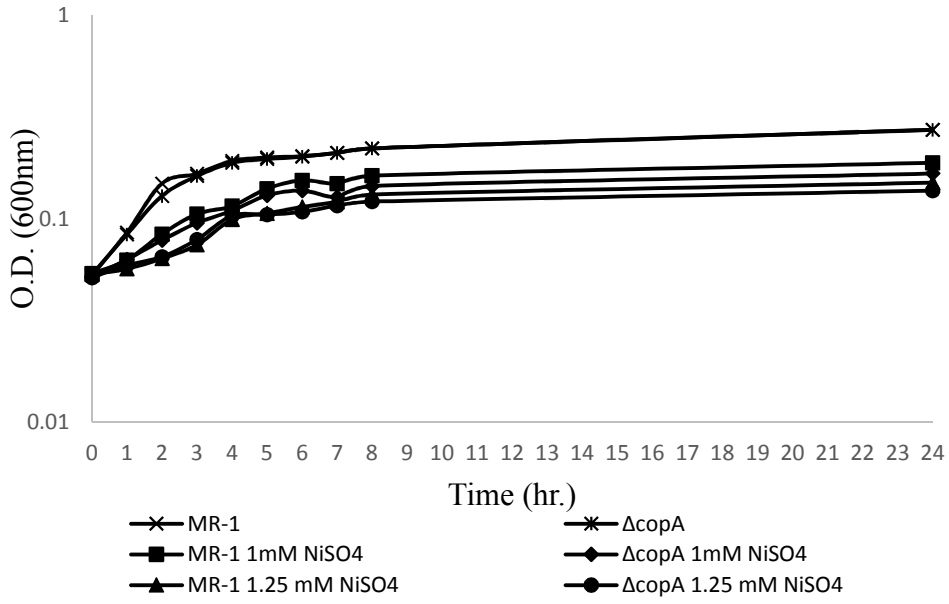
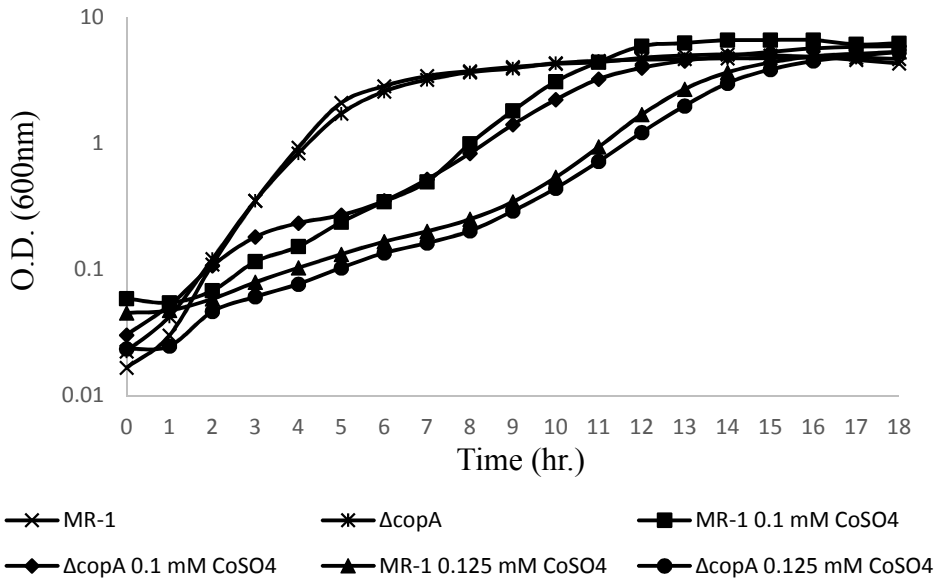


Figure 3.9. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_1689 (copA) mutant in the presence of* $NiSO_4$.

A.



B.

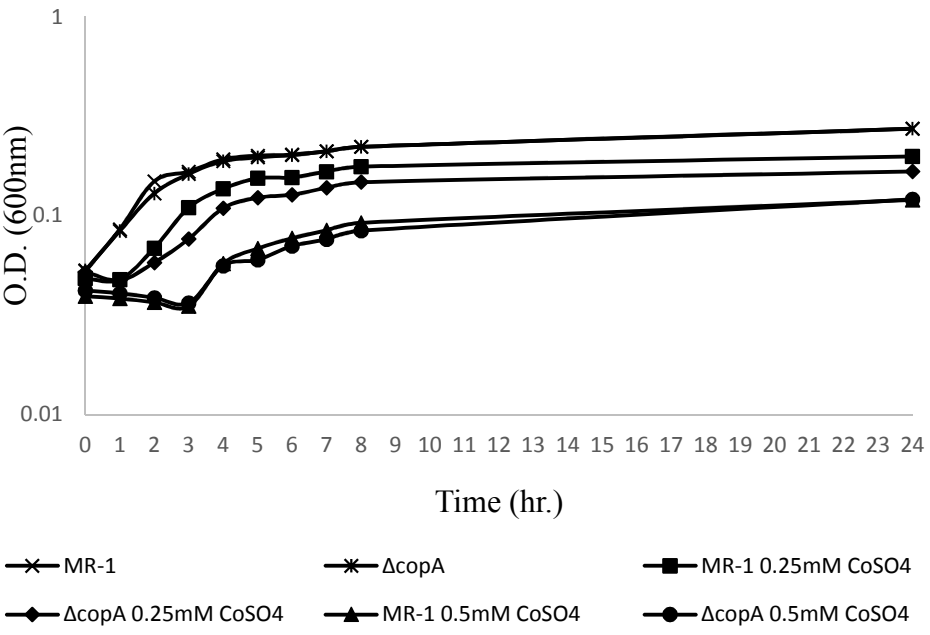


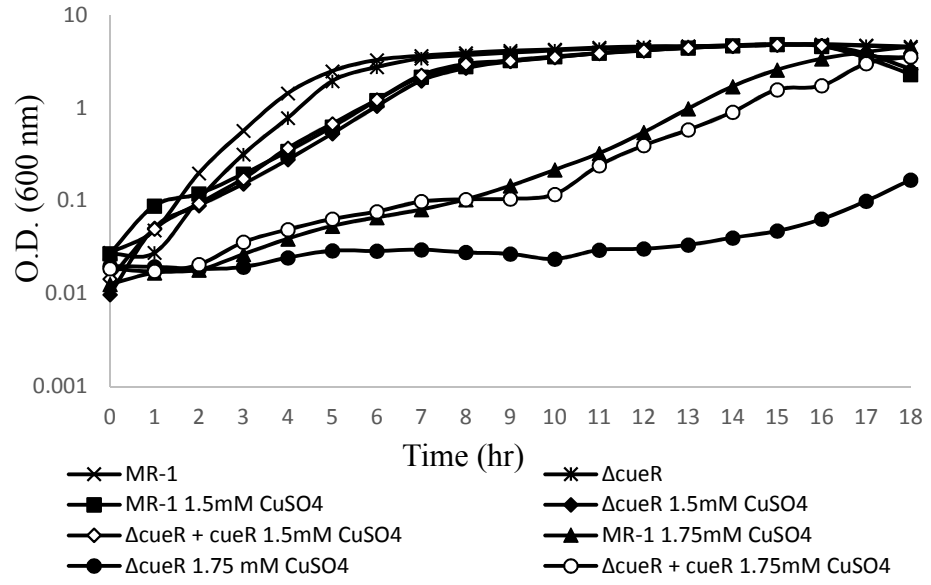
Figure 3.10. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_1689 (copA) mutant in the presence of* $CoSO_4$.

3.4. CueR, a CPx-type ATPase regulator is involved in transport of copper.

In *E. coli*, the CPx-type ATPases are regulated by metal activated homologues of MerR. Two examples of these regulators include the Copper efflux Regulator - CueR and the Zinc Responsive Transcriptional Regulator – ZntR. Analysis of the *S. oneidensis* genome indicated the presence of a CueR-like transcriptional regulator upstream of CopA – the CPx type ATPase that is involved in copper translocation We hypothesized that CueR may regulate the expression of *copA* and that loss of this gene would result in a phenotype similar to the *ΔcopA* deletion strain. To test this hypothesis, a deletion mutant lacking *cueR* was generated and the strain was tested for growth deficiencies in the presence of metals described above.

The *ΔcueR* mutant showed a severe growth defect in the presence of 1.75 mM $CuSO_4$ aerobically and 0.25 mM $CuSO_4$ anaerobically. No growth was detected when the mutant strain was grown with 0.5 mM $CuSO_4$ anaerobically (Fig. 3.11). These results are similar to the results we obtained for the *ΔcopA* mutant (Fig. 3.7). Growth was restored to wild type levels by complementation of the mutant with a plasmid that carries SO_1689. Growth of the deletion strain under all other conditions tested did not reveal any significant difference between wild type and mutant *ΔcueR* strain (Fig. 3.12 – 3.14). These results suggest that CueR regulates a copper efflux pump and its loss causes an increased sensitivity to copper.

A.



B.

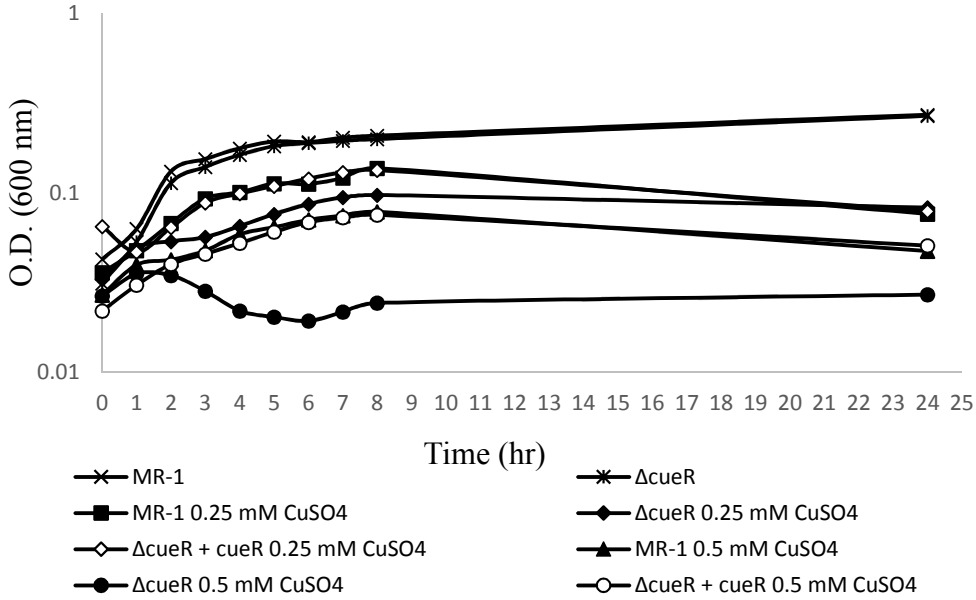
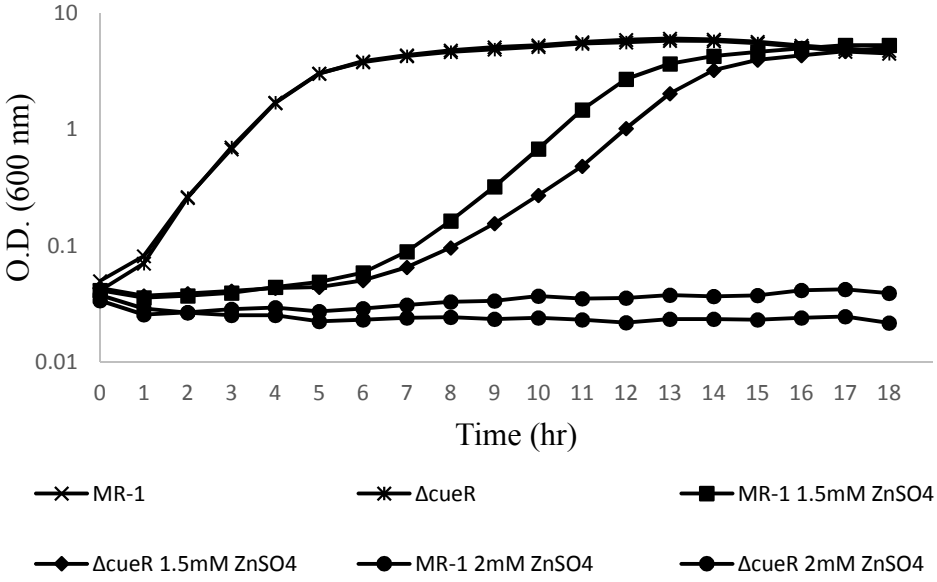


Figure 3.11. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_1687 (cueR) mutant in the presence of $CuSO_4$.* ***(A.)*** *Deletion of cueR causes severe growth defect in the presence of 1.75 mM $CuSO_4$ when grown aerobically.* ***(B)****Anaerobically, mutant cells grew slower than wild-type when grown with 0.25 mM copper, while no growth was detected in the mutant strain in the presence of 0.5 mM copper.*

A.



B.

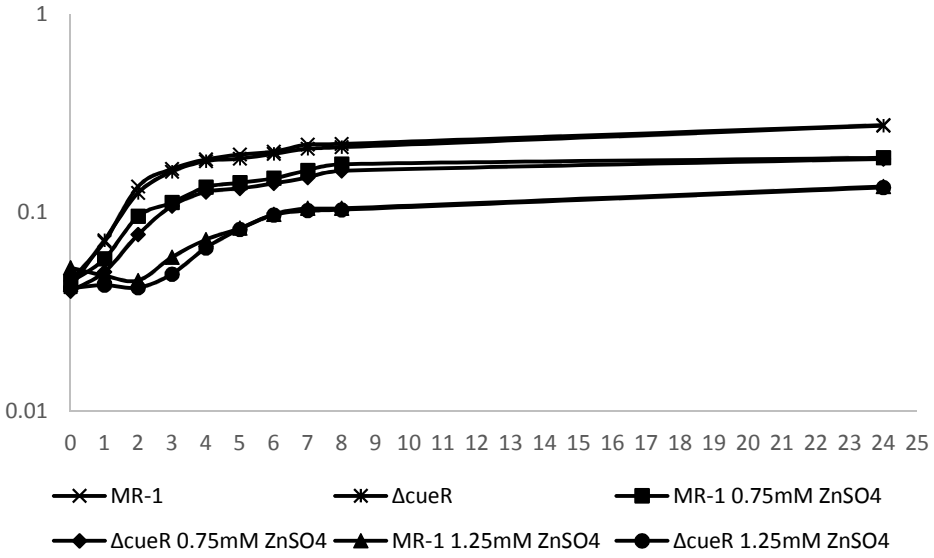


Figure 3.12. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_1687 (cueR) mutant in the presence of* $ZnSO_4$.

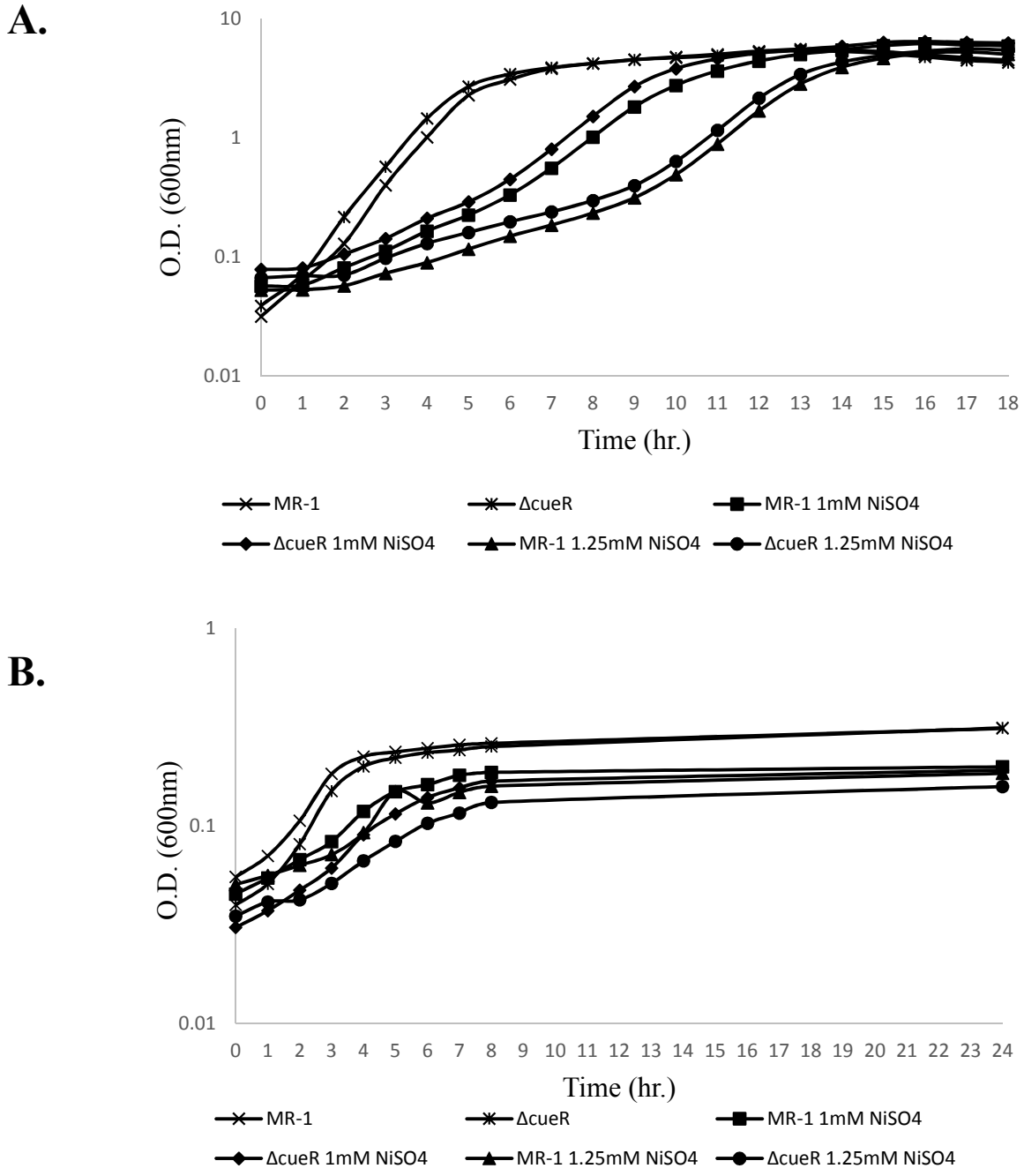
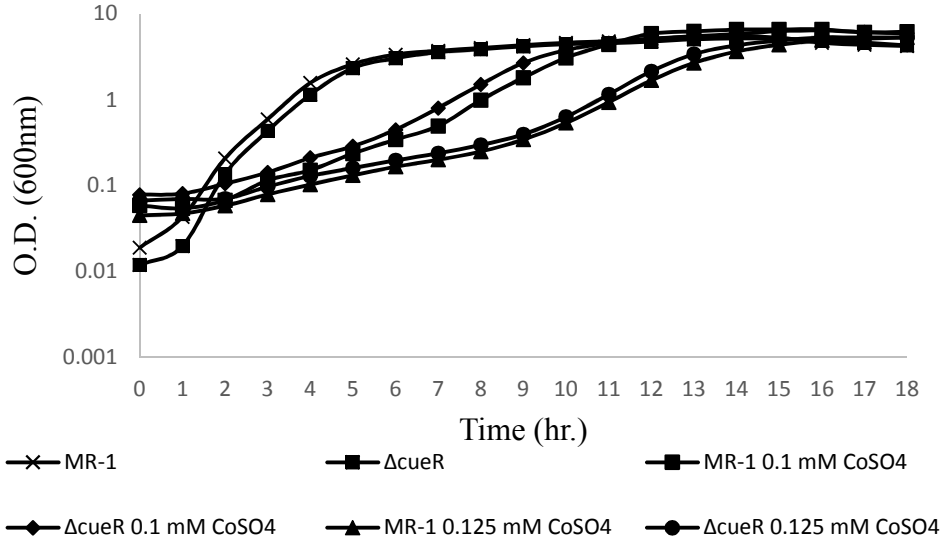


Figure 3.13. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_1687 (cueR) mutant in the presence of $NiSO_4$.*

A.



B.

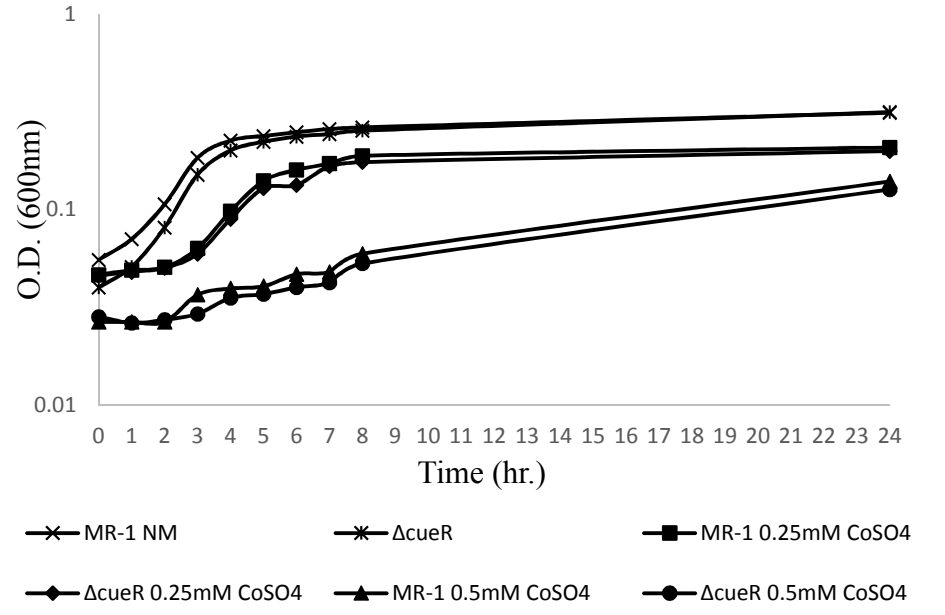


Figure 3.14. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_1687 (cueR) mutant in the presence of* $CoSO_4$.

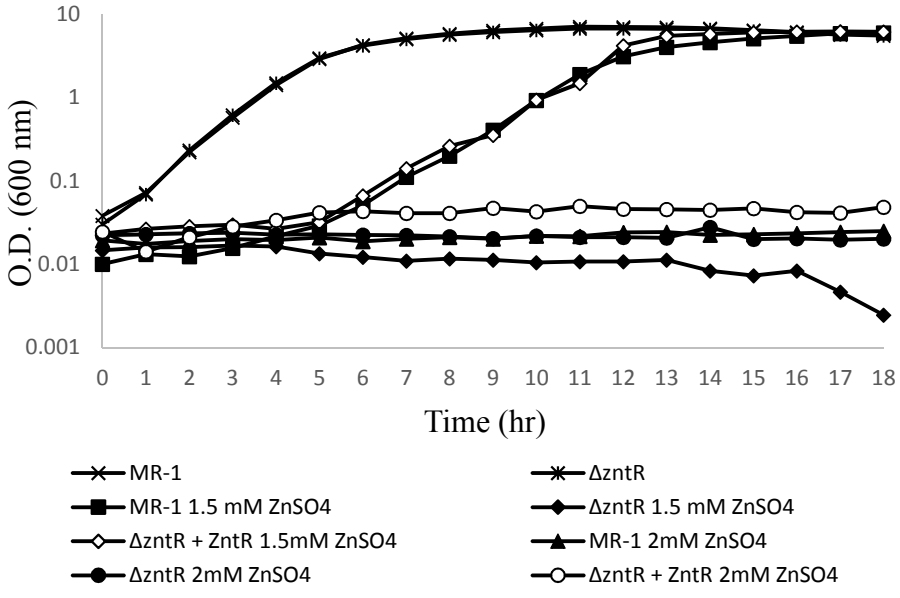
3.5. Mutants lacking the predicted CPx-type regulator ZntR are severely deficient in growth with zinc and copper

In addition to CueR, the *S. oneidensis* genome encodes a protein similar to the CPx-type ATPase regulator – the Zinc Responsive Transcriptional Regulator, ZntR. In *E. coli*, ZntR regulates *zntA*, an efflux pump involved in Zinc transport. However, sequence analysis of *S. oneidensis* genome did not identify any *zntA*-like genes. Interestingly, *zntR* is located in an operon that includes SO_0444 which encodes a predicted Zinc/Cadmium efflux pump. To test the hypothesis that the *S. oneidensis* ZntR is a zinc responsive transcriptional regulator, we generated a deletion mutant SO_0443 (*zntR*) and analyzed it for growth deficiencies using the growth conditions described above.

As expected, when this mutant was tested in the presence of zinc, we observed a deficiency in growth compared to wild type MR-1 cells under both aerobic and anaerobic conditions (Fig. 3.15). Additionally, when grown with copper, the mutant strain was unable to grow aerobically with either 1.5 mM or 2mM $CuSO_4$, while anaerobically, it grew slower as compared to wild type *S. oneidensis* with both concentrations of copper tested (Fig. 3.16). Wild type growth levels in the presence of metals were restored upon complementation of the mutant with a plasmid that carries SO_0443. We did not observe any growth defects when the *zntR* mutant was grown with nickel, or cobalt (Fig. 3.16 – 3.17).

The results described above suggest that ZntR regulates zinc and copper efflux in *S. oneidensis* MR-1 under aerobic and anaerobic conditions. This is different from the role of ZntR in *E. coli*, where it regulates zinc and cadmium responsive efflux pumps.

A.



B.

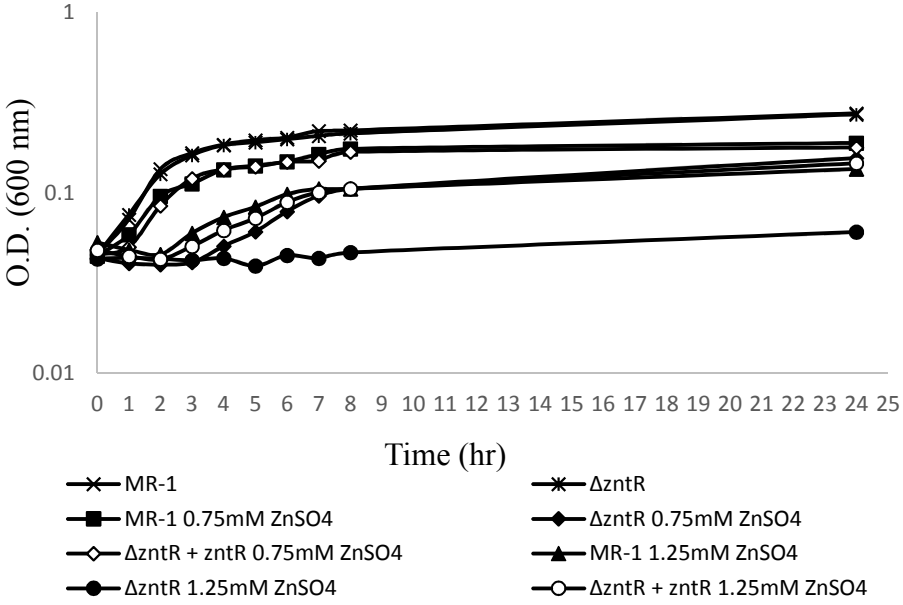


Figure 3.15. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_443 (ΔzntR) mutant in the presence of $ZnSO_4$.* ***(A.)*** *Aerobically, the mutant cells grew slower than the wild-type with 1.5 mM zinc, while no growth was observed in both wild-type and mutant strains with 2 mM zinc.* ***(B.)*** *Anaerobically, mutant cells grew slower than wild-type MR-1 in the presence of 0.75 mM zinc and did not grow with 1.25 mM zinc. Growth was restored to wild-type levels under both conditions by complementation with plasmid carrying SO_0443.*

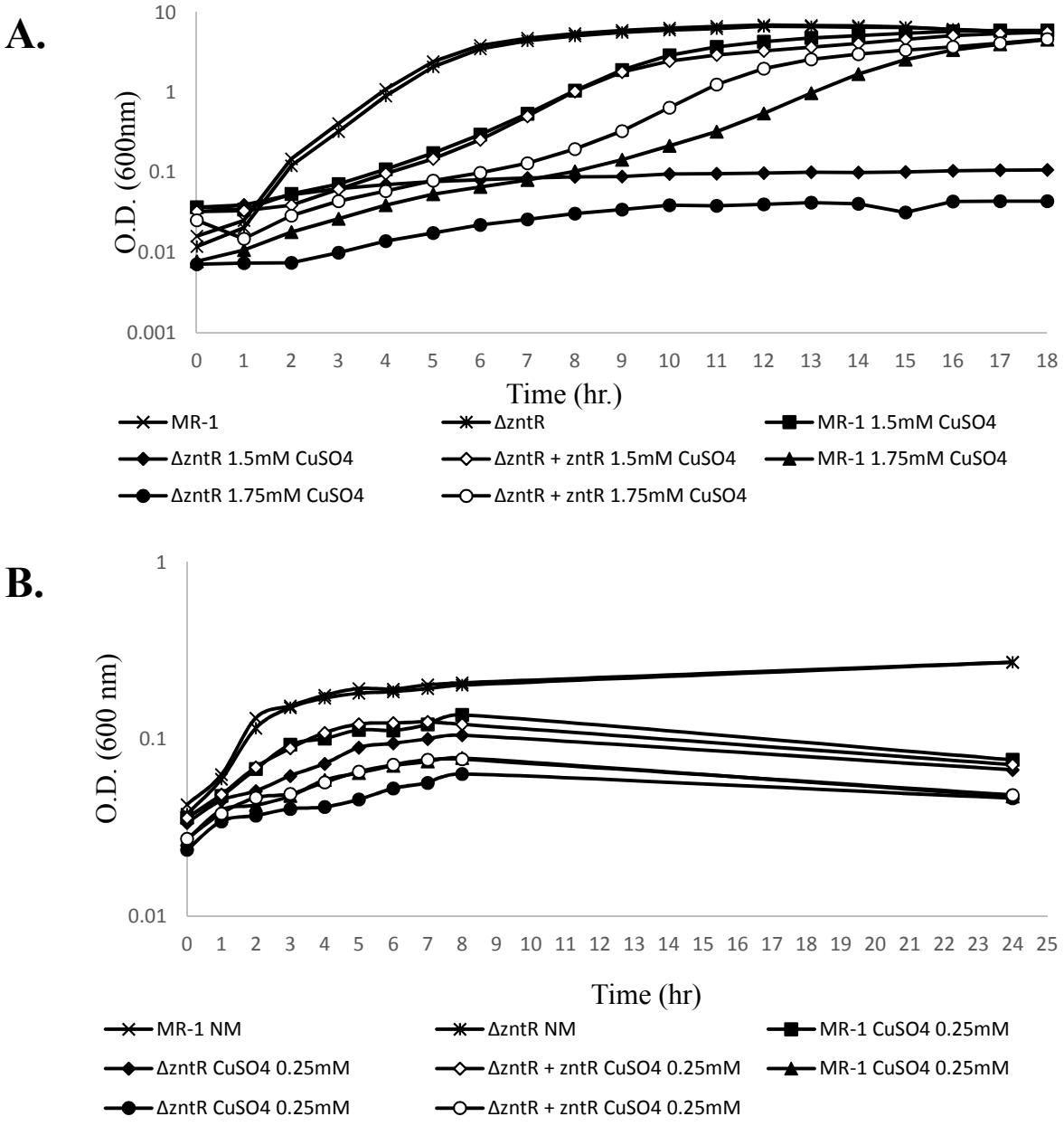
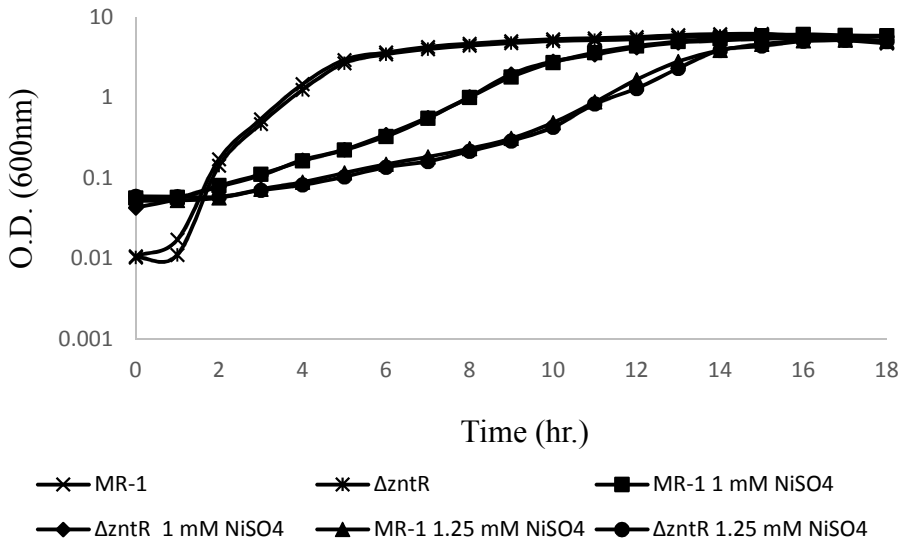


Figure 3.16. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_443 (ΔzntR) mutant in the presence of $CuSO_4$. Mutant cells were unable to grow aerobically with 1.5 mM and 1.75 mM copper. Anaerobically, the mutant cells grew slower than wild type MR-1 with 0.25mM and 0.5 mM copper. Growth was restored to wild-type levels for both conditions on complementation with plasmid carrying SO_0443.*

A.



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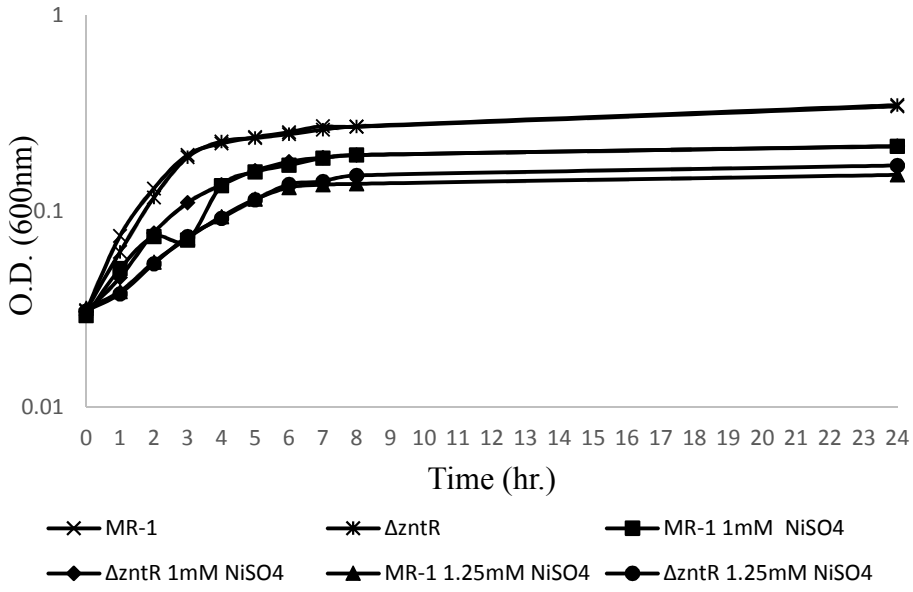
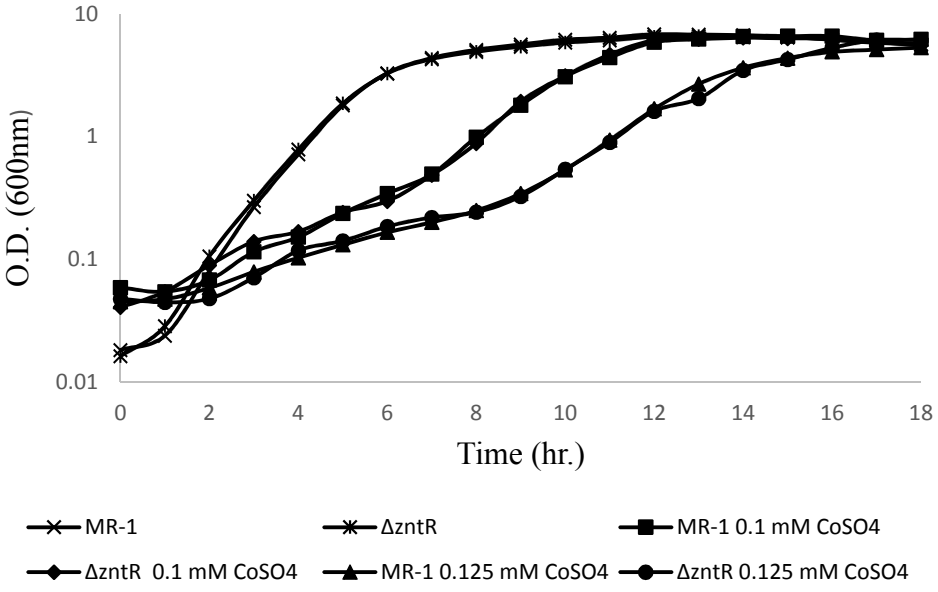


Figure 3.17. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_443 (ΔzntR) mutant in the presence of* $NiSO_4$.

A.



B.

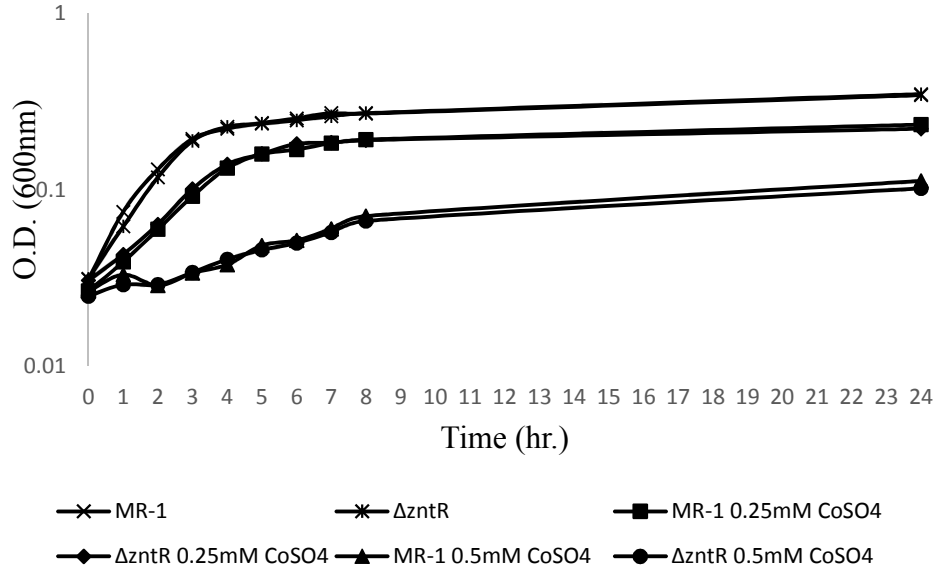


Figure 3.18. *Aerobic (A) and anaerobic (B) growth of wild-type S. oneidensis MR-1 and ΔSO_443 (ΔzntR) mutant in the presence of* $CoSO_4$.

3.6. *S. oneidensis* CopA might not be regulated by either CueR or ZntR

Since the phenotype of *copA* mutant was similar to *cueR* mutant, we predicted that *copA* may be regulated by CueR. Additionally, the results described above suggested that ZntR is also involved in the regulation of copper efflux through the regulation of a CPx-type ATPase gene such as *copA*. To test this hypothesis, I measured the *copA* promoter activity in wild type cells as well as in mutants lacking either *cueR* or *zntR* or both. The cells carrying the promoter fusion plasmids were grown overnight aerobically in LB with or without copper or zinc, and β-galactosidase activity, which reflects promoter activity, was measured.

Promoter expression analysis revealed that when cells were grown under aerobic conditions in the absence of any additional metals in the media, *copA* was expressed at similar levels in all strains tested. When grown with zinc, expression of c*opA* promoter was comparable to levels expressed when cells were grown in the absence of any metals, except in the Δ*cueRΔzntR* mutant, in which β-galactosidase activity was reduced to 53% of the activity in wild type cells. Finally, expression of *copA* promoter in strains grown with copper was drastically reduced as compared to the ones grown without any metals. Interestingly, when grown with copper, the expression of the *copA* promoter in the *ΔzntR* and *ΔcueRΔzntR* mutant was higher than its expression in wild type MR-1, suggesting that *copA* may not be regulated by either of these predicted regulators of metal efflux pumps.

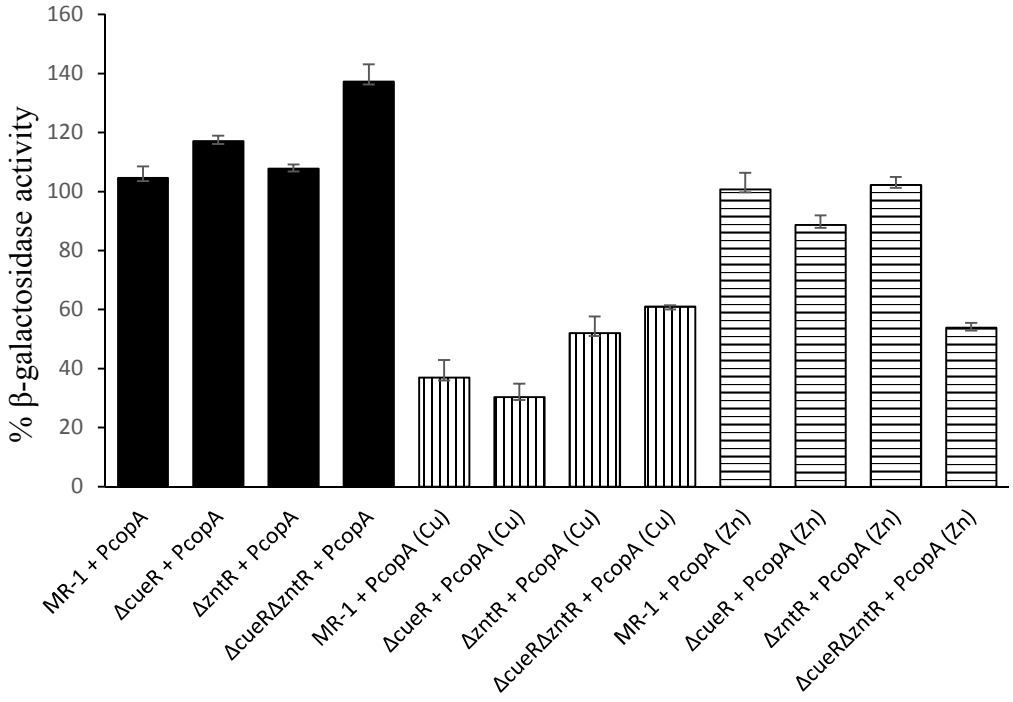


Figure 3.19. *β-galactosidase activity (P_{copA} expression) in cells grown aerobically overnight without metals, with copper and with zinc.*

4. DISCUSSION

Metals play extremely important roles in various biochemical processes. They form cores of active centers of metabolic enzymes as well as participate in electron transfer reactions during respiration. However, presence of high concentrations of metals within the cells can be detrimental because of the formation of reactive oxidative species which denature cellular proteins. Thus, the cells need to tightly regulate acquisition of metals as well as their transport to the required location. One of the primary ways by which cells achieve this is by exporting excess metal ions from the cell to the extracellular environment by using specialized efflux pumps. In this study, we have analyzed two different families of *S. oneidensis* efflux pumps – CzcCBA, a Heavy Metal Efflux RND pump and CopA, a CPx-type ATPase, to determine their specificity for metal transport. Additionally, we have also studied two predicted regulators of CPx-type ATPases – CueR and ZntR to determine their roles in *S. oneidensis* metal tolerance.

A previous study in *S. oneidensis* had identified the presence of a predicted metal efflux system belonging to the family of RND proteins encoded by SO_0518-20. Similar proteins found in other Gram-negative bacteria form an efflux pump that is involved in the transport of cobalt, zinc and cadmium. To determine the role of this predicted *S. oneidensis* HME RND pump, we generated a mutant lacking the entire efflux pump and looked at the effect of this deletion on growth with different metals. The mutant was deficient in growth with zinc and nickel when grown aerobically as well as anaerobically with fumarate as an electron acceptor. This suggests in *S. oneidensis,* the CzcCBA efflux pump is involved in the transport of zinc and nickel, unlike some other Gram-negative bacteria in which it is involved in transport of cobalt, zinc and cadmium.

Additionally, a previous grad student had generated himar mutants of two other predicted HME RND pumps found in *S. oneidensis* – SO_4598 and SO_A0154 (11). Both of these are

similar to a copper transport system found in *E. coli* and are also predicted to play a role in copper transport in *S. oneidensis*. When grown with low concentrations of copper chloride, the SO_4598 Himar mutant showed a significant growth deficiency under aerobic conditions which was restored upon introduction of a plasmid containing SO_4596-98 (11). The proteins encoded by SO_4598 and SO_0520 are very similar and thus we had predicted that both efflux systems might be involved in tolerance to copper. Interestingly, they appear to respond to the presence of different metals. This suggests that *S. oneidensis* encodes different efflux pumps for different metals/ metal concentrations. Another possible explanation for the results obtained could be due to variable sensitivity of cells to different metal salts.

In addition to the presence of multiple HME RND efflux pumps, the genome of *S. oneidensis* also encodes a CPx-type ATPase – CopA. CopA has been well characterized in other bacteria and has been shown to be involved in copper translocation from the cytoplasm against a concentration gradient. Previous studies looking at expression of *copA* in *S. oneidensis* had suggested its involvement in copper transport in this bacterium, especially under anaerobic conditions (46). To determine the role of this predicted copper transporting ATPase, we generated a deletion mutant and tested its sensitivity towards various metals. Interestingly, we observed that mutant cells lacking CopA were sensitive to copper when grown under both aerobic and anaerobic conditions conditions. This suggests that even though *copA* is expressed at low levels when grown aerobically, it plays an extremely important role in resistance to copper under these conditions. Our results also suggest that CopA shows substrate specificity towards copper and is only involved in transport of this metal in *S. oneidensis*. It is also possible that *S. oneidensis* expresses multiple efflux pumps involved in copper transport, each expressed only under specific conditions of

growth. For example, CopA may respond to high concentrations of copper while the Czc like HME RND pump (SO_4589) may respond to low copper concentrations within the cell.

In *E. coli*, the P-type ATPases involved in heavy metal transport are regulated by metal activated homologs of MerR such as ZntR, which responds to the presence of zinc and cadmium and CueR, which regulates transcription of copper efflux genes. Proteins similar to the ones encoded by *E. coli* that regulate CPx-type ATPases are also found in *S. oneidensis* and are also annotated as CueR and ZntR. To test if these predicted regulators of metal efflux proteins found in *S. oneidensis* have similar function to the ones found in *E. coli*, we generated chromosomal deletion mutants of each. Results obtained suggest that similar to *E. coli*, CueR in *S. oneidensis* regulates a copper efflux pump, potentially a copper exporting CPx-type ATPase. ZntR on the other hand regulates copper and zinc efflux, which is different from the role of ZntR in *E. coli*, where it is specifically involved in Zn and Cd resistance. Whether ZntR regulates a CPx-type ATPase or a different type of efflux pump still needs to be elucidated.

As mentioned above, our results suggest that in *S. oneidensis,* CueR and ZntR might regulate an efflux pump involved in transport of copper. In *E. coli*, CueR regulates the CPx-type ATPase CopA. Due to sequence similarity of CueR found in *S. oneidensis* to the one found in *E. coli*, we predicted that CueR might regulate CopA in *S. oneidensis*. To determine if this prediction was correct, we measured *copA* promoter activity using *lacZ* fusion plasmids in the mutants that lacked *cueR*, *zntR* or both. *copA* promoter was expressed at similar levels in all strains tested in the absence of additional metals in the medium. When tested with zinc, all strains except the *ΔcueR ΔzntR* mutant showed β-galactosidase activity levels similar to the ones grown without any metals. The β-galactosidase activity in the *ΔcueRΔzntR* mutant was reduced by almost 47%. Interestingly, when *copA* promoter activity was tested in strains grown in the presence of copper, all strains

tested showed a drastic reduction in β-galactosidase activity. Some studies have suggested that presence of copper ions in the sample can negatively affect β-galactosidase enzyme activity (55). Whether the observed reduction in β-galactosidase activity is due to reduced gene expression or due to interference of copper ions with β-galactosidase enzyme activity still needs to be elucidated. This could be done by washing the cells several times with Z-buffer before testing for β-galactosidase activity to ensure removal of all copper from the medium. Although inconclusive, our current results suggest that contrary to what we hypothesized, CopA might not be regulated by either of the regulators tested in this study. Further analysis will need to be conducted to provide a conclusive answer.

CHAPTER IV

MATURATION OF *C*-TYPE CYTOCHROMES IN *SHEWANELLA ONEIDENSIS* MR-1

1. INTRODUCTION

As mentioned above, the genome of *S. oneidensis* MR-1 encodes 42 *c*-type cytochromes which can function as intermediates in the electron transport chain or as components of terminal reductase complexes involved in aerobic and anaerobic respiration. *c*-type cytochromes are proteins that contain heme, an iron containing prosthetic group (56). The heme moiety is covalently attached to the two cysteines present in the typical CXXCH heme-binding motif. Attachment of heme to this motif is required for the maturation of *c*-type cytochromes. In α and γ-Proteobacteria, the attachment of heme to the CXXCH motif requires the function of the cytochrome *c* maturation (Ccm) system (or System I) proteins, CcmABCDEFGH (56–58). CcmABCD translocates the heme moiety across the cytoplasmic membrane and loads it into the heme chaperone, CcmE, which provides the heme to the heme-lyase complex. Heme lyases, which are also called cytochrome *c* synthases, attach the heme to the cysteine residues of the CXXCH motif in the apocytochrome *c* resulting in formation of holocytochrome *c* (Fig. 5.1) (56, 58). In *E. coli*, the heme-lyase complex is formed by CcmFH, whereas in some other Gram-negative bacteria, CcmFHI comprises the heme-lyase complex (59). In *S. oneidensis*, similar to other gram-negative bacteria, the proteins encoded by *ccmABCDE* and *ccmFGH* operons are required for maturation of *c*-type cytochromes, and mutations in these genes leads to a complete loss of cytochrome *c* maturation in *S. oneidensis*. Additionally, the product of another gene found in a gene cluster adjacent to the *ccmABCDE* and *ccmFGH* operons, *ccmI* has recently been found to be involved in the maturation of some *c*-type cytochromes such as NrfA in *S. oneidensis* MR-1 (57, 60).

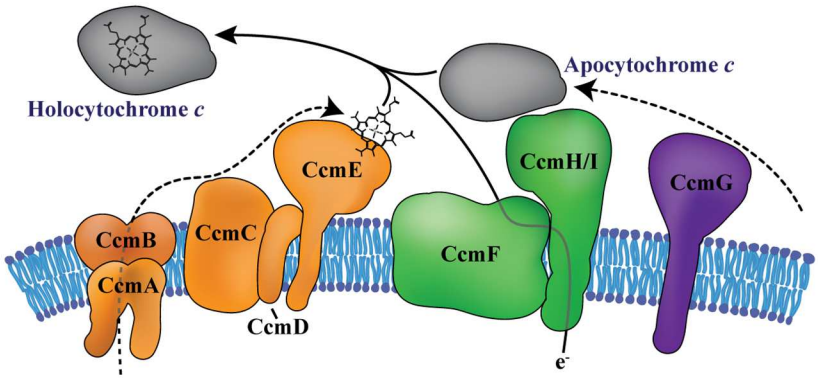


Figure 4.1 *Model of cytochrome c maturation in α and γ-Proteobacteria. The CcmABCD protein complex transports and loads heme on CcmE. The heme-lyase complex (CcmFH or CcmFHI) covalently attaches this heme to the CXXCH motif of apocytochrome c to form holocytochrome c* (61).

Some *c*-type cytochromes also show the presence of atypical sites, such as CXXCK and $CX_{15}CH$, in addition to the CXXCH motifs. Specialized heme-lyase systems are required for the attachment of heme to these atypical sites (62, 63). In *E. coli*, attachment of heme to the CXXCK atypical heme binding site of NrfA requires the heme lyase complex NrfEFG, where NrfEF forms the cytochrome *c* synthase, and NrfG is the apocytochrome *c* chaperone. In *Wolinella succinogenes*, on the other hand, heme attachment to the CXXCK site of nitrite reductase and $CX_{15}CH$ site of sulfite reductase is carried out by NrfI and CcsA1respectively (62, 64). Two atypical heme binding sites have also been identified in the *c*-type cytochromes found in *S. oneidensis* MR-1. The $CX_{15}CH$ motif is found in the outer membrane associated sulfite reductase SirA (54) and the CXXCK motif is found in the periplasmic nitrite reductase NrfA (65).

In *S. oneidensis* MR-1, a homolog of *E. coli* NrfEFG, has been found in the *sir* gene cluster – SirEFG (Fig. 4.2.). The *sir* gene cluster also encodes SirA, the terminal sulfite reductase containing the $CX_{15}CH$ atypical heme binding motif. These genes were originally annotated as *nrfEFG* but were re-annotated as *sirEFG* after their involvement in SirA maturation was discovered. The proteins encoded by *sirEFG* make up the heme lyase complex that is thought to be involved in attachment of the heme moiety to the atypical heme binding site found in SirA, SirEF comprising of the cytochrome *c* synthase and SirG functions as the chaperone. *sirG* encodes a 224 amino acid long protein SirG that is 35% identical to the N-terminus region of *S. oneidensis* CcmI. Both CcmI and SirG are predicted to belong to the family of the tetratricopeptide (TPR)-domain containing proteins. Proteins belonging to this family commonly function as chaperones and facilitate protein-protein interactions.

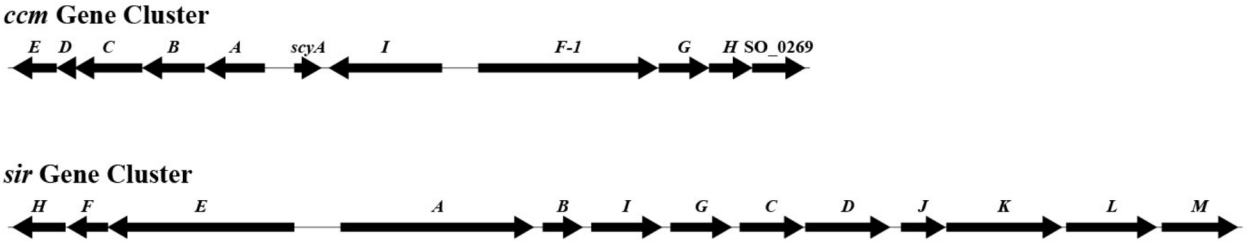


Figure 4.2. The *sir and ccm gene clusters in S. oneidensis MR-1 chromosome.*

Previous work has shown that mutants that lack either *ccmI* or *sirG* are still able to reduce sulfite, although a long lag phase is observed. However, a mutant deficient in both *ccmI* and *sirG* is completely deficient in sulfite reduction (14), suggesting the involvement of both CcmI and SirG in the maturation of the terminal sulfite reductase, SirA, in *S. oneidensis* MR-1. We hypothesize

that SirEF, CcmI and SirG form the heme lyase complex, interacting with apo-SirA to deliver the heme required for the maturation of this metalloprotein. In this study, using a bacterial two-hybrid system, the interaction of CcmI and SirG with SirA been described.

2. DETECTION OF PROTEIN-PROTEIN INTERACTION USING BACTERIAL TWO-HYBRID

The bacterial two-hybrid (BTH) system is an efficient method for detecting *in vivo* protein-protein interactions. The BacterioMatch II two-hybrid system used in this study detects these interactions based on transcriptional activation. In this method, a protein of interest (or the bait) is cloned into a plasmid (pBT) containing the full-length bacteriophage λ repressor protein and its corresponding target protein is cloned into a plasmid (pTRG) containing the α-subunit of RNA polymerase. On co-expression of the recombinant pBT and pTRG in the reporter strain, if the bait and target proteins interact, they recruit and stabilize the binding of RNA Polymerase and activate the transcription of the *HIS3* reporter gene. The transcriptional activation of this *HIS3* reporter gene is used as a test for interaction of the bait and target proteins.

The reporter strain used has a mutation in *hisB,* and the *HIS3* gene encodes a component of the histidine biosynthetic pathway that complements this mutation. In the absence of transcriptional activation of the *HIS3* gene, its gene product is produced from the reporter gene cassette at low levels which allows the reporter strain to grow in minimal medium lacking histidine. The compound 3-amino-1,2,4-triazole (3-AT) is a competitive inhibitor of the *HIS3* gene product. Thus, the reporter strain is unable to grow on minimal medium lacking histidine in the presence of 3-AT. 3-AT serves to eliminate background growth due to low levels of basal *HIS3* gene expression in the absence of interaction.

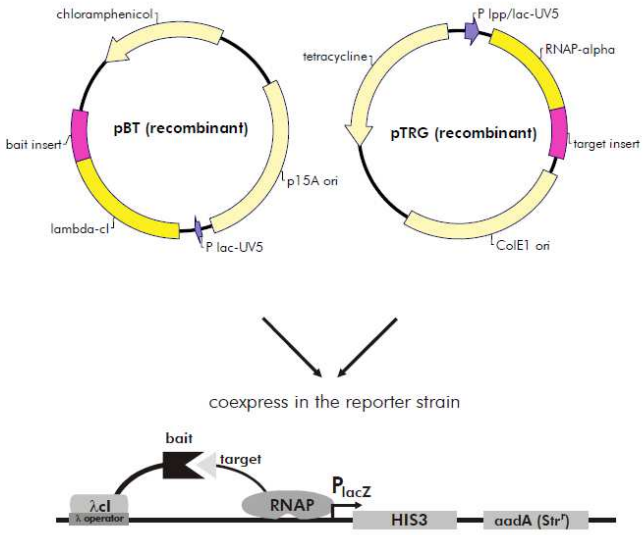


Figure 4.3. *Illustration of the BacterioMatch II two-hybrid system. The bait and target proteins are cloned into the pBT and pTRG plasmids respectively. The recombinant plasmids are co-expressed in the reporter strain. Interaction of the bait and target proteins results in the recruitment and binding of RNA Polymerase, activating the transcription of HIS3 reporter gene.*

Interaction of the bait and target proteins in the co-transformed reporter strain results in the recruitment of RNA Polymerase and upregulation of the expression of *HIS3*. This transcriptional activation increases the expression of the *HIS3* gene product which allows the reporter strain to overcome the competitive inhibition by 3-AT, thus resulting in its growth on media lacking histidine and containing 3-AT. Additionally, the reporter strain contains a second reporter gene, *aadA*, which encodes a protein that confers resistance to the antibiotic streptomycin. This second reporter can thus be used as an additional mechanism for validating the interaction between the bait and target proteins of interest.

3. MATERIALS AND METHODS

3.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 4.1. LB (Lysogeny Broth) medium was used for growth of *E. coli* XL1-Blue MRF′ Kan strain at 30°C. Screening medium (M9 non-selective, selective or dual selective) was used for the growth of the BacterioMatch II two-hybrid reporter strain at 37°C. Antibiotics chloramphenicol (25 μg/ml), tertacycline (12.5 μg/ml) and streptomycin (12.5 μg/ml) were added as needed.

3.2 Bacterial two-hybrid

Genes SO_0479 (*sirA*), SO_0482 (*sirG*) and SO_0265 (*ccmI*) were amplified by PCR from *S. oneidensis* MR-1 chromosomal DNA using primers listed in Table 5.2 and Phusion DNA polymerase (New England BioLabs, Ipswich, MA). Amplified fragments were purified using the IBI Gel/PCR DNA fragment extraction kit (IBI Scientific, Dubuque, IA). Primers used for amplification of these fragments (Table 5.3) were designed to include restriction enzyme sites that would allow the ligation of the fragments obtained by PCR amplification into target (pTRG) and bait (pBT) plasmids. The SO_479 DNA fragment was digested with BamHI and XhoI and ligated into pTRG. Similarily, SO_482 and SO_265 DNA fragments were digested with EcoRI and XhoI and then ligated into pBT. *E. coli* XL1-Blue MRF′ Kan was transformed with the resulting recombinant plasmids and screened on LB-chloramphenicol (for pBT transformants) or LB-Tetracycline (for pTRG transformants). Colonies that contained the appropriate vectors were confirmed by PCR.

The vector pairs were then co-transformed into the BacterioMatch II two-hybrid reporter strain (Agilent Technologies). Transformed cells were plated on M9 non-selective and selective (+3AT) media that contained chloramphenicol and tetracycline. Additional vectors and reporter strains were also generated with the fragments switched (i.e. *sirA* in pBT and 482 and *ccmI* in pTRG). To confirm interactions between the proteins, reporter strains were grown overnight in $M9^{+}$ His-dropout broth and serially diluted (up to 10^{-7}). 10 µl were spotted on M9, M9 + 3AT and M9 + 3AT agar supplemented with 12.5 µg streptomycin per ml and allowed to grow at 37°C for up to 48hrs.

Table 4.1. Bacterial strains used in this study

Strain	Description	Source
Escherichia coli		
XL1-Blue MRF´ Kan	Host strain for propagating pBT and pTRG recombinants	Agilent Technologies
BacterioMatch II validation reporter strain	Interaction assays, expression testing, validation of screen isolates	Agilent Technologies
EC1182	BacterioMatch II two-hybrid reporter strain with pBT-LGF2 and pTRG-GAL11P	This study
EC1123	XL1-Blue MRF´ Kan with pBTH5	This study
EC1122	XL1-Blue MRF´ Kan with pBTH3	This study
EC1124	XL1-Blue MRF´ Kan with pBTH2	This study
EC1179	XL1-Blue MRF´ Kan with pBTH6	This study
EC1180	XL1-Blue MRF´ Kan with pBTH4	This study
EC1181	XL1-Blue MRF´ Kan with pBTH1	This study
EC1128	BacterioMatch II reporter strain with pBTH5 and pBTH2	This study
EC1131	BacterioMatch II reporter strain with pBTH3 and pBTH2	This study
EC1117	BacterioMatch II reporter strain with pBTH6 and pBTH1	This study
EC1125	BacterioMatch II reporter strain with pBTH4 and pBTH1	This study
EC1178	BacterioMatch II reporter strain with empty pBT and pTRG	This study

Table 4.2. Plasmids used in this study

Plasmid	Description	Source
pBT	Bait plasmid, p15A *ori*, λcI, *lac-UV5 promoter*, Cm^R	Agilent Technologies
pTRG	Target plasmid, ColE1*ori*, RNA Pol α-subunit, *lpp/lac-UV5* promoter, Tc^R	Agilent Technologies
pBT-LGF2	Interaction control plasmid encoding the dimerization domain (40 amino acids) of the Gal4 transcriptional activator protein	Agilent Technologies
pTRG-GAL11^P	Interaction control plasmid encoding a domain (90 amino acids) of the mutant form of the Gal11 protein	Agilent Technologies
pBTH1	SO_0479 (*sirA*) fragment in pTRG	This Study
pBTH2	SO_0479 (*sirA*) fragment in pBT	This Study
pBTH3	SO_0482 (*sirG*) fragment in pTRG	This Study
pBTH4	SO_0482 (*sirG*) fragment in pBT	This Study
pBTH5	SO_0265 (*ccmI*) fragment in pTRG	This Study
pBTH6	SO_0265 (*ccmI*) fragment in pBT	This Study

Table 4.3. List of primers used in this study

Name	Sequence	Description
TRGCMIEF	GCATCAGAATTCCAACATTTAGGTGCCTTT GAAAATATAGGC	SO_0265 (CcmI) DNA Fragment for ligation into pTRG
TRGCMIXR	GATCTTACTCGAGTTGTACTTGAGTATCCAG TACTAAGTTTGCGG	
TRG482BF	GCGGCCGGATCCGGACGTTATAGCGATTGG	SO_0482 (SirG) DNA Fragment for ligation into pTRG
TGR482XR	CTGGCGCTCGAGATATCCACTTTCATTCAAT TTTATTTG	
BT479BF	CCAGCGGGATCCGCTAAATCGGATGGTAAA GTG	SO_0479 (SirA) DNA Fragment for ligation into pBT
BT479XR	CCGATCTCCTCGAGCATTTTAGCGTTGTAGC CATTACC	
BTCMIEF	GCATCGAATTCCCTGAAAATATAGGC	SO_0265 (CcmI) DNA Fragment for ligation into pBT
BTCMIXR	CGCACTCGAGGATGTACTAAGTTTGC	
BT482EF	GCATCGAATTCCGTTATTAGCGATTGG	SO_0482 (SirG) DNA Fragment for ligation into pBT
BT482XR	CGCACTCGAGGAATCAATTTTATTTG	
TRG479BF	GCGGCCGGATCCGGGATGGTAAAGTG	SO_0479 (SirA) DNA Fragment for ligation into
TRG479XR	CCGATCCTCGAGCTGTAGCCATTACC	

4. RESULTS

Both the Ccm and Sir systems are required for the proper maturation of the *S. oneidensis* sulfite reductase SirA.

Based on studies of heme maturation systems in other bacteria, such as *E. coli* and *W. succinogenes*, we hypothesized that heme ligation to the atypical $CX_{15}CH$ motif of SirA requires a specific and dedicated heme lyase complex. To identify the proteins involved in maturation of SirA, the role of CcmI and SirG in sulfite reduction was tested. Previous studies that investigated the role of CcmI in maturation of the nitrite reductase NrfA also indicated that loss of CcmI may result in a partial or incomplete defect in SirA enzyme activity (66). Accordingly, it was found that mutants that lack either SirG or CcmI reduced sulfite, although a lag phase of 24 hr (without SirG) and 48 hr (without CcmI), with respect to the Wild-Type, was observed before H_2S production was detected. However, a mutant that lacked both SirG and CcmI was completely deficient in sulfite reduction, which suggested that SirG and CcmI may be able to partially compensate for loss of one another. Introduction of *sirG* into the Δ*sirG*Δ*ccmI* mutant partially restored sulfite reduction, with reduction rates similar to that of the single Δ*ccmI* mutant. Similarly, introduction of *ccmI* into the Δ*sirG*Δ*ccmI* mutant resulted in sulfite reduction rates similar to that of the Δ*sirG* mutant. These results indicated that, contrary to nitrite reduction where CcmI was essential, neither CcmI nor SirG were essential for sulfite reduction. Both proteins appear to have a partially redundant function, and complete loss of sulfite reduction was observed only when both proteins were absent (14).

To confirm that both SirG and CcmI directly interact with the apo-SirA during maturation, bacterial two-hybrid analysis was performed. Constructs were generated to assess the interaction between SirA and SirG, and between SirA and CcmI. The bacterial two-hybrid assay indicated

that both SirG and CcmI interact with SirA independently of one another and in the absence of other components of the *S. oneidensis* MR-1 Ccm and Sir maturation systems. Reciprocal assays were performed in which SirA served as both the bait (pBT) or the target (pTRG) protein. The results obtained were the same regardless of the orientation of the two-hybrid assay, and further supported the observation that both SirG and CcmI are involved in direct maturation of the SirA sulfite reductase (Fig. 4.4).

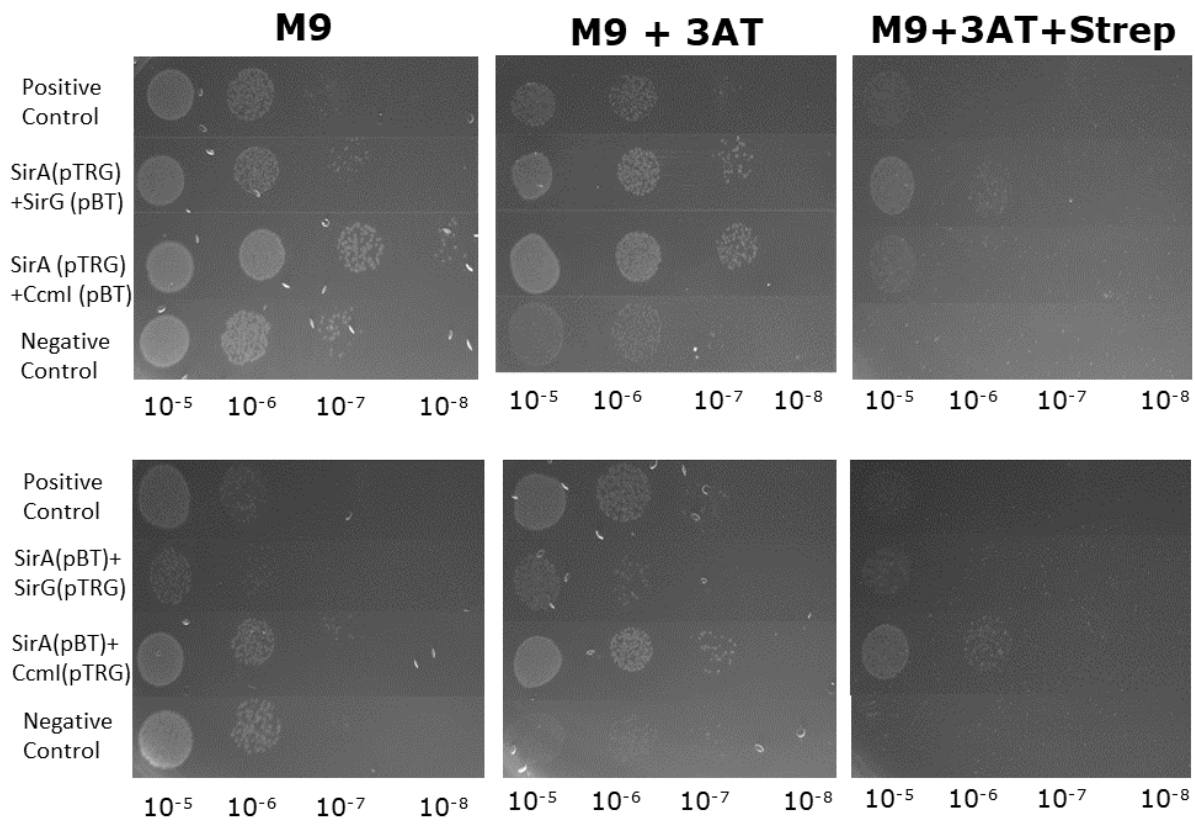


Figure 4.4. *The SirG and CcmI heme lyases interact with SirA. The bacterial two-hybrid assay was performed with the BacterioMatch II Two-Hybrid System Kit, according to manufacturer instructions. Both CcmI and SirG were shown to interact with SirA. Gal11 and LGF2 served a positive control for interaction. Reciprocal assays in which each protein served as either Bait or Target, were performed and similar results were obtained in either case.*

5. DISCUSSION

Maturation of *c*-type cytochromes requires specialized systems for the attachment of heme to the CXXCH motifs of apocytochromes via covalent bonds. These maturation systems contain an apocytochrome *c* chaperone and a cytochrome *c* synthetase – making up the heme-lyase complex. In some Gram-negative bacteria such as *Rhodobacter capsulatus* and *Pseudomonas aeruginosa*, CcmI, CcmH, and CcmF fulfill these roles, while in *Escherichia coli* CcmH carries out the functions of both CcmI and CcmH (56, 58, 67, 68). The *S. oneidensis* MR-1 genome encodes 42 *c*-type cytochromes, and all of these show the presence of the typical CXXCH heme-binding motif (69, 70). For the maturation of all *c*-type cytochromes in *S. oneidensis*, the presence of the Ccm or System I proteins CcmABCDEFGH is essential, where CcmABCDE transport and present the heme to the heme-lyase complex, which attached the heme to the appropriate enzyme. The heme-lyase complex in *S. oneidensis* is comprised of CcmFGHI, where CcmFGH forms the cytochrome *c* synthetase and CcmI functions as the cytochrome *c* chaperone (57, 59).

In addition to the typical CXXCH heme-binding domain found in all *c*-type cytochromes, some also contain atypical heme binding motifs such as CXXCK, which is found in nitrite reductase and $CX_{15}CH$ in the sulfite reductase. Attachment of heme to each of these non-conventional heme-binding motifs require specialized heme-lyase systems, such as the NrfEFG heme lyase complex in *E. coli* required for the maturation of NrfA (47). The terminal sulfite reductase, SirA, found in *S. oneidensis* MR-1 shows the presence of one of these atypical heme binding motifs – $CX_{15}CH$. This terminal sulfite reductase is encoded by *sirA*, a gene found in the *sir* gene cluster. In addition to encoding the components of the Sulfite reductase complex, the *sir* gene cluster also houses the genes that encode SirEFG, which forms the heme lyase complex

involved in the maturation of SirA, where SirG functions as the chaperone and SirEF make up the cytochrome *c* synthetase.

Analysis of mutants lacking either SirG or CcmI or both revealed that both chaperones were involved in maturation of SirA (14) and we hypothesized that they each interacted with the terminal reductase SirA during its maturation. A bacterial two-hybrid assay was used to show the interaction between the terminal reductase and the two chaperones. The results of this assay indicate that SirA interacts with SirG and CcmI independently of each other, even in the absence of other components of the cytochrome *c* maturation systems. Based on these results and previous studies conducted in our lab on maturation of SirA (14), we predict that SirEFG along with CcmI forms a four-subunit heme lyase complex that functions in the maturation of the *S. oneidensis* MR-1 terminal sulfite reductase SirA.

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EDUCATION

Ph.D. **in Biological Sciences,** Microbiology
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AWARDS

Chancellor's Teaching Award, University of Wisconsin, Milwaukee 2017 – 2018
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PRESENTATIONS

Banerjee R; "Metal resistance in *Shewanella oneidensis* MR-1"
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PUBLICATIONS

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RESEARCH EXPERIENCE

Doctoral Research: University of Wisconsin, Milwaukee 2011 – 2019

Development of genetic tools for analysis of respiration in *Porphyromonas gingivalis*

- Developed a genetic system for generation of transposon mutants in *P. gingivalis* and used it for isolation and study of mutants with growth deficiencies. Also developed a markerless chromosomal deletion system to make targeted gene deletions in this bacterium. This system will be used to make targeted gene deletions to study the role of predicted anaerobic reductases.

Metal resistance in *Shewanella oneidensis* MR-1

- Made targeted gene deletions to identify and characterize their role in maintaining metal homeostasis in the metal reducer *S. oneidensis.* Studied effect of deletions on growth in presence of various heavy metals.

Maturation of *Shewanella oneidensis* sulfite reductase

- Using a Bacterial two-hybrid system, showed the interaction and involvement of two chaperones – CcmI and SirG in maturation of a c-type cytochrome, SirA, the terminal sulfite reductase in *S. oneidensis* MR-1.

TEACHING EXPERIENCE

University of Wisconsin, Milwaukee

Teaching Assistant: General Microbiology – BioSci 383 – Laboratory 2014 - 2019

- Typical class size (Laboratory): 16 – 18 students/lab section
- Course provided students with a theoretical and practical foundation in Microbiology that can serve as a basis for further studies in Biological Sciences or for professional training in Health Sciences.

Lecturer: Foundations of Biological Sciences II (Microbiology) – BioSci 152 2016

- Typical class size (Lecture): 150 – 200 students
- Introduction to Microbiology, Plant and Animal Biology. Second half of a two-semester introductory course sequence for majors in Biological Sciences, Microbiology, Conservation and Environmental Science, and other natural science majors.

Teaching Assistant: Foundations of Biological Sciences I – BioSci 150 (Laboratory) 2011- 2014

- Typical class size (Laboratory) – 24 students/lab section
- Fundamental principles of Biology (Ecology, Evolution, Genetics, Cell and Molecular Biology). First half of a two-semester introductory course sequence for majors in Biological Sciences, Microbiology, Conservation and Environmental Science, and other natural science majors.

INTERNSHIPS

Quality Control and Quality Assurance Intern at Elder Pharmaceuticals November 2007
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- Conducted Microbiological analysis of pharmaceutical preparations (produced inhouse). Tests conducted included Sterility testing of finished products as well as raw materials used during production, Analysis of water used during manufacturing process for presence of coliforms using Most Probable Number (MPN) technique, microbiological assay of vitamins and antibiotics produced.

- Tested finished pharmaceutical preparations (including tablets, liquids and gel/creams) for stability and quality. Tests performed included testing for content of active ingredients, hardness, disintegration and dissolution tests for tablets and leakage tests for strip and blister packaging

Microbiology Intern at Padmaja Aerobiologicals Pvt. Ltd., Navi Mumbai, India Summer 2007

- Conducted Microbiological testing of Food, Water, Pharmaceutical preparations and Environmental Samples. Tests performed included - Total bacterial and fungal count testing for presence of coliforms and bacterial identification, Sterility testing, test for presence of Sulfite reducing bacteria, Nitrifying bacteria and *Legionella* spp.

TECHNICAL SKILLS

- Experienced in Microbiology techniques such as aseptic techniques, establishing and maintaining anaerobic and aerobic bacterial cultures, enumeration of bacteria using serial dilution and plate counts, bacterial identification tests, Antimicrobial Susceptibility Testing
- Experience in handling pathogenic bacteria (BSL-2).
- Proficient in Microscopy and various microbial staining techniques and Molecular Biology techniques including nucleic acid extraction, PCR, DNA Transformation and Conjugation, Restriction Enzyme digestion, primer design, Southern Blot as well as protein expression and analysis using SDS and Native PAGE and Western Blots.
- Mentoring and training undergraduate and graduate students in basic Microbiology and Molecular Biology techniques.
- Skilled in using Microsoft Office programs such as Word, Excel, PowerPoint and Outlook.

RELEVANT COURSEWORK

- Microbial Diversity and Physiology, Biological Electron Microscopy, Laboratory Techniques in Molecular Biology, Molecular Biology of Microorganisms, Bacterial Pathogenesis.