Identification of a putative P-Type ATPase Pump that may confer Gold- and Copper-resistance in *Stenotrophomonas maltophilia* Oak Ridge strain 02 (*S. maltophilia* 02)

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Signature

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Abstract

Stenotrophomonas maltophilia 02 (*S. maltophilia* 02) was isolated from a heavy-metal contaminated site in Oak Ridge, TN. It is a multi-metal resistant bacterial strain that grows in the presence of toxic levels of Cu, Pt, Hg, Au, Cd, Pb, Cr and Se salts. The *Salmonella* specific set of proteins, GolT, GolB and a small cytoplasmic metal binding protein confer resistance to gold. GolS detects the presence of gold salts/ions in the environment and activates the expression of the resistance response. The first line of defense against the toxic ion is GolT, which is rapidly expressed after GolS activation by Au. GolT is homologous to P1B-type metal ion transporters which exports monovalent metal ions from the cytoplasm. Using the amino acid residues sequence of GolT against the protein sequences from the *S. maltophilia* 02 genome, the Basic Local Alignment Search Tool (BLAST) analysis identified a *S. maltophilia* 02 putative P-type ATPase that is 80% similar to *Salmonella* GolT. BLAST analysis of the *S. maltophilia* 02 proteins suggested that it is related to a copper resistance P-type ATPase. The *cueR* gene encodes a putative transcriptional regulator gene located downstream of the putative S02 P-type ATPase gene. The overall goal is to determine if the ATPase and *cueR* genes are involved in copper- and/or gold-resistance. The cloned genes did not confer resistance to copper or gold in *E. coli*. Next, RT-PCR was used to see if they were expressed in response to copper and gold in *S. maltophilia* 02. RNA from cultures of *S. maltophilia* 02 grown in the absence of metal and in the presence of gold and copper was purified and converted to cDNA. PCR amplification of the cDNA using primers for the ATPase gene and c*ueR* showed that gold and copper induced the expression of both genes. Thus, both genes appear to be involved in copper and gold resistance in *S. maltophilia* 02.

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CHAPTER 1

1.0 INTRODUCTION

1.1 Background of the Y-12 Plant and East Fork Poplar Creek

First isolation of *Stenotrophomonas maltophilia* Oak Ridge strain 02 (*S. maltophilia* 02) was from East Fork Poplar Creek. This creek was contaminated with heavy metals from the Y-12 facility in Oak Ridge, TN. The Y-12 facility processed uranium during World War II to make nuclear bombs and lithium during the Cold War to make hydrogen bombs (Williams RL et al., 1993). Large amounts of mercury (Hg) were used to process lithium. These were spilled into the Poplar Creek and the surrounding area. In addition, the Y-12 facility used four S-3 ponds to dump off acidic wastes contaminated with heavy metals. Unfortunately, most of the wastes leached into the ground and into the creek. Therefore, in 1983 the ponds usage was discontinued. The ponds were drained, capped, and the remaining contents treated (Williams RL et al., 1993).

1.2 Properties of *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia is a non-fermentative Gram-negative rod that exists in a variety of water bodies. It uses nitrate as the terminal electron acceptor to grow without oxygen (Crossman et al., 2008). It moves with the help of polar flagella. Other characteristics are that catalase is positive and oxidase is negative (0.7–1.8 × 0.4–0.7 μm). This distinguishes it from most other genera and is positive for extracellular DNase. Nosocomial infections in immunocompromised patients can be caused by *Stenotrophomonas maltophilia* strains (Elting et al., 1990).

1.3 *Stenotrophomonas maltophilia* Oak Ridge strain 02

S. maltophilia 02 is a multi-metal resistant bacterial strain that thrives in the presence of toxic levels of copper, mercury, gold, cadmium, lead, chromium and selenium ions (Holmes et al., 2009).

Heavy metals are common pollutants in the environment. They disrupt the normal biome by causing genetic mutations, damaging cells and cell membranes, and interfering with enzyme function and cellular metabolism. Heavy metals react with molecules in soil, water and air forming toxic products (Hughes et al., 1983). They are non-biodegradable, toxic, inorganic and hard to remove from the environment. Therefore, there is a possibility to use bacteria resistant to heavy metal to counter the toxic effects of these inorganic metals.

1.4 Properties of Gold

Gold (Au) is a metal and can exist in six oxidation states but only +1 (Au (I)) and +3 (Au (III)) states are commonly found in nature. Gold is a soft Lewis acid that forms stable complexes with ligands that have free electron pairs (Nies. 1999). In the Periodic Table of Elements gold (Au) is a transition metal belonging to Group IB which lies along with Copper (Cu) and Silver (Ag). It has one s-orbital electron. It has a large ionization potential and electron affinity due to its larger atomic size.

1.5 Uses for Gold

Gold is the most useful and memorable metal ever mined. It holds a special place in the human mind. It is easy to work with, does not tarnish, can be mixed with other metals to make alloys used in electronics and construction, can be hammered into thin sheets, has high malleability , can be melted and designed into highly detailed shapes. Gold has a wonderful color and brilliant luster. Applications for compounds containing gold are as follows; in dentistry, in jewelry making, in surgical instruments and implants, in aerospace, in medical diagnostics or imaging as radiotherapy, anti-arthritis drugs and in malignant neoplasm medications (Guo and Sadler, 1999). In addition, it has antibacterial properties and can be used for biodegradation and bioremediation (Rajbanshi, 2008).

1.6 Environmental Heavy Metal Pollution

Industrial activities such as metal processing, mining, electroplating, leather tanning, washing and carpet dyeing are a major source of heavy metals. Contamination of wastewater due to toxic heavy metals pollutes the environment and therefore has fatal effects on aquatic and terrestrial life (Moten and Rehman, 1998). Cyanide leaching used in extracting gold results in land, water, and air pollution. Also, accumulation of heavy metals in sediments and soil can be because of fluvial deposition of emissions from dust and gold mining. These metals cause severe health problems in man through the food chain (Sabah and Fouzul 2012).

1.7 Multi-metal resistance in bacteria

Some bacteria can be tolerant to different types of metals. First, *Thiobacillus ferrooxidans* tolerates zinc (Zn), nickel (Ni), copper (Cu), cobalt (Co), manganese (Mn), and aluminium (Al) at concentrations of 10 g/1 and tellurium (Te), arsenic (As), silver (Ag) and selenium (Se) at a concentration range between 50 to 100 mg/1 (Tuovinen O. et al., 1971). Toxicity levels of metals were determined by how the bacteria oxidized iron or thiosulfates.

Seventy-one strains of *Pseudomonas aeruginosa* isolated from soil are resistant to various heavy metals and antibiotics (Marques A.M. et al., 1979). Every strain was resistant to different concentrations of Cd, Pb, Cr, Mo, and uranium (U) and resistant to antibiotics including ampicillin, cephalotin, chloramphenicol, furadantin, kanamycin, nalidixic acid, streptomycin, and tetracycline. These authors also suggested that persistence of resistant bacteria to antibiotic and presence of bacteria with the resistance (R) factors in soil resulted from selective pressures from heavy metals (Marques A.M. et al., 1979). Passing from one bacterium to another through bacterial conjugation spreads antibiotic resistances between bacterial species.

According to Nakahara et al., 1977, the clinical isolates of *Escherichia coli, Klebsiella pneumoniae, P. aeruginosa,* and *Staphylococcus aureus* were resistant to salts of Hg, Pb, Zn, Cd and As. These were carried on R plasmids. Among these isolates, 89.6% of the *E. coli* strains and 94.0% of the *K. pneumoniae* strains were resistant to mercury (Nakahara H. et al., 1977). Another transmissible arsenite resistant encoding plasmid was commonly found in *K. pneumoniae* and *Salmonella*. Many isolates of *K. pneumoniae* have arsenite resistance genes. And it is closely related to other genes encoded by plasmids, which determine the use of lactose, Hg tolerance, Te resistance, and antibiotic resistance (Smith H.W., 1978).

The small subspecies of *Corynebacterium flaccumfaciens oorti* has a conjugated plasmid 46-MDa (pDG101) with genes for resistance to arsenite, arsenate, and antimony (III) (Hendrick C.A. et al., 1984). Derivatives and transconjugate strains sensitive to the donor arsenite do not contain the pDG101 plasmid. Since the plasmid was transferred from the bacteria to other four strains of *C. flaccumfaciens*, the transduction conjugated strains were more resistant to arsenite. In addition, there is a recent discovery of chromosomal arsenic resistance mechanism in *Alcaligenes faecalis* and *Thiomonas* sp. bacteria. Arsenate is used in this approach as a terminal electron acceptor in heterotrophic growth in stringent supply of oxygen. Some bacteria use the classic arsenate resistance genes to tolerate very high levels of arsenate during respiration (Silver and Phung, 2005).

R factors, R773 and R46, found in *E. coli* both confer arsenic (As) resistance. It is reported that when exposed to salts of arsenite they can synthesize inducible polypeptides. The R773 derivative produces a 64-kDa polypeptide, while the R46 derivative synthesizes a 16-kDa polypeptide (Mobley L.T.H. et al., 1984). Plasmid encoded resistance is widespread in many types of bacteria.

This resistance is due to the detoxification of the Hg compounds into volatile compounds that are less toxic to the environment. Hg resistance in bacteria mostly belongs *to Pseudomonads, Mycobacterium, Acinetobacter, Bacillus, Staphylococcus, E. coli, Salmonella, Thiobacillus, Arthrobacter, Citrobacter, Vibrio, Flavobacterium* and *Klebsiella* species (Trevors et al., 1985). Since multiple tolerances are common phenomena among heavy metals resistant bacteria, these bacteria could be potential agents for bioremediation of heavy metal pollution (Rajbanshi et al., 2008). Multiple tolerance only occurs with toxic compounds based on similar toxicity mechanisms. These resistant microorganisms can be manipulated for bioremediation of heavy metals in various environments. By treating the toxic heavy metals effluents with microorganisms through processes such as biosorption, bioaccumulation and bioprecipitation.

To adapt to the presence of toxic heavy metals microbes have acquired numerous mechanisms. Bacteria, fungi and protozoa are the first types of microbes to be tolerant to heavy metal toxicity. They survive through metal sorption, mineralization, absorption, accumulation, extracellular deposition and enzymatic oxidation or reduction of harmful heavy metals to less toxic substances (Hughes and Poole, 1991; Nies, 1992; Urrutia and Beveridge, 1993).

1.8 Gold resistance mechanisms

Salmonella has a specific set of proteins such as P-type ATPase, GolT, GoIB and a small cytoplasmic metal binding protein. GolS belongs to the MerR family of protein regulators, which are highly selective for Au ions. Detection of gold salts in the environment and activation of the resistance response is carried out by GolS (Pontel et al., 2014 and 2007). GolT acts as the first line of defense against the toxic ion. Its gene is rapidly expressed upon activation by GolS (Checa et al., 2007). GolT is homologous to P1B-type metal ion transporters; thus, it can export gold monovalent metal ions from the cytoplasm (Harrison et al., 2007; Robinson and Winge, 2010). The genome of *S. maltophilia* has recently been sequenced. Using this sequence information, Basic

Local Alignment Search Tool (BLAST) analysis identified a putative P-type ATPase that is 80% similar to *Salmonella* GoIT (Fig. 1). BLAST analysis of the identified *S. maltophilia* 02 protein suggested that it is related to a copper-translocating P-type ATPase (Fig. 2). Since *S. maltophilia* 02 is resistant to Au(I) and Cu (II) (Holmes et al., 2009), we hypothesize that the identified protein may confer gold and copper resistance. Therefore, my thesis work investigates the role of this protein in copper and gold resistance.

1.9 Objectives

1. To clone the putative gold resistance genes from *S. maltophilia* 02 into *E. coli*.
2. Use minimal inhibitory concentration (MIC) experiments to see if the cloned genes confer resistance to gold and copper in *E. coli*.
3. Use RT-PCR to see if the P-type ATPase gene is expressed in response to gold or copper.

1.10 Hypothesis

BLAST analysis using GolT from *Salmonella* as a query against the *S. maltophilia* 02 genomes identified a protein that was 80% similar to GolT and BLAST analysis of the identified P-type ATPase matched a copper-translocating P-type ATPase. If the P-type ATPase gene is expressed, we expect the cloned gene for the P-type ATPase to confer copper and gold resistance in *E. coli*. In addition, we expect RT-PCR experiments to show that the gene for the P-type ATPase and maybe the genes for *tonB* and *cueR* are expressed at a higher level in the presence of copper and gold than in the absence of metal.

CHAPTER 2

2.0 METHODOLOGY

2.1 Bacterial strains

S. maltophilia 02 was purchased from the American Type Culture Collection in Manassas, Virginia and grown at 30^{O}C.

2.2 Growth medium

The composition of a liter R3A-Tris medium consists of 1 g yeast extract, 1 g Difco protease peptone no, 3, 1 g casamino acids, 1 g glucose, 1 g soluble starch, 0.5 g sodium pyruvate, 0.1 g $MgSO_4$ $7H_2O$, and 10 mM tris pH 7.5 per liter (Holmes et.al 2009). When needed, R3A-Tris broth was supplemented with 16 g agar bacteriological AMRESCO (Solon, OH), 50 µg/ml kanamycin AMRESCO (Solon, OH) or 100 µg/ml ampicillin.

LB (Lennox) Broth (20 g/l) was composed of 10 g/l tryptone, 5 g/l yeast extract and 5 g/l sodium chloride were obtained and prepared according to the manufacturer instructions from Growcells.com (Irvine, CA. USA), A Subsidiary of Molecular Biologicals International Inc. When needed, LB broth was supplied with 1.6% agar bacteriological AMRESCO (Solon, OH), 50 µg/ml kanamycin AMRESCO (Solon, OH) or 100 µg/ml ampicillin.

2.3 Genomic preparation protocol

The genomic DNA was prepared and purified according to the Promega (Madison, WI) Wizard® Genomic DNA Purification Kit protocol. 1 ml of an overnight culture was pelleted by centrifugation for 2 minutes at 13,000 × g, and the supernatant was discarded. The cells were lysed by adding 600 µl Nucleic Lysis solution and gently mixed by pipetting. The mixture was then incubated at 80^{o}C for 5 minutes and cooled to room temperature. 3 ul of RNase solution (Amresco) was added, mixed, incubated at 37^{o}C for 15 minutes and cooled to room temperature. 200 µl of

protein precipitation solution was mixed in by vortexing for 20 seconds, incubated on ice for 5 minutes and centrifuged at 13,000× g for 3 minutes. The DNA was precipitated by transferring the supernatant into a clean 1.5 ml tube with 600 µl of isopropanol, thoroughly mixed, centrifuged for 2 minutes at 13,000×g and then the supernatant was discarded. Air dried the pellets for 10-15 minutes then rehydrated with 100 µl of Rehydration Solution and incubated overnight at 4 °C.

2.4 Gel electrophoresis

1% agarose gels were prepared by adding 1.3 g of BioExcell® Agarose LE (Worldwide Medical Products, Bristol, PA) into 130 ml of 1× TBE (Amresco, Solon, OH) and thoroughly mixed by swirling after every minute of microwaving until the solution was clear. The solution was poured into a graduated cylinder and thoroughly mixed with 13 µl Gel Green Nucleic Acid Stain (Embi Tech, San Diego, CA) using a stir bar. The gel was poured into the trays and the comb was inserted to form the wells. The gel was placed and run in a RunOne™ Electrophoresis system (San Diego, CA), where 10× TBE Buffer (Amresco, Solon, OH) diluted to 1× (0.089 M Borate and 0.002 M EDTA) and was poured on the gel. The gel's wells were loaded with the mix of 2 µl of Amresco Agarose Gel Loading Dye and 3 µl of each digested and undigested sample. 10 µl of 1 kb DNA Ladder (NEB, Beverly, MA) was added to each gel. 100 volts was used to separate the DNA fragments, and the gel was imaged using an Embi Tech PrepOne™ Sapphire (San Diego, CA) system.

2.5 Gel extraction

Gel extraction procedure was carried out according to the QIAquick® Gel Extraction Kit protocol (QIAGEN, GmbH, Hilden). The DNA fragments were extracted from the agarose gel and weighed. Added 3 volumes of buffer QG to1volume of gel, and then incubated at 50 °C for 10 minutes until the gel dissolved completely by vortexing after every 2-3 minutes of incubation. Added 10 µl of 3 M sodium acetate (pH 5.0) and 1 volume of isopropanol into the sample and mixed. The mixture

was transferred into a QIAquick spin column placed in a 2 ml collection tube and centrifuged at room temperature for 1 minute at 13,000× g. The flow through was discarded and the QIAquick column was returned to the same tube. Added 750 µl of PE buffer to the spin column and centrifuged for 1 minute. The flow through was excluded and the QIAquick column set into a clean 1.5 ml microcentrifuge tube. 50 µl of EB Buffer (10Mm Tris Cl, pH 8.5) was added into the center of the QIAquick membrane to elute the DNA and centrifuged for 1 minute at room temperature. The purified DNA was then analyzed on a 1% agarose gel.

2.6 QiaQuick PCR purification

The PCR clean-up was done according to MinElute PCR purification kit protocols (QIAGEN, GmbH, Hilden). 5 volumes of PB Buffer were added to 1 volume of PCR reaction and then thoroughly mixed with 10 µl of 3 M sodium acetate, pH 5.0 and mixed. The mix was transferred into a MinElute column in a collection tube and centrifuged for 1 minute at 13,000 rpm and 25°C. The flow-through was discarded and the MinElute column returned into the same collection tube. To wash, 750 µl PE Buffer was added to the MinElute column, centrifuged for a minute and flow-through was discarded. The MinElute column was centrifuged again for a minute from the same collection tube to completely remove the ethanol residual from PE Buffer. The MinElute column was then transferred into a new 1.5 ml microcentrifuge tube. To elute DNA, 10 µl Buffer EB was added to the center of the MinElute membrane, and the column was incubated at room temperature for a minute. It was then centrifuged for another minute at 25°C and 13,000 rpm.

2.7 GoTaq Polymerase chain reactions (PCR)

GoTaq PCR reactions contained 10 µl of 2X Go Taq, 2.5 µl of 4 µM Forward primer, 2.5 µl of 4 µM Reverse primer, 3 µl of nuclease free water and 2 µl of DNA template.

The reaction was assembled on ice, gently mixed, centrifuged briefly to let the content settle at the bottom incubated and transferred to a Mastercycler personal (Eppendorf). After the reactions were

incubated at 95°C for 2 minutes, they were incubated at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 4 minute 15 seconds for 35 cycles. The reactions were complete after a final 10-minute incubation step at 72°C. In addition to the 60°C annealing step, other annealing temperatures included 50°C, 55°C, 60.8°C, and 66°C, depending on the primer pairs used in the reaction.

2.8 Q5 PCR

Q5 polymerase chain reaction contained 12.5µl of 2X Q5 Reaction Mix (NEB), 1.25 µl of 10 µM Forward Primer, 1.25 µl of 10 µM Reverse Primer, 8 µl of Nuclease-Free Water and 2 µl of DNA template in a 25 µl reaction per tube. The reaction was assembled on ice, gently mixed, and centrifuged briefly to let the contents settle at the bottom and transferred into Mastercycler personal (Eppendorf, Hauppauge, NY). After an initial incubation of 98°C for 30 seconds, the reaction was run under the Q5 program in the Mastercycler Personal (Eppendorf, Hauppauge, NY) with 35 steps of 98°C for 10 seconds, 66°C for 15 seconds, 72°C for 2 minutes, followed by a single step of 72°C for 2 minutes. The completed reactions were held at 10°C.

2.9 PCR Product

Q5 polymerase does not add a 3'- adenine base to the end of its PCR products. After obtaining the PCR product from Q5 reactions, it was purified using the MinElute PCR Purification Kit. Then, 5 ul of purified DNA was added to 5 ul of 2X GoTaq mix, and the mixture was incubated at 72°C for 10 minutes. The PCR product was then placed on ice and used for TA cloning.

2.10 Ligations using the StrataClone Kit

Used StrataClone PCR Cloning Kit to clone PCR products. Ligation mixture was systematically prepared by gently mixing 3 µl of StrataClone™ blunt cloning buffer, 2 µl of PCR product (5-50 ng) and 1 µl of StrataClone Vector Mix amp/kan. The cloning reaction mixture was incubated at room temperature for 5 minutes and then placed in ice.

2.11 Transformations

Thawed the StrataClone SoloPack competent cells on ice. 1 µl of the ligation reaction was gently mixed by inversion and incubated for 20 minutes on ice. The transformation mixture was then heat shocked at 42°C for 45 seconds and placed on ice for another 2 minutes. It was then removed from the ice and mixed with 250 µl of SOC medium, pre-warmed to 42°C. The transformation mixture was then incubated at 37°C for 1 hour on a shaker to allow the cells to recover and express plasmid encoded antibiotic resistance. The transformant was plated on LB agar plate containing 100 µg/ml ampicillin (Amresco, Solon, OH) and 40 µg/ml X-gal (Amresco). For the expression of the recombinant/plasmid, the transformant (Single white colony) was selected from the plate and inoculated into 5 ml of LB broth containing 50 µg/ml kanamycin (Amresco).

2.12 Plasmid preparation

Plasmid DNA was purified according to the instructions for the Promega Wizard® Plus SV Minipreps DNA Purification System (Madison, WI). 5 ml of overnight culture of bacterial cells was pelleted by centrifugation at 7,000 × g for 5 minutes at 4°C and the supernatant was discarded. 250 µl of Cell Resuspension Solution was added to resuspend the pellet by pipetting. 250 µl of Cell Lysis Solution was added and mixed by inverting the tube 4 times. 10 µl of Alkaline Protease Solution was added and mixed by inverting 4 times and incubated for 5 minutes at room temperature. 350 µl of Neutralization Solution was added and immediately mixed by inverting the tube 4 times. It was then centrifuged at 14,000 × g for 10 minutes and room temperature.

A spin column was inserted into a 1.5 ml collection tube and the cleared lysate was transferred into a spin column by decanting. It was centrifuged at 14,000× g for 1 minute and room temperature. The flow through was discarded and the spin column was reinserted.

750 µl of Wash Solution was added into the spin column, centrifuged for 1 minute, and the flow through was discarded. The wash procedure was repeated with 250 µl of Wash Solution and centrifuged at 14, 000 × g for another 2 minutes at room temperature.

The spin column was inserted into a new sterile 1.5 ml microcentrifuge tube. 100 µl of nuclease free water was added into the spin column to elute the plasmid DNA and then centrifuged for 1 minute at 14,000 × g room temperature. The spin column was discarded, and the purified plasmid DNA was stored at -20°C.

2.13 DNA Sequencing

DNA sequencing was done using the Genome Lab™ Dye Terminator Cycle sequencing with Quick Start Kit (Brea, CA). The plasmid DNA concentration was measured by a Nano Drop 2000C Spectrophotometer (Thermo scientific, Waltham,MA) that determined the amount of DNA and nuclease free water to be used. 2 µl of sterile water was first blanked, followed by 2 µl of DNA and clicked "measure" button. After determining the volume of DNA that was required, nuclease free water was added into the DNA to bring the total volume to 10 µl. The mixture was heated in a Mastercycler Personal (Eppendorf, Hauppauge, NY) for a minute at 96°C and then cooled at room temperature. 2 µl of 1.6 µM M13 F5' GTAAAACGACGGCCAGT 3' and M13 R5' GGAAACAGCTATGACCATG 3' primer and 8 µl of DTCS quick start mix was added to the sample. The sample was placed in a Mastercycler Personal (Eppendorf, Hauppauge, NY) set at 96°C for 20 second, 50°C for 20 second and 60°C for 4 minutes for 30 cycles and then held at 4°C.

2.14 Sequencing Reaction Clean Up

The sequencing samples were cleaned up to get rid of impurities and contaminants. 5 µl of stop solution was prepared by mixing 2 µl of 3 M sodium acetate with pH 5.2, 2 µl of 100 mM EDTA with pH 8.0 and 1 µl of glycogen. The sample was transferred into a clean 0.5 ml tube and mixed thoroughly. 60 µl of ice cold 95% ethanol was added to the mixture and it was centrifuged at

14,000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet was washed twice with 200 µl of ice cold 70% ethanol then centrifuged after every wash at 14,000 rpm for 2 minutes at 4°C. Carefully aspirated the excess supernantant and precipitated the DNA in a CentriVap DNA concentrator (Labconco Corporation, Kansas City, MO) for 10 minutes. The dried DNA pellet was resuspended with 40 µl Sample Loading Solution that was later analyzed with a Beckman Coulter CEQ 2000XL DNA Analyzer (Fullerton, CA) by Mr. Ed Budde. Use BLAST (Basic Local Alignment Search Tool) to analyze the DNA sequence.

2.15 Minimal Inhibitory Concentrations (MIC)

Replica plated *E. coli* in different concentration of copper sulfate and gold chloride to determine the minimal inhibitory concentration of copper and gold plates, respectively. The gold chloride concentrations were 10 µM, 20 µM, 30 µM, 40 µM and 50 µM whereas copper sulfate concentrations were 0.1 Mm, 0.5 Mm, 1 Mm, 2 Mm, and 3 Mm, respectively.

2.16 Copper and gold growth curves

S. maltophilia 02 was grown overnight at 30˚C in LB broth. To make a dilution of 1/20, 1.0 ml of overnight culture was added to 19 ml of fresh LB medium and mixed. 6 ml of culture was then added to 3 test tubes labelled 0, 200 µM gold and 1 mM copper. Growth was measured by determining the optical density of 100 µl of cell using a cuvette and the NanoDrop spectrophotometer. Tubes were incubated in a roller drum at 30˚C and NanoDrop readings were taken after every 30 minutes for 5 hours. After 1.5 hours of growth 22.4 µl of sterile water was added to the 0 µM tube, 22.4 µl of 50 mM gold chloride stock added to the 200 µM gold tube and 5.6 µl of 1 M copper sulfate stock added to 1 mM copper tube. 200 µl of RNA protect was added to three 1.7 ml tubes labelled no metal, gold and copper. After 2.5 hours of growth 100 µl of cells were added to 200 µl of RNA Protect Reagent (Qiagen). The samples were vortexed for 5 seconds

and incubated at room temperature for 5 minutes. The supernatant was poured off and excess supernatant was dabbed off on a paper towel. Pellets were frozen at -80˚C.

2.17 RNA Preparations

The bacterial culture/ RNA protect bacterial reagent pellet was thawed out. TE/proteinase K/lysozyme buffer was prepared by mixing 10 µl of Qiagen proteinase K to 100 µl of TE buffer (30 mM tris, 1 mM EDTA, pH 8.0) containing 15 mg/ml lysozyme. The cells were resuspended in 110 µl of TE/proteinase K/lysozyme buffer by pipetting up and down gently and then incubating at room temperature for 10 minutes. It was vortexed at 2 minutes interval. 350 µl of RLT buffer containing 10 µl β-mercaptoethanol/ ml RLT buffer was added and microfuged at ≥10,000 rpm for 2 minutes. Then the supernatant was transferred to new 1.7 ml tubes. To wash the spin column 250 µl of 95- 100% ethanol was added and microfuged at ≥10,000 rpm for 15 seconds. 700 µl of RW1 buffer was added to the RNeasy spin column and centrifuged for 15 seconds at ≥8,000 x g and the flow through was discarded. Again, 500 µl RPE buffer was added to the RNeasy spin column and centrifuged for 2 minutes at ≥8,000 x g to wash the spin membrane and the flow through was discarded. The empty spin column was centrifuged at ≥10,000 rpm for 1 minute to remove all the wash solution. The RNeasy spin column was placed in new 1.5 ml collection tube, and then 30 µl RNase-free water was centrifuged at ≥10,000 rpm through the column for1 minute to elute RNA. This step was done twice.

During DNase treatment, 6 µl of 10X TURBO DNase buffer and 1 µl of TURBO DNase was added to the eluted RNA and mixed by gently flicking the tube and incubated at 37˚C for 20 minutes. 7 µl of DNase inactivation reagent was added and mixed by pipetting and then incubated for 5 minutes at room temperature before centrifuging for 1.5 minutes. Supernatant was transferred to a new RNase free tube and then the RNA concentration was measured using the NanoDrop. Purified RNA was stored at -80˚C.

2.18 RT-PCR: cDNA Synthesis

93 ng of RNA was mixed with 2 µl of Random Primer Mix (60 µM stock solution) and nuclease free water added to make a final volume of 8 µl. RNA/primer mix was denatured at 65˚C for 5 minutes and then placed on ice. 10 µl of ProtoScript 11 Reaction Mix and 2 µl of ProtoScript 11 Enzyme Mix was added to treated samples while 2 µl of nuclease free water was added to the control tubes instead of enzyme. This was incubated in the thermal cycler for 1 cycle at 25˚C for 5 minutes, 42˚C for 60 minutes, 80˚C for 5 minutes and held at 4˚C. stored at -20˚C.

2.19 List of primers and function

Primers	Sequence	Annealing temperature	Purpose
CuR -ATPase2_1	5'-CCAACCAGATCTCCACCAA-3'		PCR amplification
CuR -ATPase2_2	5'-GGTGATCAATGCCACCAAGT-3'		PCR amplification
S02_GAPDH_F S02_GAPDH_R	5'-AAACCGCGCAGAAGCACATCGA-3' 3'-GCCGGCGTAGGTCTTGTCGTTC-5'	62˚C	RT-PCR amplification
S02_ATPase_F S02_ATPase_R	5'-CCGATCCGATCAAGCCGAGCAC-3' 3'-GGTACCAGCGTTGTCACCGGTG- 5'	62˚C	RT-PCR amplification
S02_TonB_F S02_TonB_R	5'-AACAACTGCGGTGTGGGCAACA-3' 3'-AGGTTCGGCATCACGTCCCAGA-5'	62˚C	RT-PCR amplification
S02_CueR_F S02_CueR_R	5'-GTGAAGCTGCCAAGGCCTCCAG-3' 3'-TCGGTTCTATCAGCGGCGGGAA-5'	62˚C	RT-PCR amplification

M13 F M13 R	5’ GTAAAACGACGGCCAGT 3’ 5’ GGAAACAGCTATGACCATG 3’	50°C	Sequencing

Table 1: *List of primers and function*

CHAPTER 3

3.0 RESULTS

3.1 BLAST Analysis

The *Salmonella* GolT sequence was used to identify a translated *S. maltophilia* 02 gene that could serve a similar function. BLASTP analysis of *Salmonella* GolT against the *S. maltophilia* 02 protein database identified a *S. maltophilia* 02 P-type ATPase that was 80% similar (Figure 1). Therefore, the *S. maltophilia* 02 putative P-type ATPase sequence could confer gold resistance.

```
>contig_6_1647_[345960_-_348509]_
          Length = 850

 Score = 1175 bits (3039), Expect = 0.0
 Identities = 603/745 (80%), Positives = 658/745 (88%)

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           T+ L +EGMTCASCV RVE+ + AVPGV+ A+VNLATERATVRG A    ++AAI+K GY
Sbjct: 99  TVELAVEGMTCASCVGRVERALLAVPGVSQASVNLATERATVRGVADTAALVAAIDKVGY 158

Query: 70  EARPIETAGQGEDDSEEKKEAERVRLKRDLILASVLALPVFVLEMGSHLIPGMHEWVIKT 129
            A PIE   Q ++++ EKK+AER  LKRDLI+AS LALPVFVLEMGSHLIPGMHEWV+
Sbjct: 159 AAHPIEAGVQSDEEAAEKKDAERAELKRDLIVASALALPVFVLEMGSHLIPGMHEWVMAH 218

Query: 130 IGLQQSWYWQFALTLLVLTIPGRRFYLKGFPALARLAPDMNSLVAVGTAAAFGYSLVATF 189
           IG+Q SWY QFALTLLVL IPGRRFY KGFPAL RLAPDMNSLVAVGTAAAFGYS+VATF
Sbjct: 219 IGMQTSWYLQFALTLLVLAIPGRRFYQKGFPALLRLAPDMNSLVAVGTAAAFGYSVVATF 278

Query: 190 TPDLLPEGTVNVYYEXXXXXXXXXXXGRFLEARAKGRTSEAIKRLVGLQARVAHVLREGR 249
            P LLP GTVNVYYE           GRFLEARAKGRTSEAIKRLV LQA+VAHV+R+GR
Sbjct: 279 APRLLPPGTVNVYYEAAAVIVALILLGRFLEARAKGRTSEAIKRLVNLQAKVAHVIRDGR 338

Query: 250 IVDIPVDEVVLGDCVEVRPGERIPVDGEVTEGRSFVDESMITGEPIPVEKSAGSAVVGGT 309
            VDIPV+EV  GD VEVRPGER+PVDGEV EGRS++DESMI+GEPIPVEK  GS+VVGGT
Sbjct: 339 TVDIPVNEVQSGDVVEVRPGERVPVDGEVVEGRSYIDESMISGEPIPVEKQPGSSVVGGT 398

Query: 310 VNQKGALTLRATAVGGQTMLAQIIRLVEQAQGSKLPIQAVVDKVTLWFVPMVMLIAALTF 369
           VNQKGALT+RATAVG QTMLAQIIR+VEQAQGSKLPIQAVVDKVTLWFVP VML A  TF
Sbjct: 399 VNQKGALTVRATAVGAQTMLAQIIRMVEQAQGSKLPIQAVVDKVTLWFVPAVMLAALATF 458

Query: 370 VVWLAFGPSPALTFALINGVAVLIIACPCAMGLATPTSIMVGTGRGAEMGVLFRKGEALQ 429
            VWL FGPSPAL+FAL+N VAVLIIACPCAMGLATPTSIMVGTGRGAEMGVLFRKGEALQ
Sbjct: 459 AVWLIFGPSPALSFALVNAVAVLIIACPCAMGLATPTSIMVGTGRGAEMGVLFRKGEALQ 518

Query: 430 LLKDAKVVAVDKTGTLTEGRPVLTDLDVASGFERREVLAKVAAVESRSEHPIARAIVVSA 489
           LLKDAKVVAVDKTGTLTEGRP LTD ++  GF R  VLA VAAVESRSEHPIARAIV +A
Sbjct: 519 LLKDAKVVAVDKTGTLTEGRPRLTDFEITDGFNRSTVLAAVAAVESRSEHPIARAIVDAA 578

Query: 490 EEEGIALPGMSGFESVTGMGVYATVDGTRVDVGADRYMREIGVDISGFATTAERLGQEGK 549
            E+GIALP M  FESVTGMGV A+VDG RV+VGADR+MR++GVDI+ FAT A  LG +GK
Sbjct: 579 TEQGIALPSMVDFESVTGMGVRASVDGARVEVGADRFMRDLGVDITLFATLAAELGTQGK 638

Query: 550 SPLYAAIDGQLAAIIAVADPIKPSTPAAINALHQLGIKVAMITGDNARTAQAIARQLGID 609
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Query: 610 DVVAEVLPEGKVEAIRRLKAAYGQVAFVGDGINDAPALAESDVGLAIGTGTDVAVESADV 669
           +VVAEVLPEGKVEA+RRLKA +G VAFVGDGINDAPALAE+DVGLAIGTGTD+AVESADV
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Query: 670 VLMSGNLQGVPNAIALSKATIRNIHQNLFWAFAYNTALIPVAAGALFPVWGILLSPVFAA 729
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Sbjct: 759 VLMSGNLQGVPNAIALSKATLGNIRQNLFWAFAYNTALIPVAAGVLYPVWGVLLSPVFAA 818

Query: 730 GAMAMSSVFVLGNALRLRRFRAPMA 754
           GAMA+SSVFVLGNALRLRRF+ PMA
Sbjct: 819 GAMALSSVFVLGNALRLRRFQPPMA 843
```

Figure 1: BLASTP of Salmonella GolT (Query - WP_023259303.1) against the S. maltophilia 02 protein database. The BLAST search identified a S. maltophilia 02 protein that is similar to GolT.

The identified *S. maltophilia* 02 P-type ATPase was then analyzed by BLASTP against the protein-protein BLAST database at the National Center for Biotechnology Information (NCBI). Figure 2 shows that it is identical to a copper-translocating P-type ATPase from *S. maltophilia,* suggesting that it confers resistance to copper.

copper-translocating P-type ATPase [Stenotrophomonas maltophilia]

Sequence ID: WP_088480648.1 Length: 833 Number of Matches: 1

See 4 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 833 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
1654 bits(4282)	0.0	Compositional matrix adjust.	833/833(100%)	833/833(100%)	0/833(0%)

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Sbjct  1    MSTPRAAAVSASPSTISLPIEGMTCASCVGRVEAALSKVEGVGSVSVNLATERADIRPSG  60

Query  61   PVDRAALIQAVERVGYDVPPATVELAVEGMTCASCVGRVERALLAVPGVSQASVNLATER  120
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Sbjct  61   PVDRAALIQAVERVGYDVPPATVELAVEGMTCASCVGRVERALLAVPGVSQASVNLATER  120

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Sbjct  361  MISGEPIPVEKQPGSSVVGGTVNQKGALTVRATAVGAQTMLAQIIRMVEQAQGSKLPIQA  420

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Sbjct  481  MVGTGRGAEMGVLFRKGEALQLLKDAKVVAVDKTGTLTEGRPRLTDFEITDGFNRSTVLA  540

Query  541  AVAAVESRSEHPIARAIVDAATEQGIALPSMVDFESVTGMGVRASVDGARVEVGADRFMR  600
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Sbjct  541  AVAAVESRSEHPIARAIVDAATEQGIALPSMVDFESVTGMGVRASVDGARVEVGADRFMR  600

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Sbjct  601  DLGVDITLFATLAAELGTQGKSPLYAAIDGRLAAIIAVSDPIKPSTPAAIAALHQLGLKV  660

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Sbjct  781  PVAAGVLYPVWGVLLSPVFAAGAMALSSVFVLGNALRLRRFQPPMADATAATH  833
```

Figure 2: A BLAST result from of the S. maltophilia 02 putative P-type ATPase (Query) against the National Center for Biotechnology Information database. Query the S02 putative P-type ATPase sequence in NCBI using blastp.

4.2 Sequence Alignment

Figure 3 shows a map of the copper-translocating P-type ATPase gene and its surrounding genes as derived from the annotated *S. maltophilia* 02 genome (CP056088). A gene for *tonB* is located downstream of the ATPase gene, and a gene for cueR is located upstream.

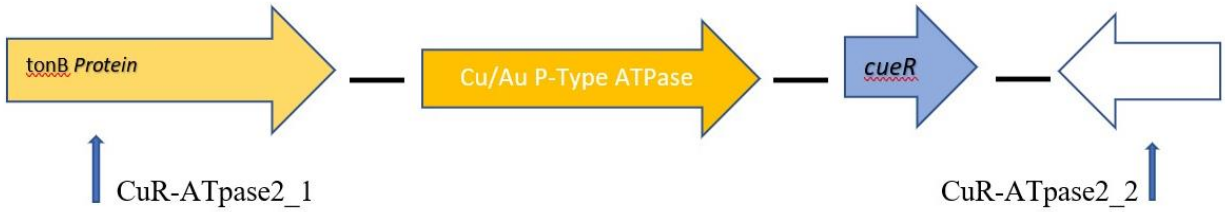

Figure 3: Map of the S. maltophilia 02 chromosomal region that contains the putative P-type ATPase regulon.

This map of the Cu/Au P- type ATPase was imported from Genome Compiler. Mapping this regulon was to identify and design specific primers that could be used to target the whole regulon. Results showed an outer membrane gene for *tonB* protein and a downstream *cueR* a gene for a putative transcriptional regulator. The gene for the Cu/Au P-Type ATPase and *cueR* were cloned using CuR-ATpase2_1 and CuR-ATpase2_2 primers at 72 °C annealing temperature.

3.3 Cloning of the putative copper-translocating P-type ATPase and *cueR* genes

The primers CuR-ATpase2_1 and CuR-ATpase2_2 (Table 1.0) was used to amplify the putative region of copper/gold resistance from the genomic DNA by PCR to obtain the expected 4.2 kb extended fragment (Fig. 4). It was then cloned into the pSCA-Kan/Amp plasmid and introduced into *E. coli* using a StrataClone PCR cloning kit.

Partial sequencing and Basic Local Alignment Search Tool (BLAST) analysis of cloned fragment showed that one end contained a gene that was related to *tonB,* while the other end was related to a gene for the P-type ATPase. These results confirmed that the correct fragment was cloned.

Lane # 1 2 3 4 5 6

1. *S02* genomic DNA 3µl
2. *S02* genomic DNA 3µl
3. DNA ladder 1kb extended 10µl
4. *S02* genomic DNA 3µl
5. Control

Expected 4.2kb bands

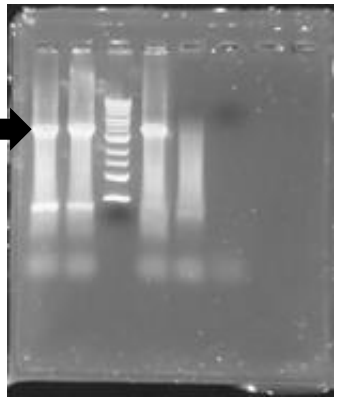

Figure 4: PCR results for S. maltophilia 02 genomic DNA amplification using CuR-ATPase2_1 and CuR-ATPase2_2 at 62°C annealing point.

3.4 Minimal inhibitory concentrations (MIC) results on agar plates

Next, the cloned fragment was tested for copper and gold resistance in *E. coli*. Colonies of *S. maltophilia* 02, *E. coli* containing the cloned fragment and *E. coli* containing the vector were spotted on R3A-tris plates. After growing the plates overnight, they were replica plated on R3A-tris agar containing different concentrations of copper sulfate or gold chloride. Table 2 shows that the copper MIC was 3 mM for *S. maltophilia* 02 and 1 mM for both *E. coli* strains. It also shows that the gold MIC was 40 µM for *S. maltophilia* 02 and 20 µM for both *E. coli* strains. Thus, the fragment did not confer resistance to copper nor gold.

Strain	**0.0 mM Cu**	**0.1 mM Cu**	**0.5 mM Cu**	**1.0 mM Cu**	**2.0 mM Cu**	**3.0 mM Cu**	**0.0 mM Cu**
S. malt	+++	+++	+++	+++	+	—	+++
***E. coli* + insert**	+++	+++	+	—	—	—	+++
***E. Coli* + vector**	+++	+++	+	—	—	—	+++
Strain	**0 µM Au**	**10 µM Au**	**20 µM Au**	**30 µM Au**	**40 µM Au**	**50 µM Au**	**0 µM Au**
S. malt	+++	+++	+++	+	—	—	+++

***E. coli* + insert**	+++	++	–	–	–	–	+++
***E. coli* + vector**	+++	++	–	–	–	–	+++

Table 2: Minimal inhibitory concentrations (MIC)

3.5 Copper and Gold Growth Curves

Although the copper-translocating gene failed to confer resistance in *E. coli*, it may still be expressed in *S. maltophilia* 02 in response to copper and gold salts. *S. maltophilia* 02 growth curves were performed to confirm its copper and gold resistant phenotypes and determine when to harvest cells for reverse transcriptase PCR (RT-PCR) analysis. An overnight culture of *S. maltophilia* 02 was diluted 1:20 in fresh LB media and transferred into three tubes labeled 0 µM (no metal), 200 µM gold and 1 mM copper. The cultures were incubated at 30 °C in a roller drum while taking NanoDrop readings at 600 nm after every 30 minutes. At 1.5 hours, cells were treated with sterile water (no metal), 200 µM gold chloride and 1 mM with copper sulfate. Cells were harvested for RNA purification after 1 hour exposure to metal. The growth curve in orange follows growth in the presence of gold. The growth curve in blue follows growth in the presence of copper, and the grey curve is the positive control which follows growth in the absence of metal. Since there was no significant difference between the three curves, *S. maltophilia* 02 demonstrated resistance to both metals.

	Optical Density (600 nm)		
Time (Hours)	0 µM	200µM Gold	1MM Copper
0	0.044	0.046	0.043
0.5	0.054	0.053	0.051
1	0.063	0.063	0.061
1.5	0.077	0.087	0.074
2	0.098	0.100	0.109
2.5	0.123	0.121	0.130
3	0.146	0.148	0.151
3.5	0.186	0.213	0.184
4	0.221	0.196	0.232
4.5	0.260	0.206	0.259
5	0.272	0.223	0.295

Table 3: Optimal density

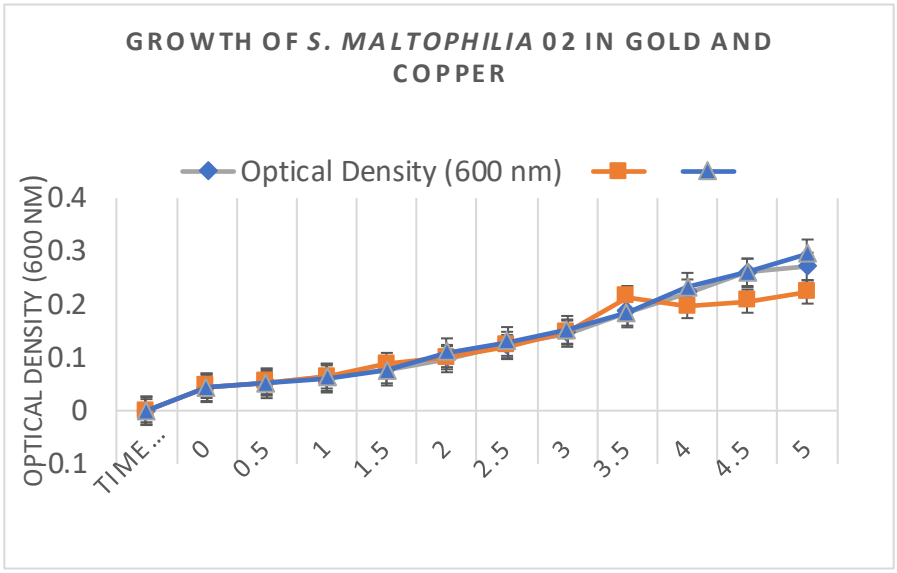

Figure 5: Copper and gold growth curves for S. maltophilia 02

3.6 Reverse Transcriptase PCR (RT-PCR)

The next step was to use RT-PCR to see if gold and copper induce the expression of the P-type ATPase gene in *S. maltophilia* 02. Equal amounts of purified total RNA were converted to cDNA and used in PCR reactions containing primers specific to GAPDH, *tonB*, the P-type ATPase gene and *cueR*.

Gel electrophoresis for the ATPase gene samples and *cueR* demonstrated higher expression levels of both the P-type ATPase gene and *cueR* in the presence of both copper and gold than in the absence of metal (Figure 6a, lanes 3, 4 and 8, 9). The P-type ATPase gene was expressed in response to gold and copper, showing a higher transcription level in the exposed sample than in the unexposed sample.

Figure 6b shows results for RT-PCR in GAPDH and *tonB*. There was consistent expression in GAPDH (Figure 6b lanes 1, 2, 3 and 4) which was the house-keeping gene (positive control) as

well as in *tonB* (Figure 6b lanes 6,7,8 and 9), suggesting that *tonB* is not expressed in response to copper and gold.

a)

1. P-type ATPase – Genomic DNA
2. P-type ATPase – No metal
3. P-type ATPase – Copper
4. P-type ATPase – Gold
5. 100 bp ladder
6. *cueR* – Genomic DNA
7. *cueR* – No metal
8. *cueR* – Copper
9. *cueR* – Gold

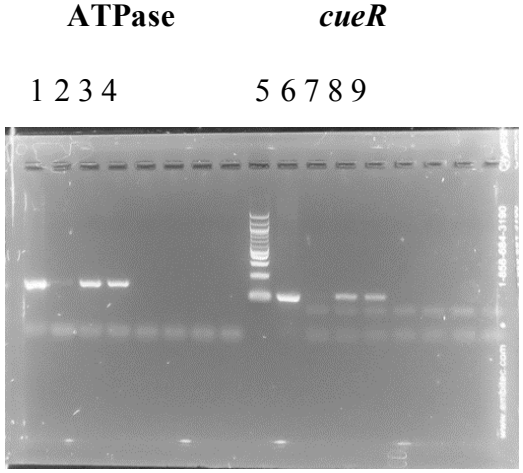

Figure 6: RT-PCR results for ATPase & cueR genes

b)

1. GAPDH – Genomic DNA
2. GAPDH – No metal
3. GAPDH – Copper
4. GAPDH – Gold
5. 100 bp ladder
6. *tonB* – Genomic DNA
7. *tonB* – No metal
8. *tonB* – Copper
9. *tonB* – Gold

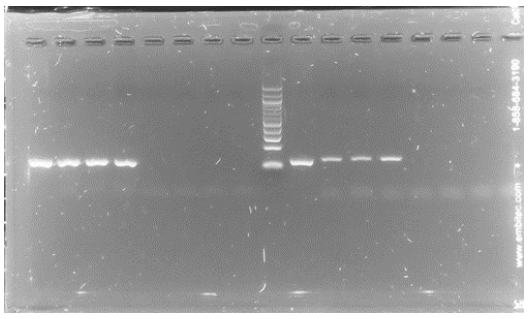

Figure 7: RT-PCR results for GAPDH & tonB genes.

3.7 Multiple Sequence Alignment

The amino acid residues sequence of *S. maltophilia* 02 CueR was aligned with *Salmonella* GolS and *E. coli* CueR to show that they have similar protein domains (Figure 7). A dimerization helix, a metal binding loop and a 2-turn α-helix makes up the metal binding domain. The amino acids residues that are highlighted in blue and green are identical to amino acid residues in the helices of *E. coli* CueR and *S. maltophilia* 02 CueR. DNA binding domain and metal binding domain are connected by the coupling domain helices. The amino acids highlighted in green shows the dimerization helix. These similarities indicate that in the presence of copper and gold salts, they may have similar functions in regulating gene expression.

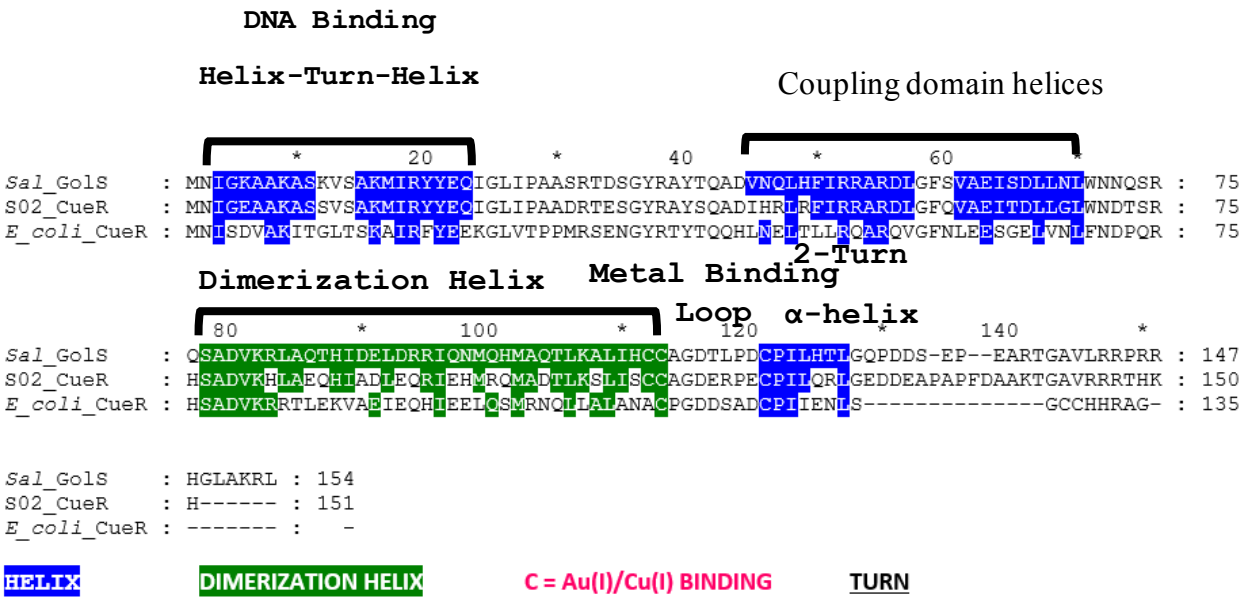

Figure 8: Alignment of the Salmonella GolS amino acid residue sequence against the S02

The promoter for the *S. maltophilia* 02 P-type ATPase gene was compared with the promoter regions of *Salmonella* GolT and *E. coli copA gene* to identify the putative RNA polymerase and CueR DNA binding regions (Figure 8). The nucleotides at -35 are similar. This region is

highlighted in black. The putative GolS or CueR binding sites are underlined. The last three ATG base pairs at the far right mark the beginning of ATPase translation, and *cueR* is transcribed immediately after the ATPase gene.

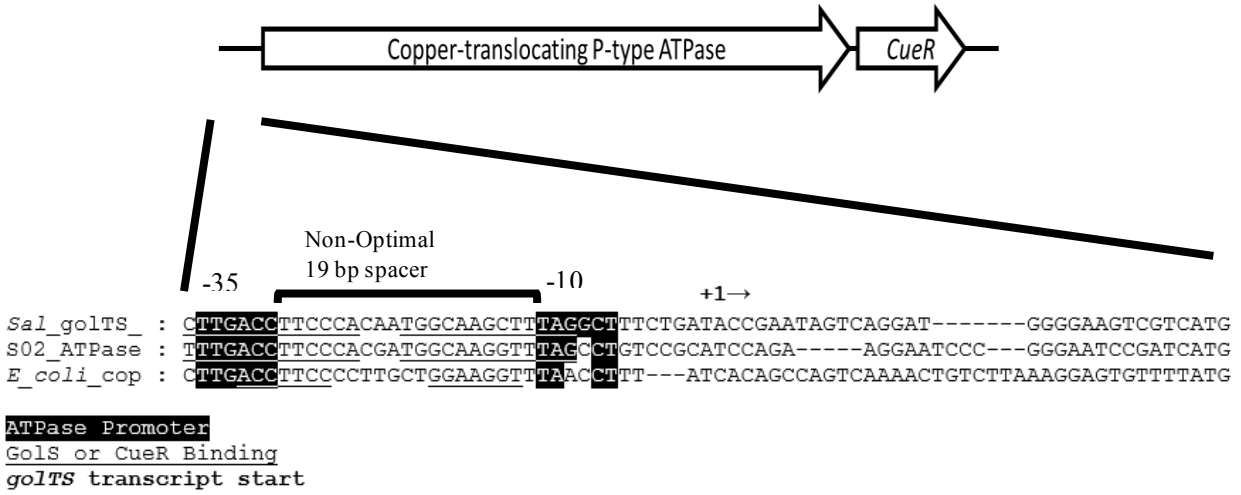

Figure 9 Cu(I)/Au(I)-Translocating ATPase Promoter (Audero et al., 2010 and Baya et al., 2021)

3.8 Prediction of Transmembrane Protein Helices

The *S. maltophilia* P-type ATPase was aligned with GolS (WP_023259303.1) to show that they have amino acid motifs that are similar to other P-type ATPases transmembrane helices and other P-type ATPase features (Figure 9). The *S. maltophilia* 02 P-type ATPase sequence was analyzed using Blastp to find motifs responsible for transmembrane helices. The results were viewed using MSA viewer and showed P-type ATPase motifs responsible for phosphorylation on amino acid D. The underlined regions show amino acids residues in transmembrane helices as predicted by TMHMM (Krough A. et al., 2001) and amino acids highlighted in yellow show predicted copper binding site. Highlighted amino acid residues in green indicate amino acid residues involved heavy metal binding. The predicted putative ATP binding sites are found inside the cell and are

highlighted in maroon. Metal transport is highlighted in purple in the predicted transmembrane protein helix.

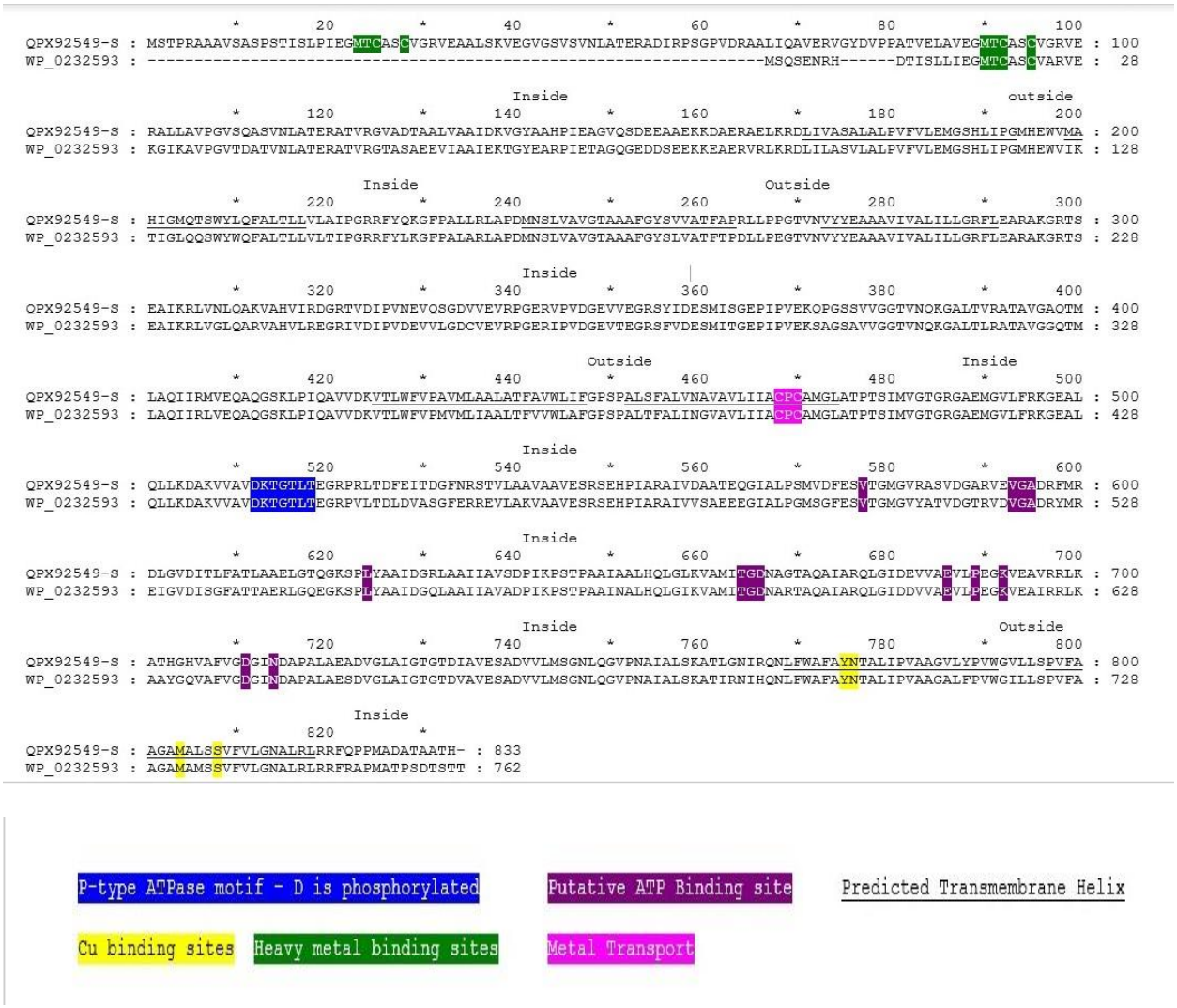

Figure 10: Prediction of transmembrane helices and other P- type ATPase motifs in the S. maltophilia 02 copper-translocating P-Type ATPase.

CHAPTER 4

DISCUSSION AND FUTURE WORK

The purpose of this experiment was to identify a P-type ATPase pump that can confer resistance to gold and copper in *S. maltophilia* 02. In RT-PCR results section, we observed higher expression of the P-type ATPase in response to both copper and gold (Figure 6a). There was increased expression of the P-Type ATPase gene in the presence of both copper and gold. This was expected because CueR controls the expression of the cup- locus Rmet_3523/ Rmet_3525 (CueAR CupC), which encodes a P-type ATPase similar to the regulators in GolT and for the metal chaperones (Jian et al., 2009). CupR selectively detects gold ions due to the presence of two groups of copper resistance cop on chromosome CHR_1 and plasmid pMOLl30, which is why the presence of GolT partially compensated for the lack in copper efflux (Osman et al., 2010).

The cloned fragment does not confer resistance in *E coli*. Perhaps *E. coli* may not have been able to produce the P-type ATPase protein, or may require other protein (GesABC) to obtain gold resistance. Other studies suggest that *S. maltophilia* 02 putative P-type ATPase, which is resistant to both copper and gold, may be due to the development of regulatory circuits in bacteria which replicates the genes that produce the transcription factor code (Martinez et al., 2010). These new factors can initially recognize the same binding sites. Therefore, regulate the same target genes as the original regulator, or they can adapt and act to different signals and improve their binding sites for both copper and gold.

For future work, an alternative approach is to make a knockout mutant. This will be done by replacing the gene for the P-type ATPase in the cloned insert (Fig. 3) with an antibiotic resistance

gene and moving the modified fragment to a conjugatable suicide plasmid. This will be mated into wild type *S. maltophilia* 02 so that a double crossover will replace the P-Type ATPase gene with the gene for antibiotic resistance. Then, this mutant will be tested for impaired resistance to gold and copper using minimal inhibitory concentration (MIC) experiments.

RT- PCR results only reported qualitative expression of copper and gold in *S. maltophilia* 02 analyzed by a 2% agarose gel. To obtain the true measure of expression differences, we can perform quantitative-PCR (qPCR) to the determine expression levels of both copper and gold in ATPase and *cueR* genes. Due to 2020 COVID-19 pandemic, this experiment has started but has not yet been completed. By knowing the *cueR* gene segment that activates the expression of gold and copper, it is possible to identify the protein responsible for the sensitive phenotype. In addition, other gold resistance genes such as *gesABC* genes in *S. maltophilia* 02 may play a role in copper and gold resistance in *S. maltophilia 02*.

In summary, the sequence alignment results confirm that P-type ATPase is 80% similar to GolT *Salmonella enterica*. Expression of ATPase and *cueR* was induced in response to gold and copper, while expression of *tonB* was not induced in response to gold or copper. GAPDH (positive control) shows constant expression in the presence of copper and gold. Finally, *cueR* from S. *maltophilia* 02 appears to be a protein from the MerR-family.

REFERENCES

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2. Crossman LC, Gould VC, Dow JM et al (2008) The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. Genome Biology 1-13.
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