Identification and characterization of a gold sensitive transposon mutant in *Stenotrophomonas maltophilia* OR02.

by

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ABSTRACT

The Y-12 plant in Oak Ridge, Tennessee was a nationwide protection site throughout the second World War as well as the cold war. The plant processed uranium to make nuclear bombs and later in the 1950's during the cold war, shifted to make hydrogen bombs by lithium processing. These procedures led to heavy metal waste deposits in East Fork Poplar Creek and the surrounding stratosphere. In addition, four S3 ponds that stored acidic wastes leached heavy metals into the creek. *Stenotrophomonas.maltophilia* 02 (*S. maltophilia* 02*)* was isolated from this site and demonstrated resistance to the salts of Au (III), Hg (II), Zn (II), As (III), Cd (II), and as well as Se (IV). Transposon mutagenesis generated a *S. maltophilia* 02 mutant that was sensitive to gold. Sequencing of the mutated region revealed that the transposon inserted itself seven base pairs after the gene for a hypothetical protein and 65 base pairs downstream of genes for a DNA binding protein and a two-component sensor histidine kinase. Reverse transcriptase-PCR showed that the transposon interference did not interfere with the gene expression in of any of these genes. The transposon insertion interferes with gold-resistance by some other mechanism.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER I-INTRODUCTION:

1 Background:

1.1 The Y-12 plant and East Fork Poplar Creek

The Oak Ridge reservation, which was first part of the Manhattan project, is a U.S. Department of Energy research, development, and production office established in Oak Ridge, Tennessee, in 1943[6]. One of its essential missions was to separate uranium-235 (^{235}U) from other isotopes of uranium (^{234}U and ^{238}U) for making nuclear weapons during World War-II. During Cold war in the 1950's, it switched to processing lithium to produce hydrogen bombs.X-10, the Oak Ridge National Laboratory, K-25, the gaseous diffusion plant, and the Y-12 Weapons Plant make up the three major working facilities located on this Reservation [1].

The Y-12 plant, which is located near the origin of East Fork Poplar Creek (EFPC), used tons of mercury (Hg) to process lithium for hydrogen bombs. Unfortunately, much of it leaked from sites between 1950 and 1963, leading to the contamination of soil, building foundations, and subsurface channels. It was estimated that, 100 metric tons of mercury-laden wastewater was directly discharged into the creek [10]. Since the 1980s, mercury accumulation and change to the EFPC biological system have been a concern [2].

1.2 *Stenotrophomonas maltophilia* Oak Ridge strain 02 (*S. maltophilia* 02)

S. maltophilia is a global, aerobic, non-fermentative, gram-negative bacillus that is firmly identified to the *Pseudomonas* species [3]. In view of the Greek origin, the name implies "a unit feeding on few substrates" stenos, which means narrow or slender, trophos, one who feeds, and monas meaning a unit. *Maltophilia* signifies "proclivity for malt," because of the Greek origin maltum (malt) and philia (affinity) [3]. *S. maltophilia* was first isolated in 1943 from the human pleural fluid and was named *Bacterium bookeri.* It was subsequently classified under the genus *Pseudomonas,* then, to *Xanthomonas,* and afterward to *Stenotrophomonas* in 1993 by Palleroni and Bradbury. It is one of the most challenging pathogens because it is one of the most underestimated multi-drug resistant organisms in the hospital. Its closest genetic relatives are plant pathogens [5]. It is normally found in soil, water, plant matter, and hospital settings.

S. maltophilia is the only species of *Stenotrophomonas* that causes nosocomial infections in humans [3,6]. In hospital settings, *S maltophilia* colonizes intravenous fluids and patient respiratory, and urine secretions. *S maltophilia* bypasses normal host defenses to cause human infections [6]. It infects immunosuppressed patients with cystic fibrosis, HIV, venous catheters, mechanical ventilators, trauma, and long-term hospitalization [3]. Its capacity to produce a biofilm, delivering protections from host defenses along with antimicrobial agents. This fact is because of its positively charged surface and fimbrial attachments [4]. Thus, high morbidity and death have also been related to *S. maltophilia* infections in severely immunocompromised and weakened individuals [3].

Lewis and Zaas, 2014 [4] showed that *S.maltophilia* is an aerobe that occurs on commonly used media for academic research institutions, such as blood and MacConkey agars. Standard biochemical tests showed that it is non-lactose fermenting, oxidase-negative, and catalase-positive [4]. It also demonstrates positive tests for mobility, glucose utilization without gas production, lysine, urea, DNase, catalase, methyl red, Voges-Proskauer, citrate, ornithine, H_2S, adonitol, lactose, arabinose, sorbitol, dulcitol, phenylalanine, indole, and oxidase [4,5].

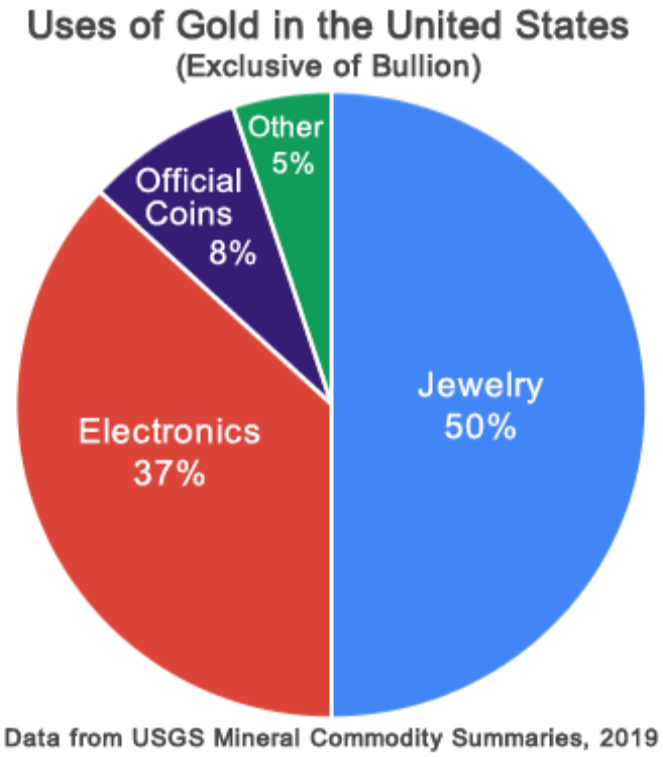
The multi metal resistant bacterial strain, *Stenotrophomonas maltophilia* Oak Ridge strain 02 (*S. maltophilia* 02), was isolated from East Fork Poplar Creek. Minimal inhibitory concentration (MIC) experiments showed that it is resistant to salts of the Hg (II), Cd (II), Zn (II), Cu (II), Au (III), Cr (VI), As (III), and Se (IV) [5,6].

A gold-sensitive mutant of *S.maltophilia* 02*,*A12, was generated using the EZ-Tn5™ transposome [6] . The minimal inhibitory concentration (MIC) by liquid culture experiments for A12 was about 170 μM gold chloride, whereas the wild type *S. maltophilia* 02 grew well into high concentrations of gold salts close to 190 μM. Basic local alignment search tool (BLAST) and DNA sequencing analysis showed that the transposon inserted itself 7 base pairs after the stop codon for a hypothetical protein and 65 base pairs before the start codon for a DNA binding protein and a two-component sensor histidine kinase (Figure 1) [6].

1.3 Properties and uses of Gold:

Gold is the heaviest metal according to King and is excellent at conducting power and heat [7]. An ounce of gold leaf can cover an area of 187 square feet in extremely thin sheets [6]. Gold is present in low concentrations in igneous rocks, and the earth's hull contains about 0.005 parts per million gold [6]. Only tellurium, selenium, and bismuth are synthetically combined, remaining primarily in the indigenous state [7].

Owing to its unique properties, gold is one of the most important minerals mined from the Earth. It is very easy to work with because it is a good conductor of electricity, does not tarnish, and has a beautiful color and a brilliant luster [7].



Jewelry and ornaments made from gold have been used for thousands of years. Every year, jewelry accounts for around 78% of the world's gold production [7].

As a medium of exchange and money, it has long been highly valued and in very limited supply. Gold's rarity, versatility, and desirability make it a valuable medium of exchange for the long-term worth [6, 7]. Gold is crucial in the production of electronics; it is an important component of most desktop and laptop computers. The dental industry, medicine, glass manufacturing, and aerospace industry all use gold [7].

1.4 Environmental Pollution

- **Heavy Metal Toxicity**:

About 40 different elements are heavy metals. Due to their ability to denature proteins, heavy metals can cause toxic effects on cells, even though some of them are essential for growth. Microbial resistance to heavy metals is, however, a common phenomenon, and is of particular importance to microbial ecology particularly in connection with the functions of microbes in metal-contaminated environments and polluted ecosystems [8].

The biological availability of metals is highly dependent on how they bind to other environmental constituents in a system. In the presence of heavy metal ions and other environmental constituents, a metal can be wholly or partially be removed by sequestering proteins to eliminate their toxic effects [29]. Mercury is often associated with oil spills. According to a previous study, mercury levels in oil-polluted marine habitats were 4000 times higher than those in the sediment and 300,000 times higher than those in the water. Mercury resistance was detected in the organisms isolated, while its effects on resistant behavior were unknown [8].

According to studies conducted by Gadd, Griffiths (1977), pH can significantly play an important role on the availability and toxicity of heavy metals in the environment. In addition, the presence of other ions, such as calcium and magnesium, can lower the biological activity of heavy metal ions [8].

Gold toxicity:

Gold nanoparticles have unique properties that make them useful for applications in chemistry, physics, biology, and medicine. Gold nanoparticles enhance antimicrobial properties when conjugated to small molecules such as antibiotics, medicines, vaccines, and antibodies. However, the results are sometimes unclear and contradictory due to the lack of standard experimental procedures for toxicological effects. Different research has employed different approaches, administration routes and doses, leading to different conclusions [3].

Merchant (1988) proposed that gold poses minimal hazardous harm to humans at normal amounts found in nature. The human body has trace amounts of gold, which is restricted to hair, skin, and nails. Gold occurs in six oxidation states the most prevalent state in the human body is gold (I). Oxidized species such as gold (III) undergo hydrolysis under physiological settings and have a significant redox potential. Gold is non-essential in the human body. It harms microbes by causing oxidative stress. The development of effectual therapeutic agents requires the stability of gold-incorporating drugs under physiological conditions remains challenging [6].

1.5 Bacterial gold sensing and resistance

Checa, and Soncini, (2011) discovered that gold ions are transported into bacterial cells by non-specific intake. Gold salts are toxic to bacteria even at very low concentrations. Thus, bacteria such as *Salmonella enterica* and *Cupriavidus* use gold-specific MerR-Type transcriptional regulators to sense micromolar concentrations. Beside the copper sensor CueR, the Au-selective metalloregulatory proteins can distinguish Au(I) from Cu(I) or Ag(I) [9,10]. As well as preferring Au(I) binding over Cu(I) or Ag(I) in *Salmonella enterica,* GolS also distinguishes its target recognition sites in its regulated promoters minimizing cross-activation of CueR-controlled operators [29]. Using a specific Au sensory device could alleviate this problem for species with resident Cu-homeostasis systems, which might impede the acquisition of Cu in Au-rich environments without effecting the removal of the harmful ions [10].

1.6 Background behind the *S. maltophlia* 02 A12 mutant.

Transposon mutagenesis

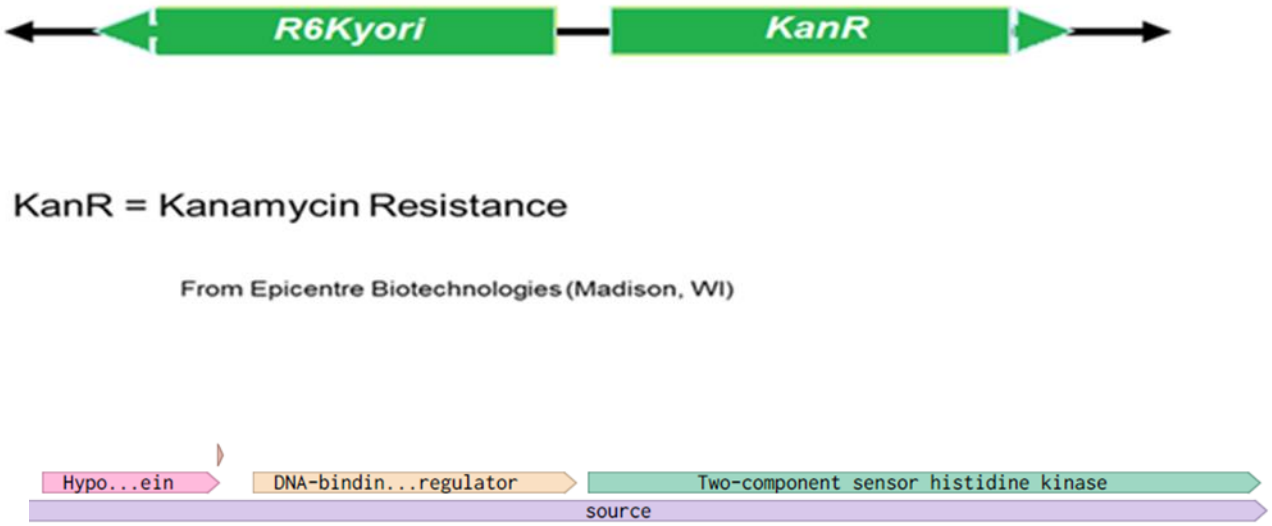


Figure 1: Map of transposon insertion site in the A12 mutant. The figure showing hypothetical protein, DNA-binding protein (cpxR) and two-component sensor histidine kinase (cpxA).

Figure 1 shows a map of the chromosomal region that was interrupted by the transposon in the A12 mutant. The *cpxA* (Two-component sensor histidine kinase) and *cpxR* (DNA-binding protein) genes are part of the Cpx-two component signal transduction system. The two-component signal transduction system enables the bacteria to sense, respond and adapt to environmental changes. On the receipt of an input signal, the histidine kinase auto phosphorylates a conserved histidine residue. The phosphoryl group is then passed on to a

conserved aspartate residue on the response regulator. The regulator protein activates gene expression in response to the toxic gold concentration [16].

The introduction of this EZ-Tn5 transposome by electroporation randomly incorporated itself into the genome of *S. maltophilia* 02 resulting in the gold-sensitive mutant A12. The transposon inserted itself seven base pairs after the stop codon of a gene for hypothetical protein (purple arrow) located downstream of genes for a DNA binding protein and a two-component sensor histidine kinase [11]. The objective is to see if the insertion interfered with the gene expression of the hypothetical protein, DNA binding protein or the histidine kinase [11].

CHAPTER II -HYPOTHESIS/SPECIFIC AIMS

Experimental Design

S.maltophilia 02 wild type and mutant strains were grown in the presence and absence of gold, and cells were harvested one hour after exposure. Then, RNA was purified and converted to cDNA and used in PCR reactions to see if there is a decrease in expression of the hypothetical protein, the DNA binding protein, and the histidine kinase.

Specific Aims:

- To detect if the hypothetical protein, DNA binding protein and histidine kinase are involved in gold resistance. Growth curves will be used to define the resistance and sensitive phenotypes and to select the best time point for RNA purification.
- To separate PCR amplified cDNA on a 2 % agarose gel to see if there is a change in the expression of the histidine kinase [11,13].
- RT-PCR (Reverse transcriptase-PCR) may reveal if introducing the transposon interfered with the expression of the putative protein, *cpxR* or *cpxA* (PhoQ) [12].
- In the presence of gold, agarose gel electrophoresis will indicate the level of expression for these genes [6]. The presence of the PCR product in the wild type and its absence in the A12 mutant will indicate that transposon insertion disrupted gene expression.

CHAPTER III- METHODS

3.1 Bacterial Strains

Stenotrophomonas maltophilia Oak Ridge strain 02 (*S. maltophilia* 02) (ATCC 53510) was obtained from the American Type Culture Collection AATC (Manassas, VA). The *Escherichia coli* EC100DTM pir-116 (EC6P095H) was obtained from Epicenter (Madison, WI), the A12 mutant was generated from *S.maltophilia* 02 through transposon mutagenesis [6].

3.2 Growth Medium and Gold Salts

Growcells (Irvine, CA) supplied the Lennox LB medium that contained 10 grams of tryptone, 5 grams of yeast extract and 5 grams of sodium chloride per liter of medium. LB medium was supplemented with 1.6% Agar (Amresco Inc., Solon, OH) for solid medium. When required, the A12 strain was grown in LB medium with the addition of 800 µg/ml kanamycin (Amresco, Solon, OH). Amresco, supplied the gold salts ($HAuCl_4 \cdot 3H_2O$) [14,15,10].

3.3 Gold Growth Curves with *Stenotrophomonas maltophilia* 02

The overnight cultures of *S. maltophilia* 02 and A12 were diluted 1/50 in fresh LB broth. Then, six ml of the culture was added to 2 tubes labeled 0 and 200 µM gold. The samples were incubated in the roller drum at 30 degrees. A NanoDrop 2000c Cuvette Spectrophotometer (Thermo Fisher Scientific; Waltham, MA) was used to measure the optical density for each culture at 600 nm, every 30 minutes for 5 hours. After 1.5 hours of growth, gold chloride was added to the gold tubes and sterile water(control)

was added to no metal tubes [14]. After 2.5 hours of growth, RNA purification was carried out by using 200 µl of cells that were added to 200 µl of RNA protect reagent (Qiagen GmbH, D-40724 Hilden, Germany). Incubation was carried out at room temperature for five minutes after vortexing the mixture for five seconds. After pelleting the cells by centrifuging at 5,000 x g for ten minutes, the supernatant was drained off and the remaining supernatant was dabbed off with a paper towel. The pellet was frozen at -80°C [15].

3.4 Genomic DNA preparation:

Purification of genomic DNA was performed using the Wizard Genomic DNA purification kit from Promega (Madison, WI). 1 ml of overnight culture was centrifuged at 15,000 x g for 2 minutes to pellet the cells. The supernatant was then drained off, and the pelleted cells were gently resuspended in 600 µl of Nuclei Lysis Solution and lysed for five minutes at 80 °C [6]. After the aliquots were cooled to room temperature, they were mixed with 3 ml of RNase solution (4 mg/ml), inverted 2-5 times, and then incubated at 37 °C for 15 minutes**.** Having allowed the lysate to reach room temperature, 200 µl of protein precipitation solution was added and vortexed for 20 seconds. The samples were kept on ice for five minutes before centrifuging them at 15,000 x g for three minutes. A clean 1.5 ml tube containing 600 µl of isopropanol was mixed with the supernatant. DNA strands were visible after inverting the mixture for a few minutes. After Centrifuging at 14,000 x g DNA, pellets were collected, and the supernatant was poured off. As a next step, 300 µl of 70% ethanol was added to the tube. It was inverted several times, and centrifuged for two minutes at 14,000 x g. The supernatants were decanted, and the

pellets were air dried for 15-minute. Finally, the DNA was resuspended in 100 µl of DNA Rehydration solution and stored in a refrigerator [6,16]

3.5 RNA preparations

The RNA Protect treated pellet was thawed. A TE/proteinase K/lysozyme buffer was prepared by mixing 10 µl of Qiagen proteinase K (Qiagen GmbH 40724, Hilden, Germany) with 100 µl of T. E buffer (30Mm tris, 1mM EDTA, pH 8.0) containing 15 mg/ml lysozyme (Amresco, Solon, OH) [6]. By pipetting up and down gently, the thawed cells were resuspended in 110 µl of TE/proteinase K/lysozyme buffer and incubated for 10 minutes at room temperature. The sample was vortexed for 10 seconds every 2 minutes. Next, 350 µl of RLT buffer containing 10 µl β-mercaptoethanol/ml (VWR Chemicals, Solon, Ohio) was added to the mixture. The supernatant was transferred to new a 1.7 ml tube after microfuging the solution at 10,000 rpm for two minutes. [6,16,17]. Then, the supernatant was mixed with 250 µl of 95-100% ethanol, loaded onto a spin column and bound to the column resin by centrifugation at 10,000 rpm for 15 sec [19]. The flow-through was discarded, and the spin column was washed by centrifuging 700 µl of Buffer RW1 through it for 15 seconds at 8000 x g. After, discarding the flow-through, the column was washed with 500 µl RPE buffer at 800 x g for 15 seconds [17]. To avoid contamination from flow-through, the column was washed again for 15 seconds with a 500 µl RPE buffer at 800 x g. Spinning the empty column at 10,000 rpm for 1 minute removed residual buffer [20,21]. The spin column was placed in a new tube, and RNA was eluted by centrifuging 30 µl of RNase-free water through the column at 8000 X g for 1 minute [19]. This elution step was repeated to increase yield [6,17].

DNase Treatment

To remove residual DNA, the purified RNA was mixed with 6 µL of 10X TURBO DNase buffer and 1 µL of TURBO DNase (Thermo Fisher Scientific Baltics UAB VA Graciuno8, LT-02241 Vilnius, Lithuania) and incubated at 37° C for 20 minutes [22,12]. Then, 7 µl of DNase Inactivation Reagent (Thermo Fisher Scientific Baltics UAB VA Graciuno8, LT-02241 Vilnius, Lithuania) was added by gently pipetting it up and down**.** The mixture was incubated for 5 minutes at room temperature and centrifuged for 1.5 minutes. The supernatant was subsequently transferred to a new RNase free tube. Concentrations of RNA were determined by using Nanodrop. The purified RNA was frozen at -80 °C [13,22].

3.6 cDNA synthesis

For cDNA synthesis,86 ng of RNA was mixed with 2 µl of Random Primer Mix (60 m stock solution) and nuclease free water to make a final volume of 8 µl. The control was made with a second tube [16,12,13]. Denaturing of RNA was done at 65°C for 5 minutes and placed on ice. 10 µl of ProtoScript II Reaction Mix and 2 µl of ProtoScript II Enzyme Mix (New England, Biolabs) were added to the tubes labelled for no metal and gold. For the control tube 2 µl of nuclease-free water was added, instead of enzyme. The reactions were then incubated in the thermal cycler for 1 cycle of 25 °C for 5 minutes, 42°C for 60 minutes and,80° C for 5 minutes. The reactions were held at 4°C and then frozen at -20°C [6,17].

3.7 Polymerase Chain Reaction PCR:

PCR reactions contained 2.5 µl of 4 µM of forward primer, 2.5 µl of 4 µM of reverse primer (Table 1), 10 µl of 2x GoTaq DNA polymerase (Promega Corporation, Madison, WI), 1 µl of DNA or cDNA template and 4 µl of nuclease free water to make the total volume up to 20 µl [18,19].

A thermal cycler was used for the PCR reactions using the following program: 95 ºC for 2 minutes, 35 cycles of 95 ºC for 1 minute, 60 ºC for 1 minute and 72 ºC for 30 seconds, followed by a last 72 ºC incubation step for 10 minutes. The final reactions were held at 25ºC [6, 16, 19].

Table 1: List of Primers used for RT-PCR

Primer	Nucleotide sequence
S02_A12sen_R	5'-CCT CGG CGT TGG ATT CGA TCG G- 3'
S02_A12sen_F	5'-AGC TGC AGC GCA TGA ACA ACC T -3'
S02_A12reg_R	5'-ACG GTC TCG ATC GGC TTC AGC T-3'
S02_A12reg_F	5'-AGG ACT TCG ACC GCG ACT CGA A-3'
S02_A12Hyp_R	5'-GCT CCA TCA TCT CTG CAC GCC C-3'
S02_A12Hyp_F	5'-CTT CCG CTC TCC ATT GCC G-3'
S02_GAPDH_F	5'-AAACCGCGCAGAAGCACATCGA-3'
S02_GAPDH_R	3'-GCCGGCGTAGGTCTTGTCGTTC-5'

3.8 Agarose Gel Electrophoresis

1.3 g of BioExcell Agarose LE was added (WorldWide Medical Products; Bristol, PA) to 130 ml of 1X TBE (AMRESCO; Solon, OH) to prepare a 2% agarose gel [12,16]. 10X TBE buffer (0.089 M Tris Base, 0.089 M Borate, and 0.002 M EDTA) was diluted with water to make the 1X TBE buffer. The mixture was then microwaved until the agarose was completely dissolved, then 13 µl of Gel Green Nucleic Acid Stain (Embi Tec; San Diego, CA) was added and mixed using a stir bar and stir plate. The contents were mixed and were poured into a gel tray and a comb was inserted to form wells. The comb was then removed after the gel hardened [16,17]. The gel was transferred to a Run One Electrophoresis System and covered with 1X TBE buffer. All the gels prepared using this method were run at 100V for 30 minutes [6, 12, 17]. Quick load purple 100bp DNA ladder (New England, Biolabs) was used in this experiment. The gel was run for about 30 minutes and a picture of the gel was taken using an UltraCam Imaging Systems (Ultra-Lum, Inc. Claremont, CA) [17].

CHAPTER IV-RESULTS

4.1 Minimum Inhibitory Concentrations (MIC's):

The A12 *S.maltophilia* 02 mutant was generated by Andrew Zack [6]. Gold sensitivity of A12 mutant was measured by performing MIC experiments. Overnight cultures (18 hours) of A) *S. maltophilia* 02 and B) A12 were diluted into fresh R3A-tris medium in the ratio of (1/50). Different concentrations of gold

chloride were added to the test tubes and the cultures were incubated overnight [16]. A scatter chart of turbidity vs gold concentration was plotted as shown in the figures 2 and 3.

The turbidity was measured by a Klett Colorimeter. The MIC for the A12 mutant was found to be around 175 µM, whereas the wild type grew well at 190 µM gold and beyond. This showed that A12 is sensitive to gold chloride and *S.maltophilia* 02 is resistant as the *S.maltophilia* 02 MIC was greater than the A12 MIC (Figures 2-3). [17].

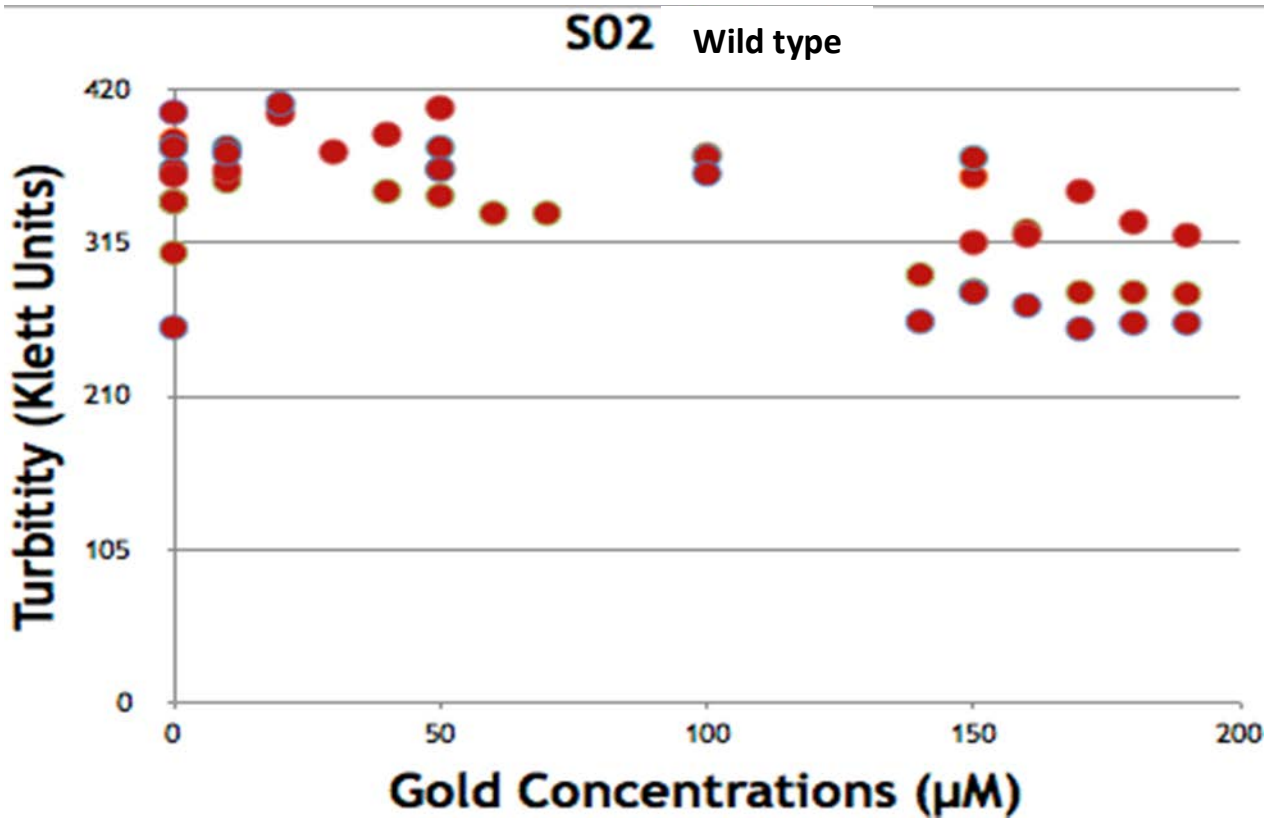


Figure 2: Stenotrophomonas maltophilia (S02) wild type, Minimum inhibitory concentration [6].

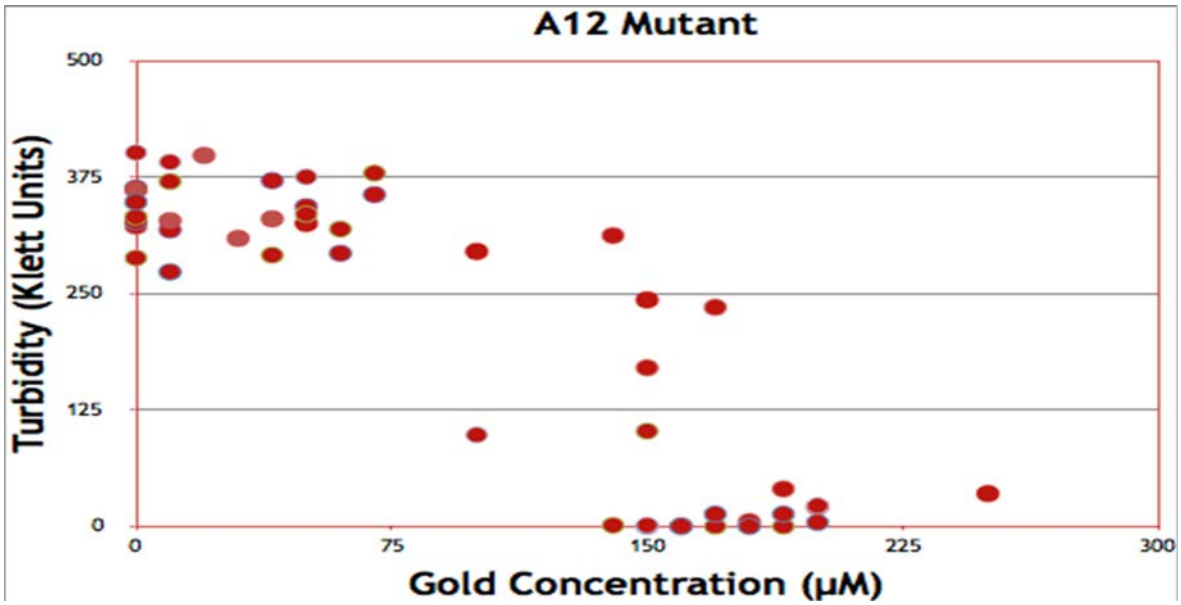


Figure 3: Stenotrophomonas maltophilia (A12) mutant Minimum inhibitory concentration [6].

4.2 Growth curve results:

S.maltophilia 02 and A12 overnight cultures were diluted 1/50 in 50 ml of fresh LB broth. After 1.5 hours of growth, sterile water (control) and gold chloride was added to concentration of 200 µM to make the gold tube and an equal volume of sterile water was added to the second tube as the control [15]. After 2.5 hours of growth, samples were taken for RNA purification.

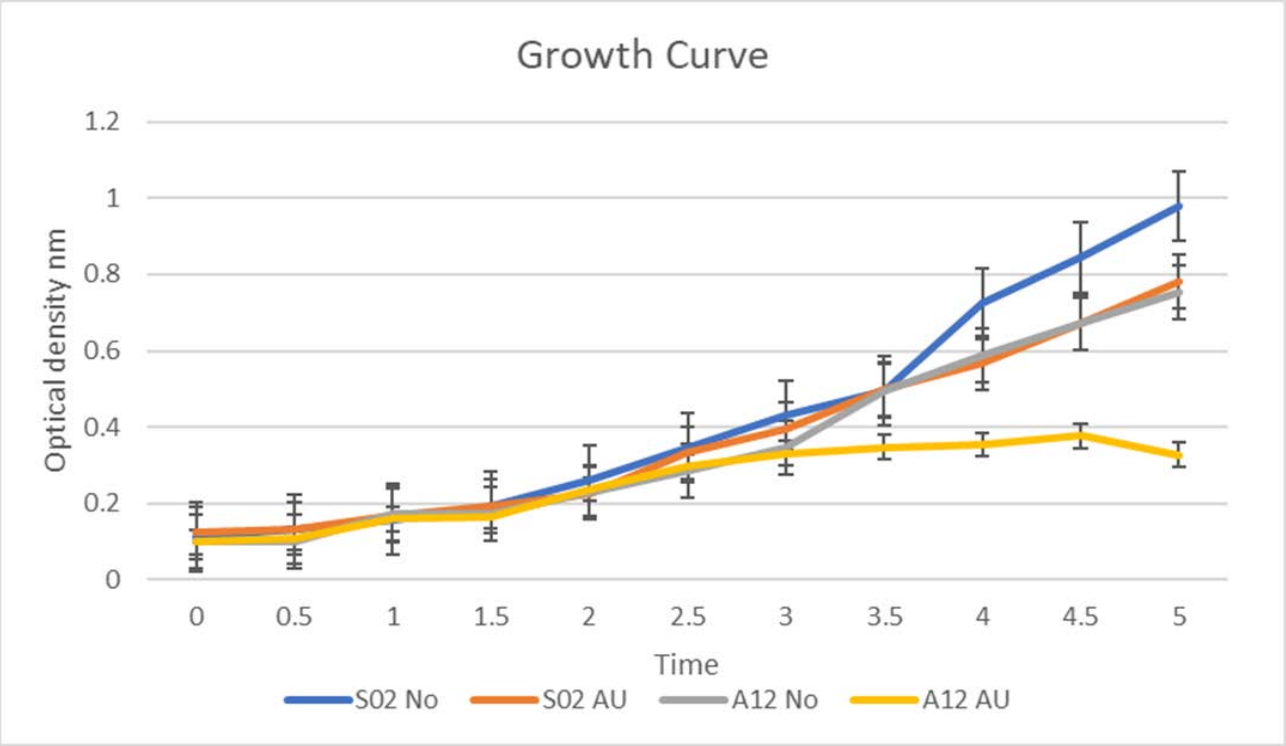


Figure 4: Growth curve graph

The blue line represents *S.maltophilia* 02 (no metal) which is the positive control curve, which grew to highest density. The orange line, *S.maltophilia* 02 (Au) with metal, also showed a higher growth density in the presence of gold than demonstrated by A12.The A12 curve (no metal) grey line is quite similar to *S.maltophilia* 02 curve with metal. In other words, A12 without metal does not grow as well as *S.maltophilia* 02 without metal. The A12 curve (with metal) yellow line shows a lower density in the

presence of gold, which suggests A12 is sensitive to gold. The student T test was used with a 95% confidence level to generate error bars denoted in black in the graph.

4.3 Rt-PCR with GAPDH primer:

RT-PCR reactions were performed to see if the transposon insertion interfered with the expression of the genes for the hypothetical protein, DNA binding protein and the histidine kinase (figure 1) [12,23]. RNA was purified from cells 1 hour after they were exposed to gold chloride. After converting it to cDNA using reverse transcriptase, PCR reactions were carried out with primers specific for the genes for GAPDH, the hypothetical protein, the DNA binding protein, and the histidine kinase. The GAPDH reactions, which served as a housekeeping positive control were separated on a 2% agarose gel (Figure 5). PCR amplified *S.maltophilia* 02 genomic DNA shows an expected 100bp band in lanes 1 and 8 of figure 5. RT-PCR reactions using cDNA from untreated *S.maltophilia* 02 and A12 cells produced 100 bp fragments (lanes 2 and 9) as expected. In addition, RT-PCR reactions using cDNA from gold treated *S. maltophilia* 02 and A12 cells produced 100 bp fragments (lanes 4 and 11). The equal intensity of the bands between lanes 2 and 4 and 9 and 11 verifies that equal amounts of cDNA were PCR amplified. Lane 6 contained a sample from a PCR reaction that contained primers but no template. It was a negative control that showed that there was no DNA contamination in any of the reagents. The separated reactions in lanes 3, 5, 10 and 12 were negative controls to test for DNA contaminated in the RNA preparations. These contained cDNA reactions that lacked reverse transcriptase. Since *Taq* polymerase only amplifies DNA but not RNA, the only way that a band will appear in these lanes is if there is DNA contamination in the RNA preparations. The lack of DNA bands in these lanes verified that there was no DNA contamination in any of the RNA preparations [24,25].

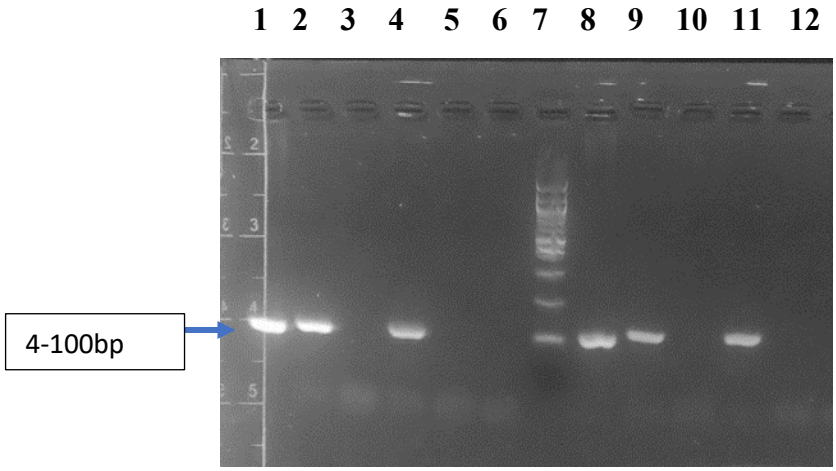
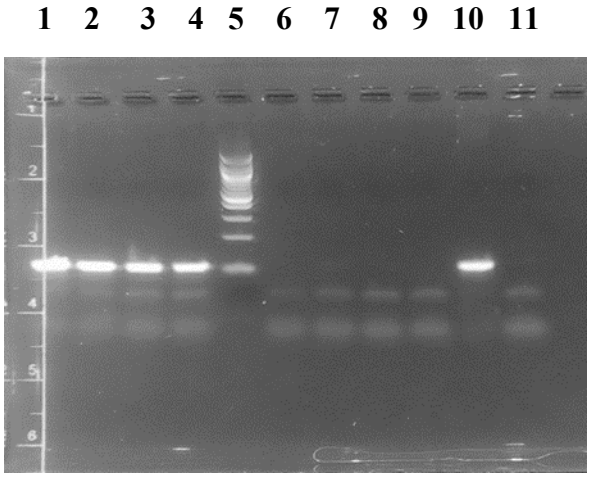


Figure 5- PCR result with GAPDH primer-Lane 1-S02 Genomic DNA, Lane 2-S02 No metal with enzyme, Lane 3-S02 No metal without Enzyme, Lane 4- S02 Gold with enzyme, Lane 5- S02 Gold without enzyme, Lane 6- Control (-), Lane 7- 100 bp Ladder, Lane 8-S02 Genomic DNA, Lane 9- A12 No metal with enzyme, Lane 10- A12 No metal without enzyme, Lane 11- A12 Gold with enzyme, Lane 12- A12 Gold without Enzyme.

4.4 RT-PCR Results:

Hypothetical protein:

PCR reactions targeting the gene for the hypothetical protein were separated using a 2% agarose gel (figure 6) Amplified *S.maltophilia* 02 genomic DNA shows an expected 100bp band in lane 10.RT-PCR reactions using cDNA from untreated *S. maltophilia* 02 and A12 cells produced 100 bp fragments (lanes 1 and 3). In addition, RT-PCR reactions using cDNA from gold treated *S. maltophilia* 02 and A12 cells produced 100 bp fragments (lanes 2 and 4). The equal intensity of the bands between lanes 1 to 4 show that the transposon did not interfere with the expression of the hypothetical protein gene. Lane 11 contained a sample from a PCR reaction that contained primers but no template. It was a negative control that showed that there was no DNA contamination in any of the reagents. The separated reactions in lanes 6, 7, 8 and 9 were negative controls to test for DNA contaminated in the RNA preparations. The lack of DNA bands in these lanes verified that there was no DNA contamination in any of the RNA preparations.



Hypothetical protein

Figure 6- Lane 1- S.maltophilia (S02) No metal with enzyme, Lane 2- S02 Gold with enzyme, Lane 3- A12 No metal with enzyme, Lane 4- A12 Gold with enzyme, Lane 5- 100 bp Ladder, Lane 6- S02 No

metal without enzyme, Lane 7-S02 Gold without enzyme, Lane 8-A12 No metal without enzyme, Lane 9-A12 Gold without enzyme, Lane 10-S02 Genomic DNA, Lane 11- Control.

Histidine kinase:

PCR reactions targeting the gene for the histidine kinase, were separated using a 2% agarose gel (figure 7) Amplified *S.maltophilia* 02 genomic DNA shows an expected 100 bp band in lane 10.RT-PCR reactions using cDNA from untreated *S. maltophilia* 02 and A12 cells produced 100 bp fragments (lanes 1 and 3). In addition, RT-PCR reactions using cDNA from gold treated *S. maltophilia* 02 and A12 cells produced 100 bp fragments (lanes 2 and 4). The equal intensity of the bands between lanes 1 to 4 show that the transposon did not interfere with the expression of the histidine kinase gene. Lane 11 contained a sample from a PCR reaction that contained primers but no template. It was a negative control that showed that there was no DNA contamination in any of the reagents. The separated reactions in lanes 6, 7, 8 and 9 were negative controls to test for DNA contaminated in the RNA preparations. The lack of DNA bands in these lanes verified that there was no DNA contamination in any of the RNA preparations.

Sensory-Histidine kinase

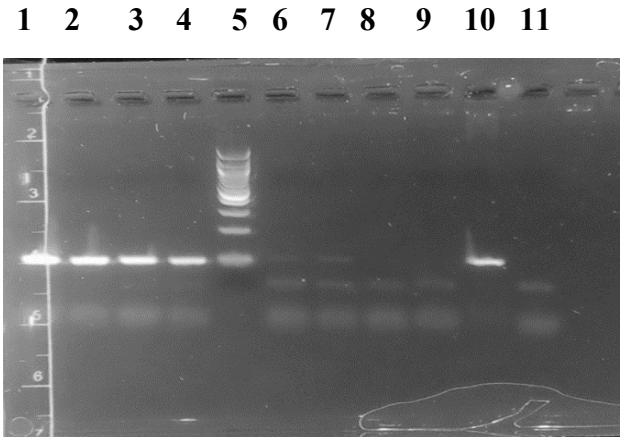
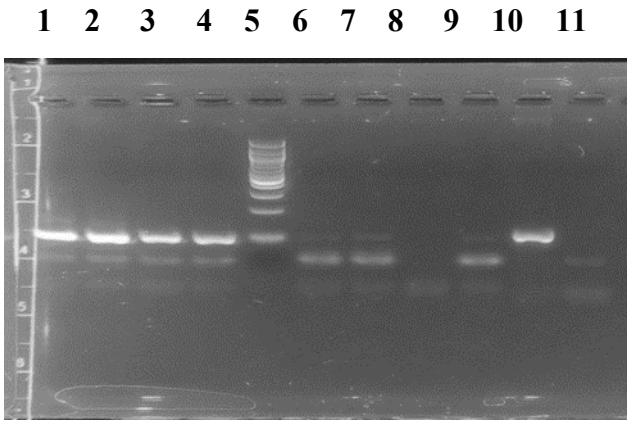


Figure 7- Lane 1-S. maltophilia (S02) No metal with enzyme, Lane 2- S02 Gold with enzyme, Lane 3- A12 No metal with enzyme, Lane 4- A12 Gold with enzyme, Lane 5- 100 bp Ladder, Lane 6- S02 No metal without enzyme, Lane 7-S02 Gold without enzyme, Lane 8-A12 No metal without enzyme, Lane 9- A12 Gold without enzyme, Lane 10-S02 Genomic DNA, Lane 11- Control.

DNA binding protein:

PCR reactions targeting the gene for the DNA binding protein, were separated using a 2% agarose gel (figure 8) Amplified *S.maltophilia* 02 genomic DNA shows an expected 100 bp band in lane 10.RT-PCR reactions using cDNA from untreated *S. maltophilia* 02 and A12 cells produced 100 bp fragments (lanes 1 and 3). In addition, RT-PCR reactions using cDNA from

gold treated *S. maltophilia* 02 and A12 cells produced 100 bp fragments (lanes 2 and 4). The equal intensity of the bands between lanes 1 to 4 show that the transposon did not interfere with the expression of the histidine kinase gene. Lane 11 contained a sample from a PCR reaction that contained primers but no template. It was a negative control that showed that there was no DNA contamination in any of the reagents. The separated reactions in lanes 6, 7, 8 and 9 were negative controls to test for DNA contaminated in the RNA preparations. The lack of DNA bands in these lanes verified that there was no DNA contamination in any of the RNA preparations.



Regulatory- DNA Binding protein

Figure 8- Lane 1- S.maltophilia (S02) No metal with enzyme, Lane 2- S02 Gold with enzyme, Lane 3- A12 No metal with enzyme, Lane 4- A12 Gold with enzyme, Lane 5- 100 bp Ladder, Lane 6- S02 No metal without enzyme, Lane 7-S02 Gold without enzyme, Lane 8-A12 No metal without enzyme, Lane 9- A12 Gold without enzyme, Lane 10-S02 Genomic DNA, Lane 11- Control.

Bands were seen with treated samples and positive control S02 genomic DNA, whereas the untreated samples and negative control did not show bands. There was no consistent difference in the results of hypothetical protein, histidine kinase, and DNA binding protein.

4.5 BLAST Analysis Basic Local Alignment Search Tool

The Basic Local Alignment Search Tool (BLAST) is a program that compares query sequences to a database of thousands of other sequences to find sequence similarities. BLAST searches a protein or nucleotide database in several ways [17]. A query can be submitted in FASTA format, as a Gi number or as an accession number. A BLAST analysis matches your query sequences to subject sequences in the database [12].

The amino acid residue sequence of *S. maltophilia* 02 hypothetical protein was evaluated using BLAST to determine if it was linked to other known proteins (Fig 9). None of the results showed a function for hypothetical protein though it was found to be 100% like other *S. maltophilia* 02 hypothetical proteins.

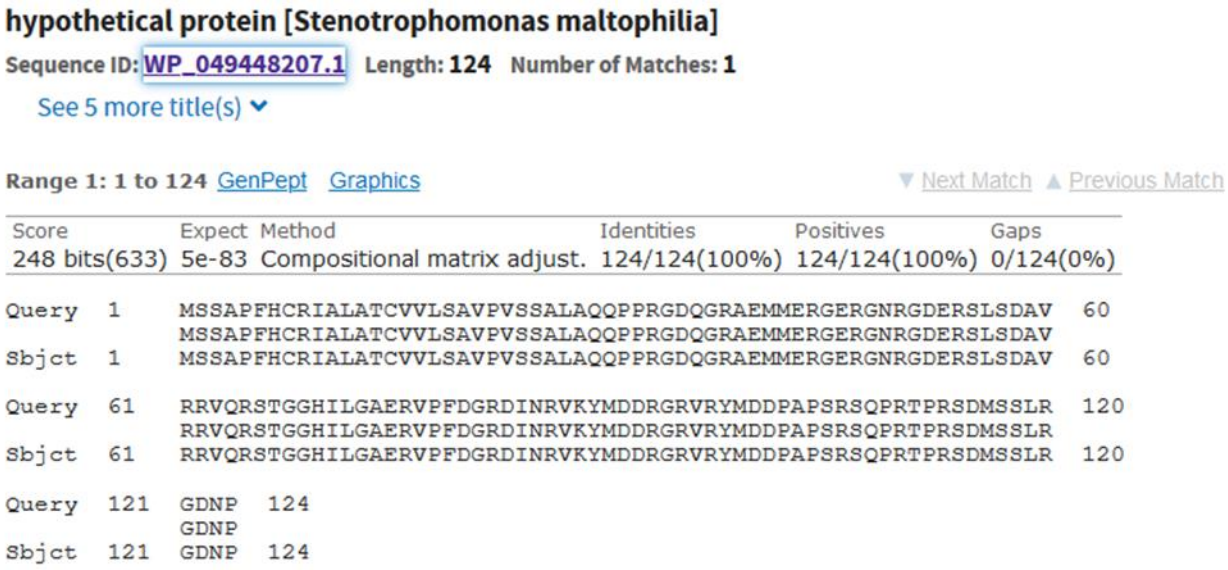


Figure 9. BLAST analysis of the hypothetical protein from S. maltophilia 02*. The hypothetical protein showed 100% similarity to other hypothetical proteins in S.maltophlia 02.*

The amino acid residue sequence of *S. maltophilia* 02 histidine kinase was evaluated using BLAST to determine if it was linked to other known proteins (Fig 10). The results showed that it is identical to *S. maltophilia* 02 PhoQ proteins.

Sensor protein PhoQ [Stenotrophomonas maltophilia]

Sequence ID: VUN93040.1 **Length:** 474 **Number of Matches:** 1

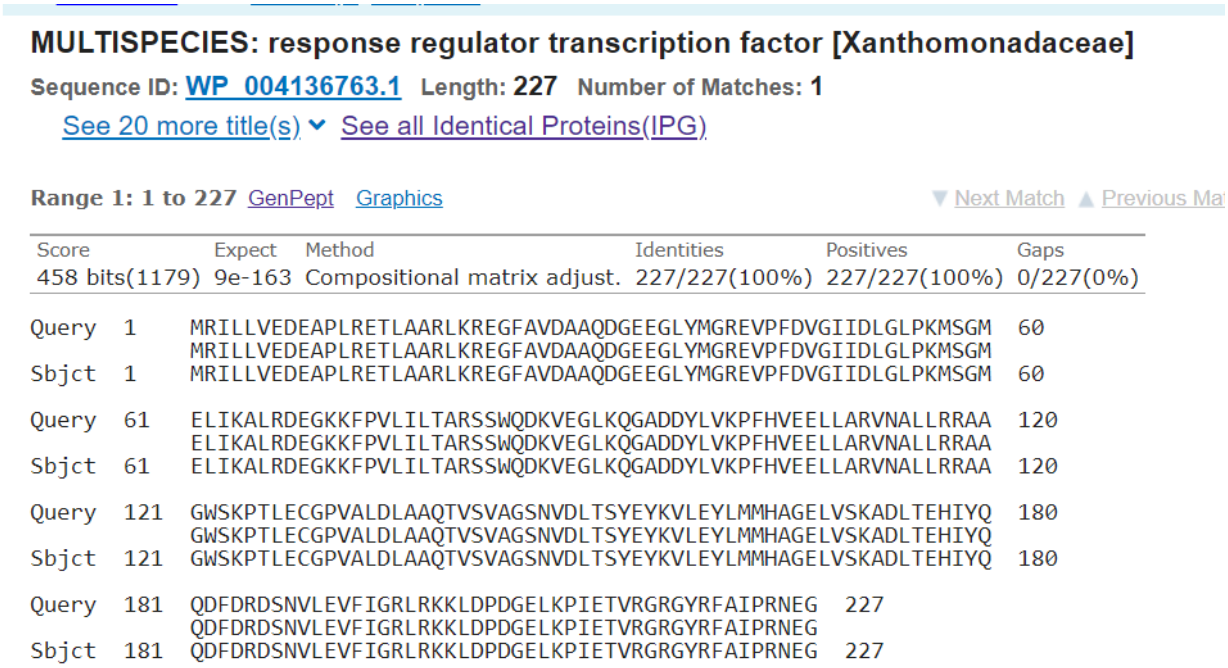
Range 1: 1 to 474 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
955 bits(2469)	0.0	Compositional matrix adjust.	474/474(100%)	474/474(100%)	0/474(0%)

```
Query  1    MSGRLWFFRRWRPRSLQARQMFAASVGLVAFLALAGYALDAAFADTAKANLRERLKNYAT  60
            MSGRLWFFRRWRPRSLQARQMFAASVGLVAFLALAGYALDAAFADTAKANLRERLKNYAT
Sbjct  1    MSGRLWFFRRWRPRSLQARQMFAASVGLVAFLALAGYALDAAFADTAKANLRERLKNYAT  60

Query  61   AYAAGIDFTRDRSLYIREQPPDSRFDVPGSGLYLQVVMPHGKGNSMSAEGPMLPTVGGGL  120
            AYAAGIDFTRDRSLYIREQPPDSRFDVPGSGLYLQVVMPHGKGNSMSAEGPMLPTVGGGL
Sbjct  61   AYAAGIDFTRDRSLYIREQPPDSRFDVPGSGLYLQVVMPHGKGNSMSAEGPMLPTVGGGL  120

Query  121  LAPRQEVFEGPLPMIQIDGSQGSVYRYGLGLVWDADADPATEFPYTIYVMEDSRALGAQL  180
            LAPRQEVFEGPLPMIQIDGSQGSVYRYGLGLVWDADADPATEFPYTIYVMEDSRALGAQL
Sbjct  121  LAPRQEVFEGPLPMIQIDGSQGSVYRYGLGLVWDADADPATEFPYTIYVMEDSRALGAQL  180

Query  181  RVFRSRVWFYLGGIGLILLLLQTVILQWSLRPLRRVITELTKVQRGETERMSERHPRELE  240
            RVFRSRVWFYLGGIGLILLLLQTVILQWSLRPLRRVITELTKVQRGETERMSERHPRELE
Sbjct  181  RVFRSRVWFYLGGIGLILLLLQTVILQWSLRPLRRVITELTKVQRGETERMSERHPRELE  240

Query  241  PLTDSINAFIESERENLERQRNTLADLAHSLKTPIAVLRTQMDSGAGDGALREELDVQLQ  300
            PLTDSINAFIESERENLERQRNTLADLAHSLKTPIAVLRTQMDSGAGDGALREELDVQLQ
Sbjct  241  PLTDSINAFIESERENLERQRNTLADLAHSLKTPIAVLRTQMDSGAGDGALREELDVQLQ  300

Query  301  RMNNLVSYQLARAASSGHKLFSAPLPIESNAEEIVRGLEKVYASKCVLCEFDIDPAARFH  360
            RMNNLVSYQLARAASSGHKLFSAPLPIESNAEEIVRGLEKVYASKCVLCEFDIDPAARFH
Sbjct  301  RMNNLVSYQLARAASSGHKLFSAPLPIESNAEEIVRGLEKVYASKCVLCEFDIDPAARFH  360

Query  361  GEPGDLQELLGNLLENAFKWANRRVLLTAQPLPAPNARRAGLLLAVDDDGPGIAPDDIGK  420
            GEPGDLQELLGNLLENAFKWANRRVLLTAQPLPAPNARRAGLLLAVDDDGPGIAPDDIGK
Sbjct  361  GEPGDLQELLGNLLENAFKWANRRVLLTAQPLPAPNARRAGLLLAVDDDGPGIAPDDIGK  420

Query  421  VLQRGVRGDERVQGHGIGLSIVQDLIKDYRGELTVGRSSELGGARFEVRLPPGP  474
            VLQRGVRGDERVQGHGIGLSIVQDLIKDYRGELTVGRSSELGGARFEVRLPPGP
Sbjct  421  VLQRGVRGDERVQGHGIGLSIVQDLIKDYRGELTVGRSSELGGARFEVRLPPGP  474
```

Figure:10 BLAST analysis of the S. maltophilia 02 *histidine kinase sequence was submitted, which showed 99% sequence similarities to S. maltophilia PhoQ sensor, which is involved in antibiotic resistances.*

The amino acid residue sequence of *S. maltophilia* 02 DNA binding protein was evaluated using BLAST to determine if it was linked to other known proteins (Fig 11). The results showed that it is identical to a *Xanthomonas* response regulator transcription factor.



MULTISPECIES: response regulator transcription factor [Xanthomonadaceae]

Sequence ID: WP_004136763.1 **Length:** 227 **Number of Matches:** 1

See 20 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 227 GenPept Graphics ▼ Next Match ▲ Previous Ma

Score	Expect	Method	Identities	Positives	Gaps
458 bits(1179)	9e-163	Compositional matrix adjust.	227/227(100%)	227/227(100%)	0/227(0%)

```
Query  1    MRILLVEDEAPLRETLAARLKREGFAVDAAQDGEEGLYMGREVPFDVGIIDLGLPKMSGM  60
            MRILLVEDEAPLRETLAARLKREGFAVDAAQDGEEGLYMGREVPFDVGIIDLGLPKMSGM
Sbjct  1    MRILLVEDEAPLRETLAARLKREGFAVDAAQDGEEGLYMGREVPFDVGIIDLGLPKMSGM  60

Query  61   ELIKALRDEGKKFPVLILTARSSWQDKVEGLKQGADDYLVKPFHVEELLARVNALLRRAA  120
            ELIKALRDEGKKFPVLILTARSSWQDKVEGLKQGADDYLVKPFHVEELLARVNALLRRAA
Sbjct  61   ELIKALRDEGKKFPVLILTARSSWQDKVEGLKQGADDYLVKPFHVEELLARVNALLRRAA  120

Query  121  GWSKPTLECGPVALDLAAQTVSVAGSNVDLTSYEYKVLEYLMMHAGELVSKADLTEHIYQ  180
            GWSKPTLECGPVALDLAAQTVSVAGSNVDLTSYEYKVLEYLMMHAGELVSKADLTEHIYQ
Sbjct  121  GWSKPTLECGPVALDLAAQTVSVAGSNVDLTSYEYKVLEYLMMHAGELVSKADLTEHIYQ  180

Query  181  QDFDRDSNVLEVFIGRLRKKLDPDGELKPIETVRGRGYRFAIPRNEG  227
            QDFDRDSNVLEVFIGRLRKKLDPDGELKPIETVRGRGYRFAIPRNEG
Sbjct  181  QDFDRDSNVLEVFIGRLRKKLDPDGELKPIETVRGRGYRFAIPRNEG  227
```

Figure 11- BLAST analysis of the S. maltophilia 02 *DNA binding transcriptional regulator cpxR showed that it is identical to a S. maltophilia OmpR, a regulatory protein with 100% sequence similarity.*

4.6 Multiple sequence alignment:

The protein sequences of the histidine kinase and homologs were compared using multiple sequence alignment (MSA). From a blastp search of a translated histidine kinase DNA sequence, ten homologs were chosen. As indicated in figure 12, clustalX was used to align the sequences and GeneDoc was used to tabulate the results. Conserved transmembrane domains were identified using the TMHMM 2.0 domain database. TMHMM is a program that predicts transmembrane helices in proteins [17]. Long and short output formats are available. The server provides basic statistics, and a list of the predicted transmembrane helices as well as the predicted location of the intervening loop regions for the long format (default). The plot depicts the inside/outside/TM helix posterior probabilities [26]. TMHMM's analysis indicated the presence of two transmembrane domains in the histidine kinase, PhoQ (Figure 13).

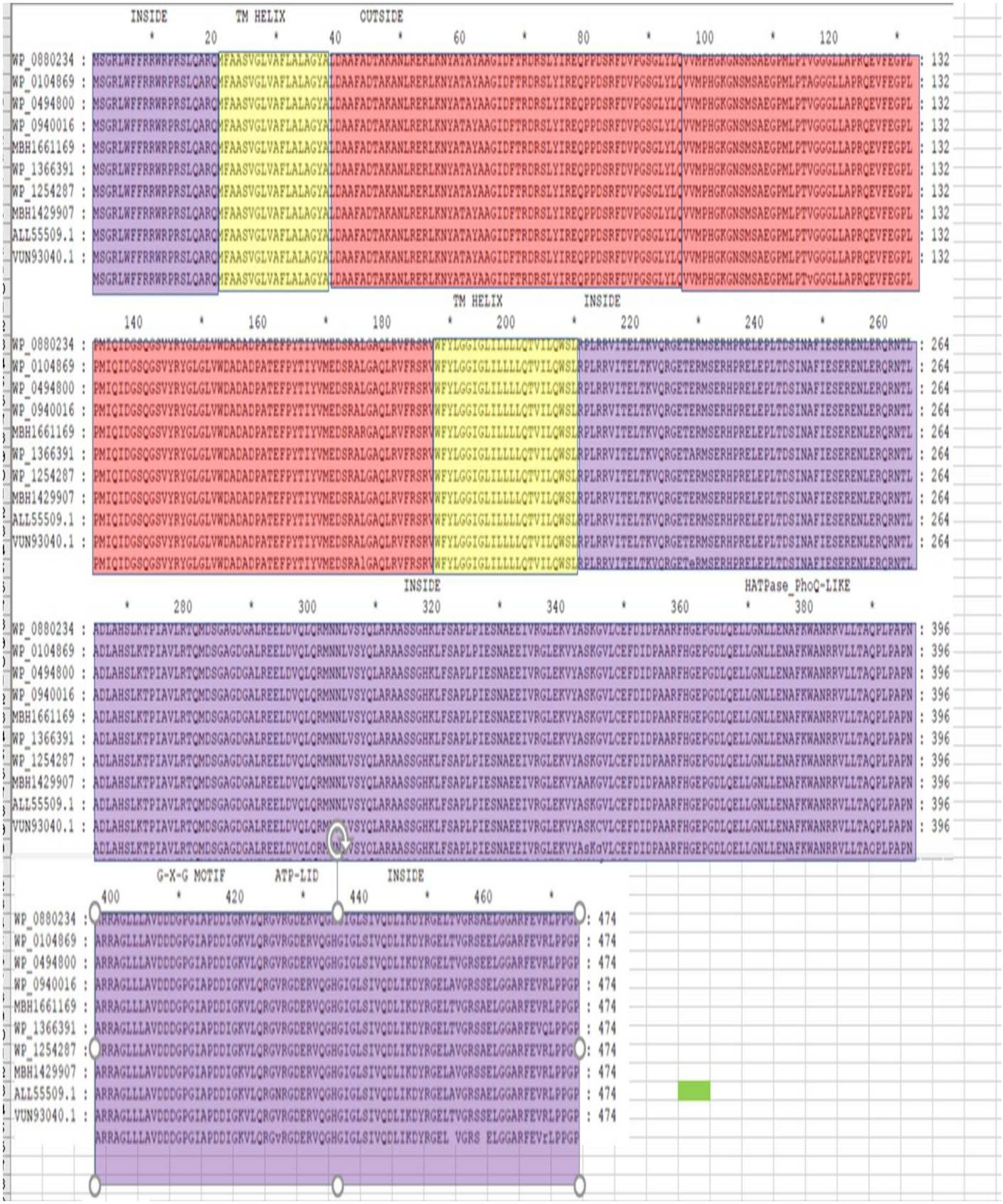

INSIDE
TM HELIX
OUTSIDE
TM HELIX
INSIDE
INSIDE
HATPase_PhoQ-LIKE
G-X-G MOTIF
ATP-LID
INSIDE
WP_0880234
WP_0104869
WP_0494800
WP_0940016
MBH1661169
WP_1366391
WP_1254287
MBH1429907
ALL55509.1
VUN93040.1

Figure 12: Multiple sequence alignment: The protein sequence alignment of histidine kinase and its homologs shown by GeneDoc output. – WP_088023432 MULTISPECIES: sensor histidine kinase [*Stenotrophomonas*], WP_125428765 sensor histidine kinase [*Stenotrophomonas maltophilia*], WP_049480014 sensor histidine kinase [*Stenotrophomonas maltophilia*], MBH1661169 sensor histidine kinase [*Stenotrophomonas maltophilia*], WP_094001618 MULTISPECIES: sensor histidine kinase [*Stenotrophomonas],* VUN93040 Sensor protein PhoQ [*Stenotrophomonas maltophilia*], WP_010486997 sensor histidine kinase [*Stenotrophomonas maltophilia*] MBH1429907 sensor histidine kinase [*Stenotrophomonas maltophilia]*, WP_136639111 sensor histidine kinase [*Stenotrophomonas maltophilia],* ALL55509 PhoQ [*Stenotrophomonas maltophilia*]. The transmembrane protein, inside, outside helix, HATPase_ PhoQ like, G-X-G Motif are located.

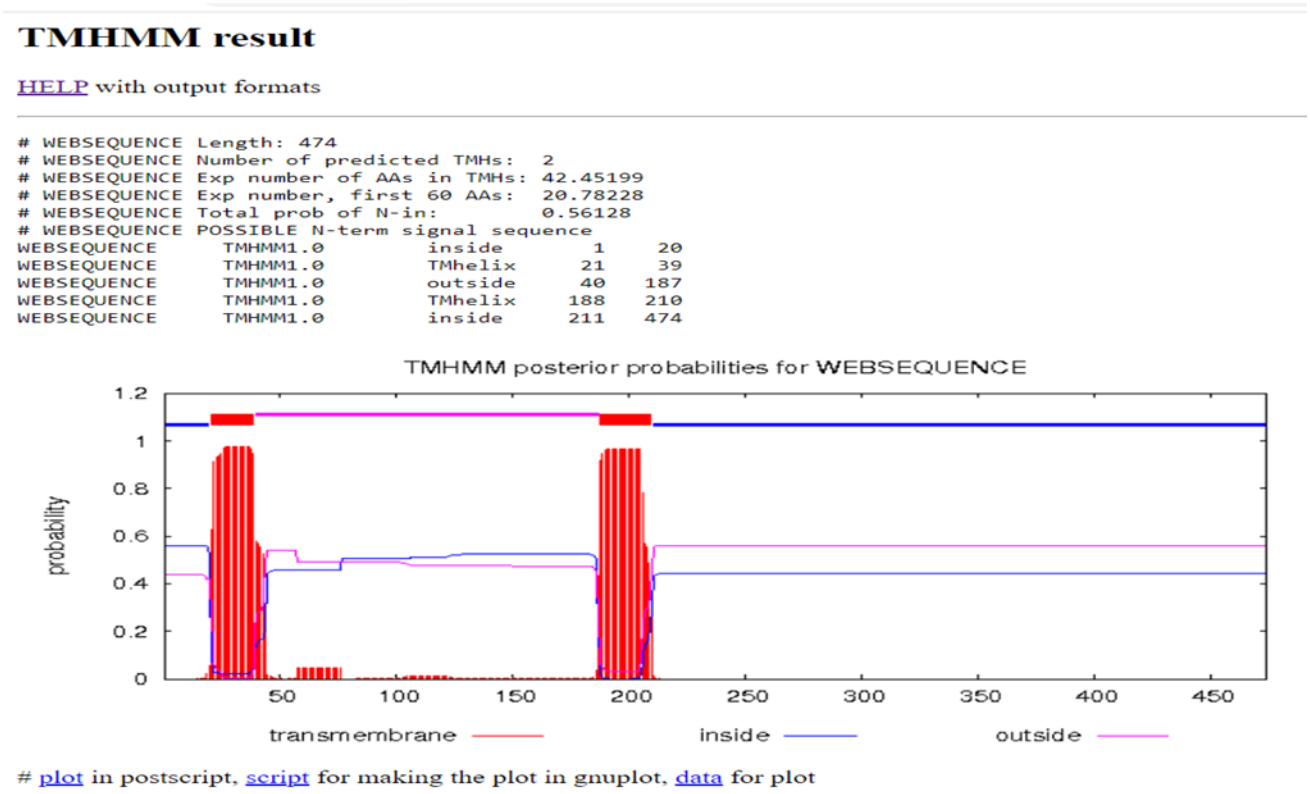


Figure 13- TMHMM result showing the number of predicted transmembrane protein

4.7 Identification of conserved domains in Histidine kinase

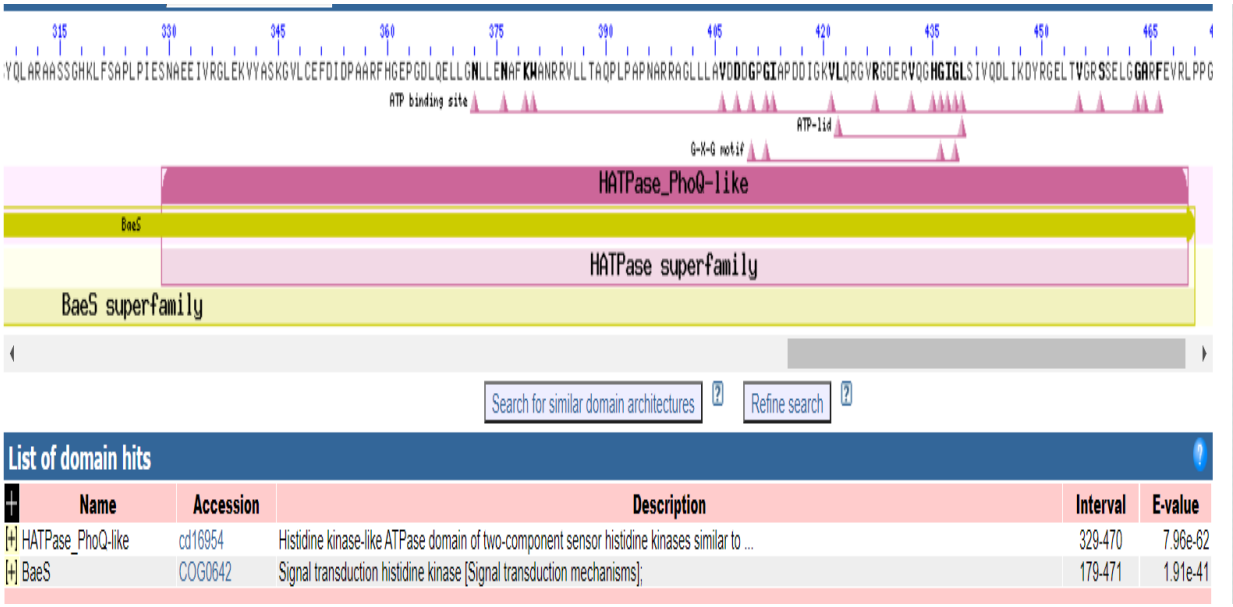


Figure 14-Identification of conserved domains in Histidine Kinase showing HATPase domain, ATP binding sites, G-X-G motif regions.

Escherichia coli PhoQ and *Providencia stuartii* AarG is as Histidine kinase-like ATPase domain of two component sensor histidine kinases.[27].

In the PhoP-PhoQ two-component regulatory system (TCS), PhoQ is the histidine kinase (HK) that controls virulence, controls pathogenicity, mediates adaptation to salt-depleted conditions, and responds to magnesium and calcium levels. The expression of the 2'-N-acetyltransferase and an intrinsic multiple antibiotic resistance (Mar) response in *Providencia stuartii* is controlled by a putative sensor kinase *Providencia stuartii AarG* [6,27]. PhoQ and AarG restore wild-type levels of resistance in *Salmonella typhimurium* [26,27]. Most of the members of this family contain an accessory HAMP sensor domain and an intracellular membrane interaction sensor domain (Figure 14). Some members of this family also have a histidine kinase dimerization and phosphoacceptor domain (HisKA) [6,27].

4.8 SignalP:

In amino acid sequences from Gram-positive prokaryotes, Gram-negative prokaryotes, and eukaryotes, the SignalP server predicts the existence and location of signal peptide cleavage sites [12]. The method combines cleavage site prediction and signal peptide/non-signal peptide prediction using a combination of artificial neural networks. Signal peptide-containing proteins are directed to the secretory route, but they are not always secreted [17]. In the *S.maltophilia* 02 sequence, the cleavage site between 44 and 45 is shown, as well as different signal peptides. (Figure 15).

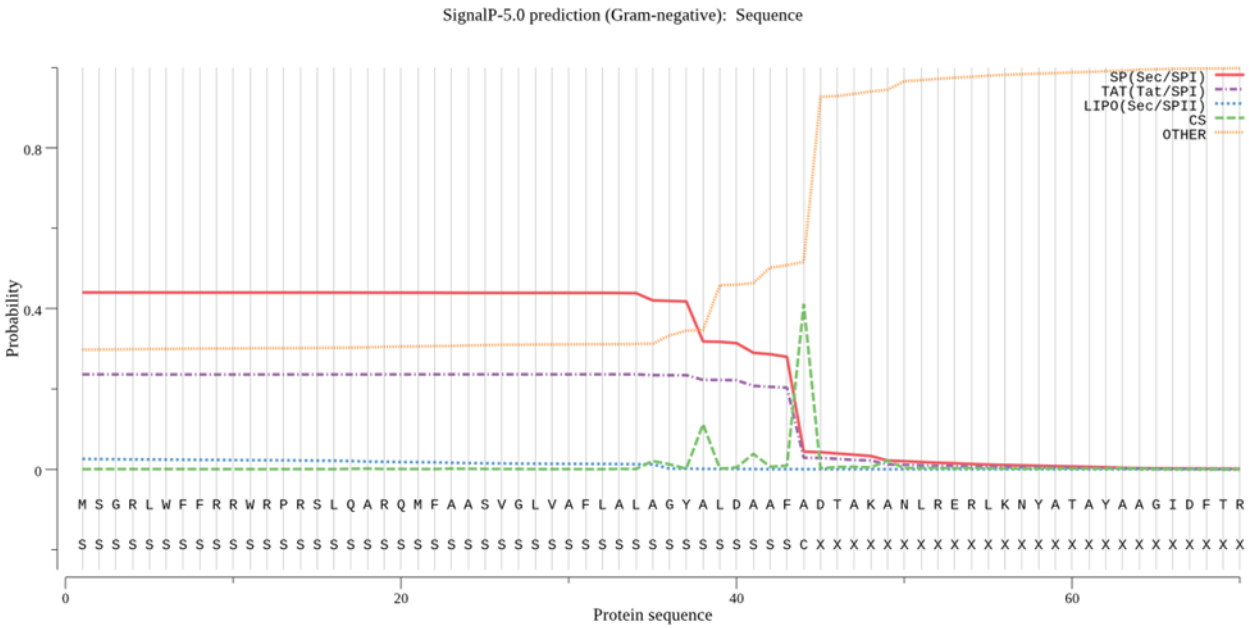


Figure 15- SignalP result showing predicted proteins. Cleavage site between pos. 44 and 45: AFA-DT. Probability: 0.4113

CHAPTER V-DISCUSSION

The Tn*5* transposome was incorporated into *S. maltophilia* 02 genome to generate a gold sensitive mutant termed as A12.Sanger sequencing showed that the transposon inserted itself after the stop codon of hypothetical protein and before the start codon for a DNA binding protein and a two-component sensor histidine kinase. I postulated that the transposon insertion interfered with the gene expression in *S.maltophilia* 02. To determine whether transposon insertion interfered with the expression of the hypothetical protein*, cpxR* or *cpxA* (PhoQ), reverse transcriptase-PCR (RT-PCR) was performed. Our refutes the hypothesis that the transposon not interfered with the gene expression of the hypothetical protein, *cpxR* or *cpxA* (PhoQ), as the PCR product in the wild type and A12 mutant were observed. There were no consistent differences between expression profiles for each gene. The *cpxR* and *cpxA* are controlled by their own promoter, distinct from hypothetical protein [6].

However, the growth curve experiment showed that the A12 mutant grows as well without gold as the wild type grows with gold [12]. Thus, the mutation impairs the growth of A12 even in the absence of gold [16,17]. Why it is impaired is not clear.

Future work:

The other method that can be used to identify, if the transposon interfered with protein expression in the mutated region is by doing protein analysis [6]. In this experiment, the wild type and the A12 mutant can be subjected to the same growth curves performed in the presence or absence of gold salts [6,16]. Protein from each sample is separated by one dimensional (1D) polyacrylamide gel electrophoresis (SDS-PAGE) instead of purifying RNA from each sample after exposure to gold [12]. SDS-PAGE can detect the hypothetical protein and other gold resistance proteins. Even though *CpxR* and *CpxA* (PhoQ) proteins are not expressed at high levels to detect protein bands [13]. The separated gold-treated wild

type sample showing the presence of a protein band and absence of the corresponding band in the separated gold-treated A12 mutant sample would indicate that the insertion of transposon interfered with the protein expression [16,17,13]. In tandem mass spectrometry, the protein bands will then be excised from the gel and identified. A number of these trials have been conducted, and failed [6,12]. There were no significant differences between expression profiles for each sample. Two-dimensional gel electrophoresis and RNA sequencing can be performed to identify the transposon interferences [6,12].

CHAPTER VI-APPENDICES

Stenotrophomonas maltophilia strain Oak Ridge strain 02 chromosome (Nucleotide sequence)

GGGGTTATCGCCGCGTAGTGATGACATATCCGACCGCGGCGTGCGCGGCTGTGAACGCGAC
GGGGCGGGGTCGTCCATGTAGCGGACCCGGCCCCGGTCGTCCATGTACTTCACCCGGTTGA
TGTCACGACCGTCGAACGGAACGCGCTCGGCGCCGAGGATGTGGCCACCGGTGCTGCGCTG
CACGCGGCGCACGGCATCGGACAGCGAACGTTCGTCGCCGCGGTTGCCGCGCTCGCCCCGC
TCCATCATCTCTGCACGCCCCTGATCGCCACGCGGCGGCTGTTGCGCCAGCGCCGATGACA
CCGGCACAGCCGACAGCACCACGCAGGTGGCCAGGGCAATGCGGCAATGGAAGGGAGCG
GAAGACAT

Hypothetical protein [*Stenotrophomonas maltophilia*] (protein sequence)

MSSAPFHCRIALATCVVLSAVPVSSALAQQPPRGDQGRAEMMERGERGNRGDERSLSDAVRR
VQRSTGGHILGAERVPFDGRDINRVKYMDDRGRVRYMDDPAPSRSQPRTPRSDMSSLRGDNP

Sensor histidine kinase [*Stenotrophomonas maltophilia*] (protein sequence)

MSGRLWFFRRWRPRSLQARQMFAASVGLVAFLALAGYALDAAFADTAKANLRERLKNYATA
YAAGIDFTRDRSLYIREQPPDSRFDVPGSGLYLQVVMPHGKGNSMSAEGPMLPTVGGGLLAPR
QEVFEGPLPMIQIDGSQGSVYRYGLGLVWDADADPATEFPYTIYVMEDSRALGAQLRVFRSRV
WFYLGGIGLILLLLQTVILQWSLRPLRRVITELTKVQRGETERMSERHPRELEPLTDSINAFIESER

ENLERQRNTLADLAHSLKTPIAVLRTQMDSGAGDGALREELDVQLQRMNNLVSYQLARAASS
GHKL

FSAPLPIESNAEEIVRGLEKVYASKGVLCEFDIDPAARFHGEPGDLQELLGNLLENAFKWANRR
VLLTAQPLPAPNARRAGLLLAVDDDGPGIAPDDIGKVLQRGVRGDERVQGHGIGLSIVQDLIKD
YRGELTVGRSAELGGARFEVRLPPGP

>WP_004136763.1 MULTISPECIES: response regulator transcription factor

[Xanthomonadaceae]

MRILLVEDEAPLRETLAARLKREGFAVDAAQDGEEGLYMGREVPFDVGIIDLGLPKMSGMELI
KALRDEGKKFPVLILTA

RSSWQDKVEGLKQGADDYLVKPFHVEELLARVNALLRRAAGWSKPTLECGPVALDLAAQTVS
VAGSNVDLTSYEYKVLEY

LMMHAGELVSKADLTEHIYQQDFDRDSNVLEVFIGRLRKKLDPDGELKPIETVRGRGYRFAIPR
NEG

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