Identification of a putative *metK* selenite resistance gene in *Stenotrophomonas maltophilia* OR02

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Abstract

Stenotrophomonas maltophilia OR02 (S02) is a multi-metal resistant strain that was isolated from a metal-contaminated site in Oak Ridge, TN. It grows in the presence of 20 mM sodium selenite and produces a red precipitate which is probably elemental selenium, and a stale garlic odor, which is probably methyl-selenide. The reduction of selenite to selenide may be dependent on the role of a glutathione reductase. Then, methyl-selenide may be produced by a thiopurine methyltransferase.

A selenite-sensitive mutant was generated by introducing the EZ-Tn5 transposome into S02. This transposon, which carried a kanamycin resistance gene, randomly incorporated itself into the S02 genome and generated thousands of kanamycin resistant transformants. Replica plating of 880 transformants yielded one selenite-sensitive S02 mutant, AX55. Liquid culture experiments showed that the minimal inhibitory concentration (MIC) for AX55 was 10 mM selenite, whereas the MIC for the S02 was 30 mM. DNA sequencing and Basic Local Alignment Search Tool (BLAST) analysis showed that the transposon interrupted the 5' untranslated region (UTR) of a putative *metK* gene. The *metK* gene encodes the protein S-adenosylmethionine (SAM) synthetase, which catalyzes the formation of SAM. These results confirm the requirement of SAM for selenite methylation, which may be catalyzed by a thiopurine methyltransferase. Furthermore, the decreased production of selenium nanoparticles by AX55 suggests the reduction mechanism is also dependent on SAM.

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Chapter I: Introduction

1.1 Y-12 plant

During World War II an electromagnetic isotope separating plant in Oak Ridge, TN, code-named Y-12, had a significant role in the processing of uranium that was used in the fabrication of nuclear weapons [1]. Due to the ever-growing threat of a nuclear war with the USSR, the plant was converted to a lithium isotope processing center for the construction of hydrogen bombs in 1951. Wastes from these processes were disposed of in four, poorly constructed S-3 ponds near the plant and East Fork Poplar Creek. The waste deposit process eventually led to the contamination of the creek and the surrounding areas. Around 920,000 kg of mercury which was used in lithium processing, as well as uranium nitrate, acid washes for metal processing, and an amalgam of other metals spilled into the creek, creating an acidic environment [1, 2]. In 1983, the use of the ponds as a means of waste disposal was discontinued. The wastes remaining in the pond were treated and removed; then, the pond was filled and capped.

1.2 *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia (*S. maltophilia*) is a gram negative, non-fermentative, aerobic bacterium [3–5]. Although *S. maltophilia* does not naturally exhibit a high level of virulence, its resistance to several antibiotics gives it the capability of causing nosocomial infections in immunocompromised individuals [4, 5]. Genetic variants have been associated with bacteremia and respiratory tract infections, such as pneumonia and cystic fibrosis. A multi-metal resistant strain, *Stenotrophomonas maltophilia* Oak Ridge strain 02 (*S. maltophilia* 02), was isolated from East Fork Poplar Creek and was able to grow in the presence of toxic levels of heavy metals and metalloids (Cu, Pt, Hg, Cd, Pb,

Cr, Au, and Se) [1]. The resistance mechanism conveyed was a detoxification process that transformed the toxic metal salts into an insoluble product. For example, some bacteria reduced selenite to elemental selenium.

1.3 Microbial Interactions with Selenium

Comparable to any other living organism, the presence of specific elements in the cellular environment of bacteria is essential for its life [6]. Of the twelve most naturally occurring elements, six organic elements (C, O, H, N, P, S) function as major constituents of proteins, nucleic acids, lipids, carbohydrates, and coenzymes. The remaining six inorganic elements (K, Mg, Ca, Fe, Na, Cl) are present as cations or anions. These ions serve as cofactors in enzymatic reactions, and are essential for microbial growth and metabolism [7]. In addition to the previously mentioned elements, some transitional elements such as Mn, Co, Ni, Cu, Zn, and Se have pivotal roles in the function of certain enzymes in the cellular environment. Metals can also exhibit cytotoxic effects on a microbial cell when present in high concentrations. This is most notably seen among transitional metals and metalloids.

Selenium (Se) is a naturally occurring metalloid that is essential for life among bacterial organisms at low concentrations [8–13]. In aquatic environments, such as East Fork Poplar Creek, Se will be present in the water-soluble oxyanion forms of selenate (SeO_4^{2-}) and selenite (SeO_3^{2}-) [8]. These forms are much more reactive than the water-insoluble elemental selenium (Se^0), or volatile methylated selenides. The mechanism of uptake of the oxyanion forms into the cell are not well understood. However, once they are brought into the cell they can be incorporated into the biosynthesis of the amino acids selenocysteine and selenomethionine via two pathways, a specific and a non-specific [14,

15]. Immediately following the uptake of selenite or selenate into the cell, the oxyanion undergoes assimilatory reduction to generate the less reactive selenide (Se^{2-}) form [16]. In a reference regarding the incorporation of the non-specific pathway products in *Escherichia coli*, selenium will replace the sulfur in the sulfur-containing groups of methionine and cysteine [8, 14]. This will generate the selenium-containing amino acids selenomethionine and selenocysteine, which will be substituted in place of methionine and cysteine in proteins respectively.

The specific pathway is a more complex process and results in the generation of selenocysteine, which is then incorporated into a polypeptide chain by an amino acid codon. The pathway requires the presence of: SelA (selenocysteine synthetase), SelB (translation/elongation factor), SelC (selenocysteyl-tRNA), and SelD (selenophosphate synthetase). It begins with the conversion of selenide and adenosine triphosphate (ATP) to generate selenophosphate [14, 15, 17]. Selenophosphate is then used as a reactant in the conversion of serine to selenocysteine that is catalyzed by SelA. The selenocysteyl-tRNA (SelC) is bound to SelB and then binds to a unique stem loop structure in the mRNA that encodes for selenocysteine at the UGA stop codon [15]. Selenocysteine is an amino acid component of formate dehydrogenase and glycine reductase, enzymes that are regularly found in bacteria [7].

1.4 Heavy Metal Resistance

S. maltophilia 02 can grow in the presence of toxic levels of metals such as Cu, Hg, Cd, Pb, and Se due to detoxification mechanisms [1]. There are five possible mechanisms that bacteria can use to process selenium derivatives: assimilatory reduction, dissimilatory reduction, methylation, oxidation, and demethylation [16, 18]. The detoxification process

carried out by of *S. maltophilia* 02 combines dissimilatory reduction and methylation [11, 16].

1.4.1 Selenite Detoxification

Previous studies have hypothesized the selenite detoxification mechanism based on the presence of specific intermediates and products. The Challenger pathway (*Figure* 1), which reduces and methylates selenite in a series of reactions to produce the volatile gas dimethylselenide (DMSe) was the first to be proposed [16]. However, this pathway was modified to incorporate the production of dimethyldiselenide (DMDSe). The modified pathway diverges from the original upon the production of the intermediate, a methaneseleninic ion (*Figure* 2). The methaneseleninic ion may either be reduced or methylated, which ultimately leads to the production of DMDSe or DMSe respectively. Another detoxification pathway was generated to accommodate the formation of elemental selenium and selenide as intermediates. This is known as the Doran pathway, and it generates DMSe as the final product (*Figure* 3).

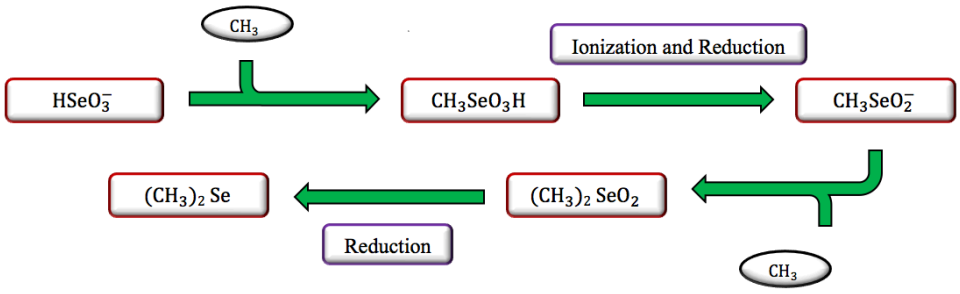

***Figure 1*:** Challenger Pathway. $HSeO_3^-$ (selenite); CH_3SeO_3H (methane selenonic acid); $CH_3SeO_2^-$ (methaneseleninic ion); $(CH_3)_2SeO_2$ (dimethyl selenone); $(CH_3)_2Se$ (dimethyl selenide).

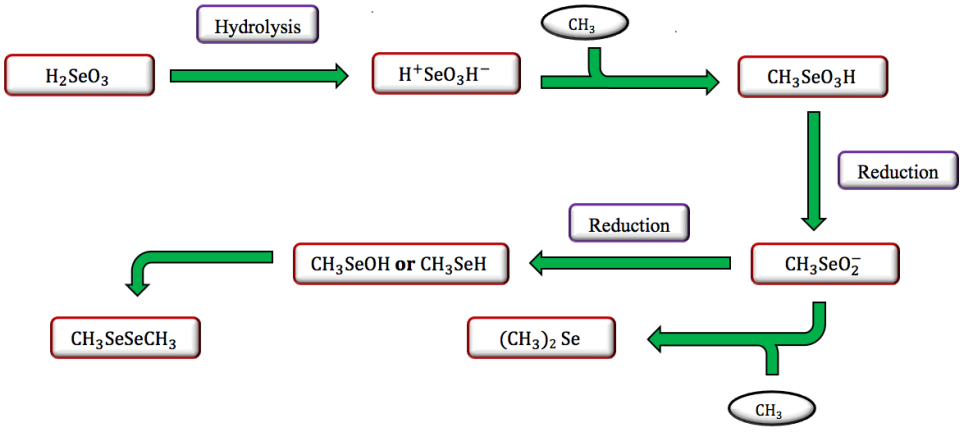

***Figure 2*:** Modified Challenger Pathway. H_2SeO_3 (selenite); $H^+SeO_3H^-$ (selenonic acid); CH_3SeO_3H (methane selenonic acid); $CH_3SeO_2^-$ (methaneseleninic ion); $(CH_3)_2SeO_2$ (dimethyl selenone); $(CH_3)_2Se$ (dimethyl selenide); CH_3SeH (methaneselenol); CH_3SeOH (methaneselenenic acid); $CH_3SeSeCH_3$ (dimethyl selenide).

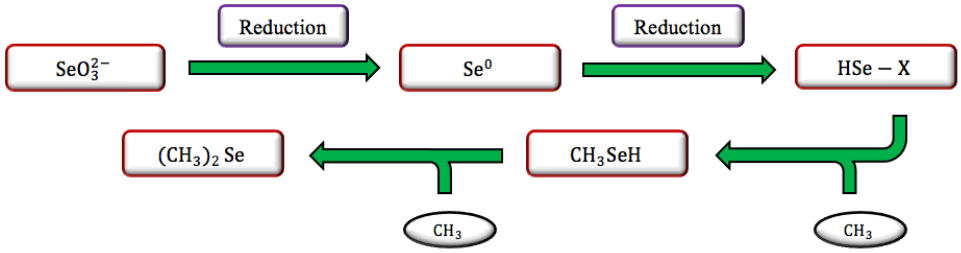

***Figure 3*:** Doran pathway. SeO_3^{-2} (selenite); Se^0 (elemental selenium); HSe - X (selenide); CH_3SeH (methane selenol); $(CH_3)_2Se$ (dimethyl selenide).

The detoxification of sodium selenite (Na_2SeO_3) was observed under aerobic conditions in a strain of *S. maltophilia* that was isolated from an agriculture drainage pond contaminated with selenium oxyanions [11]. *S. maltophilia* was supplemented with 0.5 mM of sodium selenite and grown at room temperature for a 48-hour period, which resulted in the reduction of 99% of the sodium selenite to elemental selenium. The presence of red elemental selenium began to accumulate around the 25^{th} hour when the bacteria entered a stationary phase of growth, just ten hours after the oxygen concentrations were depleted to microaerophilic conditions. The reduction of selenite results in the accumulation of elemental selenium, also known as selenium nanoparticles (SeNPs), in the cytoplasm and in the surrounding growth medium [19]. The reduced selenium species were also subjected to methylation, yielding the following volatile selenide gases: DMSe, DMDSe, and dimethylselenylsulphide (DMSeS) [11]. The ability of *S. maltophilia* to reduce the toxic selenite ion and convert it to volatile methylated species results in the complete removal of selenium from the surrounding environment. This ability may be beneficial in the bioremediation of selenium contaminated environments.

1.4.2 Reduction Mechanisms

The reduction of selenite in bacteria is thought to be dependent on the activity of a nitrate reductase, a nitrite reductase, or a glutathione reductase [5]. Nitrate and nitrite reductases are categorized by their location in the cell: membrane-bound reductases and periplasmic reductases [8, 20]. The membrane-bound nitrate reductases of *Escherichia coli* (nitrate reductase A and nitrate reductase Z) can reduce selenate; however, the reduction of selenite requires a different mechanism [21]. The periplasmic nitrate reductase (Nap) of *Rhodobacter sphaeroides*, unlike the nitrate reductases in *E. coli*, can reduce both selenite

and selenate.

The activity of nitrite and glutathione reductases were examined under aerobic conditions in a strain of *Stenotrophomonas maltophilia* (SeITe02), which was isolated from selenium-contaminated mining soil [9]. Originally, selenite reduction was thought to occur via a periplasmic nitrite reductase mechanism. However, the addition of nitrite to a broth culture (previously supplemented with selenite) during the exponential growth phase significantly decreased the total amount of selenite that was reduced by SeITe02. Nitrite and selenite are believed to compete for the same intracellular transport mechanism that will preferentially bind to nitrite. When a nitrite uptake inhibitor, 2,4-dinitrophenol (DNP) was added, selenite detoxification was not affected. It should be noted that selenite reduction is localized in the cytoplasm of *S. maltophilia*. Therefore, the addition of DNP was not to measure the selenite-reducing activity of periplasmic proteins, but to characterize a mechanism by which selenite is brought into the cell. Glutathione (GSH), a reducing enzyme that is found in high concentrations throughout α, β, and γ-classes of proteobacteria, has previously been shown to be involved in the reduction of selenite to elemental selenium [8, 9, 12, 16, 18, 19, 22]. A painter-type reaction was originally proposed, which demonstrated a high reactivity between selenite and thiol groups. The painter-type reaction led to the formation of selenotrisulfide (RS-Se-SR), which was determined to be selenodiglutathione (GS-Se-SG) by Ganther [12, 18]. Selenodiglutathione is readily reduced by glutathione reductase (GR) to generate selenopersulfide (GS-Se^-) (and reduced-GSH), which dismutates into elemental selenium and reduced-GSH.

Reaction 1:

$$3\,SeO_3^{2-} + 6\,GSH + 4\,H^+ \rightarrow 3\,GS - Se - SG + 2\,O_2^- + 5\,H_2O$$

Reaction 2:

$$3\ GS-Se-SG+3\ NADPH+3\ H^{+}\rightarrow 3\ GS-Se^{-}+3\ GSH+3\ NADP^{+}$$

Reaction 3:

$$3\ GS-Se^{-}\rightarrow 3\ GSH+3\ Se^{0}$$

The first step in the Ganther mechanism was modified by Kessi and Hanselmann (*Figure* 4) to account for the generation of superoxide anions (O_2^-). Oxidative stress proteins, such as superoxide dismutase (SOD) and catalase will protect the cell from oxidative damage by removing superoxide anions through the generation of hydrogen peroxide and oxygen (SOD), and the conversion of hydrogen peroxide to water and oxygen (catalase).

Reaction 1a:

$$2\ O_2^{-}+2\ H^{+}\rightarrow O_2+H_2O_2$$

Reaction 1b:

$$2\ H_2O_2\rightarrow H_2O+O_2$$

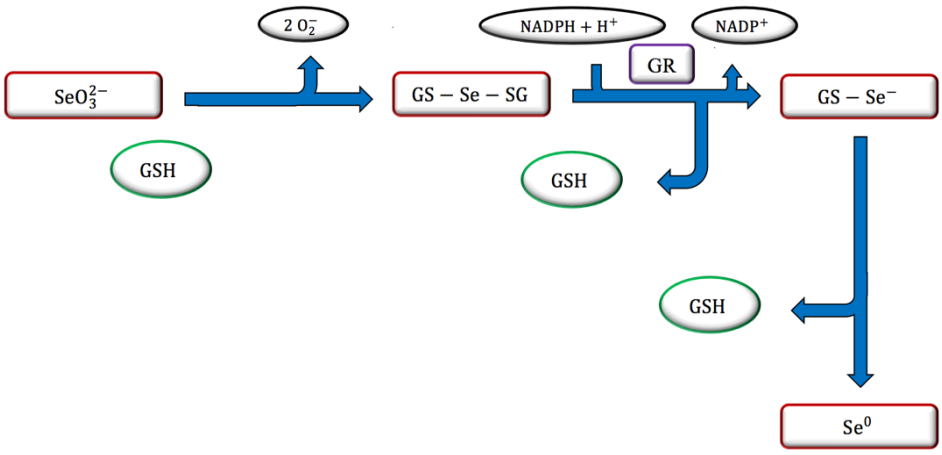

***Figure 4*:** Selenite Reduction by Glutathione. SeO_3^{-2} (selenite); GSH (glutathione); GS-Se-SG (selenodiglutathione); GR (glutathione reductase); GS-Se^- (selenopersulfide); Se^0 (elemental selenium).

Stenotrophomonas maltophilia SeITe02 utilizes the GSH/GR mechanism proposed by Kessi and Hanselmann [12] to reduce selenite into elemental selenium [9, 19]. This was identified by the requirement of GSH in SeITe02, the cellular location of elemental selenium, and the preferred coenzyme involved in the process. The role of GSH in the reduction of selenite was tested by the addition of a GSH synthesis inhibitor, buthionine sulphoximine (BSO) [9]. When added to a liquid culture supplemented with selenite at the beginning of lag phase, the reduction of selenite was nearly prevented. However, when BSO was added during the exponential growth phase and the stationary growth phase, there was no decrease in selenite reduction. These findings have corroborated the role of GSH in the beginning stages of the selenite reduction. In SeITe02, reduced elemental selenium accumulates in the cytoplasm of the cell. An *in vitro* enzymatic assay was used to test the selenite reduction capabilities of cytoplasmic, periplasmic, and membrane-associated protein fractions [9, 19]. The reduction was found to be localized to the cytoplasmic protein fractions. Further enzymatic analysis of the cytoplasmic protein fractions revealed NADPH to be the preferred electron donor in the reduction of selenite. This is supported by the mechanism proposed by Kessi and Hanselmann which requires NADPH to reduce selenodiglutathione in the GR catalyzed reaction that produces GSH, that can then reduce additional selenite [12]. Proteomic analysis also found that the presence of selenite in SeITe02 led to the expression of the oxidative stress-related proteins, catalase and glutamate-cysteine ligase (GCL) precursor [9]. GCL, known as γ-glutamylcysteine synthetase (GCS), catalyzes the first reaction of GSH biosynthesis. Further reduction of elemental selenium produces selenide, which can be incorporated into selenocysteine or converted to a methylated product [8].

1.4.3 Methylation Mechanisms

The reduction of selenite may be followed by a methylation mechanism which requires S-adenosyl-L-methionine (SAM) to produce volatile gaseous compounds [10, 13, 16, 18, 22–28]. *S. maltophilia* 02 most likely utilizes the mechanism proposed by Doran (*Figure* 5) which includes the production of elemental selenium, thereby supporting selenite reduction by the GSH/GR pathway [9, 12, 19]. In γ-proteobacteria, two types of methyltransferase enzymes have been found to catalyze the methylation of selenium derivatives: bacterial thiopurine methyltransferase (bTPMT) and tellurite methyltransferase (TehB).

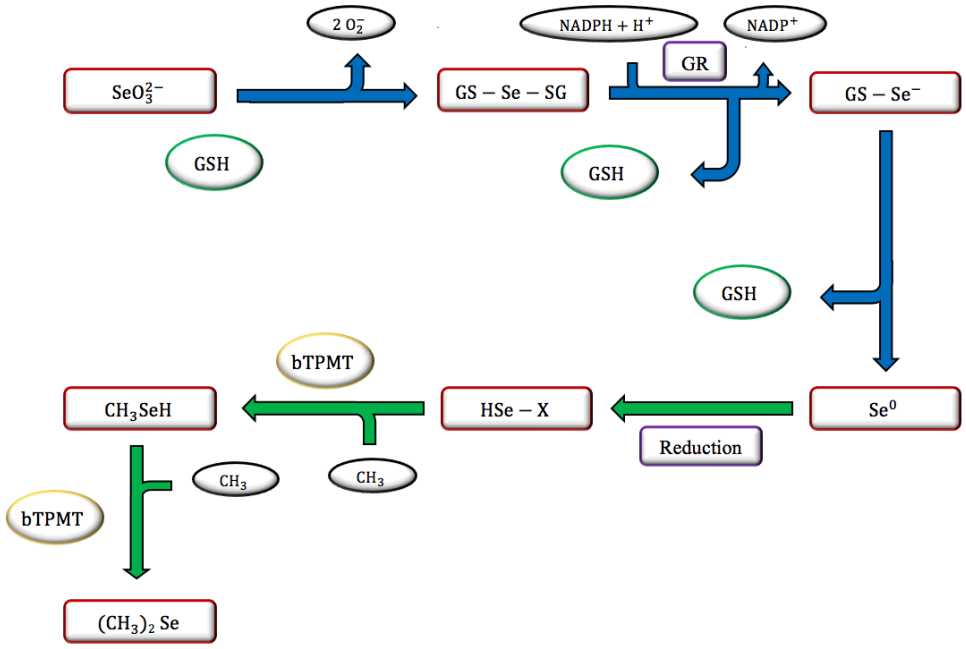

***Figure 5*:** Putative Selenite Detoxification Pathway in *S. maltophilia*. SeO_3^{-2} (selenite); GSH (glutathione); GS-Se-SG (selenodiglutathione); GR (glutathione reductase); GS-Se^- (selenopersulfide); Se^0 (elemental selenium). Se^0 (elemental selenium); HSe - X (selenide); CH_3SeH (methane selenol); bTPMT (bacterial thiopurine methyltransferase); $(CH_3)_2Se$ (dimethyl selenide).

Bacterial thiopurine methyltransferases were first identified in the tellurite-resistant bacteria, *Pseudomonas syringae* (*P. syringae*) [24]. The bTPMT of *P. syringae* was found to methylate inorganic and organic selenium derivatives into DMSe and DMDSe by cloning the gene (*tpm*) that encodes the bTPMT into *E. coli* cells [23, 24]. This was used as a DNA probe to characterize freshwater bacteria that express the capability of producing methylated products from selenite [10]. A bacterium expressing a high sequence identity to *Pseudomonas anguiliseptica* was isolated. This *Pseudomonas* species was found to generate DMSe after the addition of sodium selenite via a bTPMT (bTPMT-I), which supports the mechanism proposed by Doran (*Figure* 3) [16]. The bTPMT-I was later found to be conserved among most γ-proteobacteria by phylogenetic analysis from five different freshwater sources [13] and five different soil sources [29]. However, unlike the species of γ-proteobacteria that were identified (e.g. *Pseudomonas* species and *Xanthomonas* species), a gene encoding the bTPMT-I was not found in the genome of *E. coli*.

In *E. coli*, the methylation reaction involves the non-nucleic acid methyltransferase, TehB. TehB, which forms one half of the TehAB operon in *E. coli*, is a cytoplasmic protein that functions as a SAM-dependent methyltransferase in the detoxification of tellurite and selenite [25–28]. A similar mechanism is employed by the β-proteobacterium, *Thauera selenatis* (*T. selenatis*). Genes encoding selenium factors A (SefA) and B (SefB) were found on the same operon, which suggests a connection in the reduction and methylation mechanisms [22]. SefA was found to be involved in the assembly of selenium nanoparticles (SeNPs) following reduction in the cytoplasm. SefA is thought to stabilize the formation of SeNPs by binding to reduced elemental selenium prior to excretion into the surrounding growth medium. Conversely, instead of SeNP formation via SefA, the reduced selenium

derivative may be methylated to produce a volatile gas. SefB is a putative SAM-dependent methyltransferase that may have a role in the methylation mechanism due to its location downstream of SefA.

1.5 S-Adenosyl-L-Methionine

Found throughout all domains of life, S-adenosyl-L-methionine is a high energy compound that functions as a cofactor in trans-methylation reactions (e.g., methionine, cysteine, DNA, and phospholipid biosynthesis), trans-sulfuration reactions (e.g., cysteine and glutathione biosynthesis), and polyamine biosynthesis [6, 17, 30–32]. SAM is synthesized from L-methionine and ATP in a two-step reaction catalyzed by SAM synthetase. The first step results in the production of SAM upon the generation of a bond between methionine (sulfur, S) and ATP (adenosine carbon-5, C5'). Then, SAM is released from the active site upon the hydrolysis of tripolyphosphate (PPP_i). Primarily, SAM functions as an alkylating agent in cellular reactions due to a positively charged sulfur atom that is bound to a highly reactive methyl group.

1.5.1 SAM Cycle

The production of SAM in *S. maltophilia* is dependent upon the cytosolic concentration of methionine. Aside from transporting extracellular methionine into the cell, *S. maltophilia* can synthesize the amino acid through three main pathways: the methionine biosynthesis pathway, the methionine salvage pathway, and the SAM cycle [33–35]. In the biosynthesis pathway methionine is produced from L-serine and L-aspartate, while the salvage pathway regenerates methionine from the by-product of spermidine and spermine biosynthesis, 5-methylthioadenosine (MTA). The first step of the SAM cycle is catalyzed by a SAM-dependent methyltransferase that removes the reactive alkyl group from SAM,

generating S-adenosylhomocysteine (SAH). Then, the hydrolysis of the S-C5' bond within SAH will produce the methionine and cysteine precursor L-homocysteine (*Figure 6*). At concentrations greater than 1 mmol/L, SAH will serve as a competitive inhibitor to SAM in the methyltransferase reaction that yields SAH until the concentration decreases to a reasonable level via the reaction catalyzed by adenosylhomocysteinase. The transfer of a methyl group to homocysteine will generate methionine and complete the SAM cycle. Alternatively, homocysteine may be converted to glutathione (GSH) through the trans-sulfuration pathway.

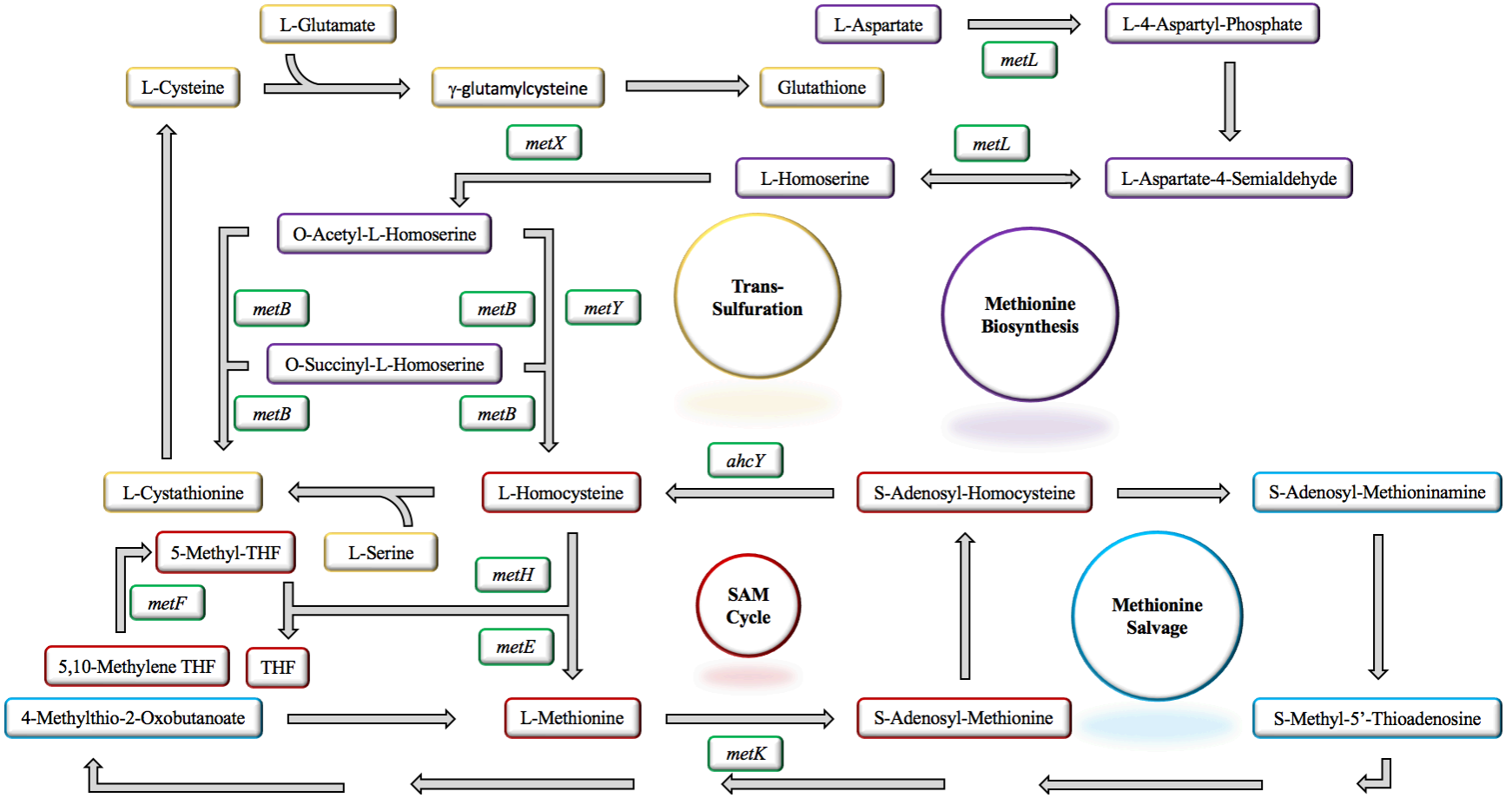

***Figure 6*:** Methionine Metabolism in *S. maltophilia* [33–35].

1.6 Transposon Mutagenesis

The EZ-Tn5^{TM} <R6Kγ*ori*/Kan-2>Tnp Transposome (Epicentre; Madison, WI) was randomly incorporated into the genome of *S. maltophilia* 02 by electroporation. The transposome contains a gene for kanamycin resistance, an *R6Kγ* replication origin, and two mosaic ends that consist of DNA sequences for transposase binding.

Transposition begins with the binding of a transposase to a 19 bp end sequence on the transposon [36]. Next, an oligomerization occurs at the end of the transposable element, creating a transposition synaptic complex. The transposition synaptic complex is then cleaved at blunt ends to generate and release the transposome. The transposome then binds to target DNA allowing for strand transfer to occur. The 3' end of the transposon is transferred to the 5' end of the target DNA, then the transposase is released. This will result in one or more transposon inserts being present in the target DNA sequence, which can interrupt gene function.

Chapter II: Hypothesis

Transposon mutagenesis was used to create a *Stenotrophomonas maltophilia* OR02 selenite-sensitive mutant, AX55. *S. maltophilia* 02 exhibits selenite resistance through a detoxification mechanism that results in the reduction and methylation of selenite. The reduction of selenite to selenide may be dependent on the role of a glutathione reductase. Then, methyl-selenide may be produced by a thiopurine methyltransferase. If a gene involved in one of these mechanisms is interrupted, it could have a negative effect on the detoxification of selenite. The Basic Local Alignment Search Tool (BLAST) identified a putative *metK* gene that encodes the protein S-adenosyl-L-methionine synthetase, which synthesizes S-adenosylmethionine involved in selenide methylation.

Chapter III: Methods

3.1 Bacterial Strains

Stenotrophomonas maltophilia (ATCC 53510™) was acquired from the American Type Culture Collection (Manassas, VA). The competent *Escherichia coli* EC100D™ *pir*-116 (EC6P095H) was acquired from Epicentre (Madison, WI), a subsidiary of Illumina, however this product is currently being distributed by Lucigen (Middleton, WI).

3.2 Growth Media

Lennox Broth (LB) was purchased through Molecular Biologicals International, Inc. (Irvine, CA). The broth is comprised of the following: 10 g/l tryptone, 5 g/l sodium chloride, and 5 g/l yeast extract. The *S. maltophilia* 02 mutant (AX55), that contains transposon inserts, was grown in LB media supplemented with 800 µg/ml of kanamycin. The LB media was supplemented with 50 µg/ml of kanamycin for the growth of competent *Escherichia coli* cells that contained a plasmid with a transposon insert. LB agar plates were prepared by the addition of 1.6% agar (AMRESCO; Solon, OH) to the LB broth.

A modified R3A medium, R3A-Tris [1, 37] , was used for replica plating and consisted of the following: 1 g/l yeast extract (AMRESCO), 1 g/l Bacto proteose peptone number 3 (Becton, Dickinson and Company; Sparks, MD), 1 g/l casamino acids (AMRESCO), 1 g/l D-glucose (AMRESCO), 1 g/l Difco soluble starch (Becton, Dickinson and Company), 0.5 g/l sodium pyruvate (Fisher Scientific; Waltham, MA), 0.1 g/l magnesium sulfate (Fisher Scientific), and 10 ml/l Tris (pH 7.5) (AMRESCO). R3A-Tris agar plates were prepared by the addition of 1.6% agar to the R3A-Tris broth.

An M-9 minimal medium supplemented with casamino acids was also used for replica plating and consisted of the following: 20 ml/100 ml of 1X M-9 salts (Fisher), 0.1

ml/100 ml of 1 mM magnesium sulfate, 1 ml/100 ml of 20% glucose, and 2 ml/100 ml of 5% casamino acids (AMRESCO). M-9 agar plates were prepared by the addition of 1.6% agar to the M-9 broth.

3.3 Preparation of Electrocompetent Cells

Electrocompetent cells were prepared by diluting 2 ml of *S. maltophilia* cells from an overnight culture (grown at 30°C with agitation at 120 rpm) into 100 ml of fresh LB media. These were then grown at 30°C with agitation (120 rpm) to an optical density (600 nm) between 0.6 and 1.0. The optical density was measured using a BioPhotometer (Eppendorf; Hauppauge, NY). The cells were then transferred into two 50 ml centrifuge tubes and placed on ice (4°C) to cool. Once cooled, the cells were pelleted at 4°C and 7,000 X g for five minutes; the supernatant was discarded. The cells were resuspended by the addition of 15 ml of ice cold water to the centrifuge tubes and briefly vortexing them. The tubes were then filled to the 50 ml mark with ice cold water. The cells were pelleted. Then, the supernatant was poured off. The cells were resuspended in 15 ml of ice cold water with vortexing. Ice cold water was added to the centrifuge tubes to the 50 ml mark, and the cells were pelleted once again. After the cells were resuspended in the remaining supernatant, they were transferred into a 15 ml centrifuge tube. Ice cold water was then added to the 15 ml mark, the cells were pelleted, and the supernatant was discarded. Finally, the cells were resuspended in 200 µl of ice cold water and 40 µl of cells were transferred into three sterile 1.7 ml microcentrifuge tubes.

3.4 Preparation of Calcium Chloride Competent Cells

An overnight culture *Escherichia coli* strain EC100D™ *pir*-116 was grown at 37°C with agitation at 120 rpm. 10 ml of the cells were added to 200 ml of fresh LB media. This

was grown at 37°C with agitation (120 rpm) to an optical density (600 nm) of 1.0. The cells were then transferred into two 50 ml centrifuge tubes and placed on ice (4°C) to cool. Once cooled, the cells were pelleted at 4°C and 5,000 X g for five minutes; the supernatant was discarded. The cells were resuspended using 15 ml of 0.15 M NaCl and pelleted once more at 4°C and 5,000 X g for five minutes; the supernatant was discarded. The cells were then resuspended using 2 ml of ice-cold transformation buffer. The transformation buffer contained 15% glycerol (v/v), 0.1 M $CaCl_2$, 10 mM Tris-HCl (pH 8.0), and 10 mM $MgCl_2$. Once suspended, 400 µl of cells were transferred into individual 1.5 ml microcentrifuge tubes and incubated on ice in a refrigerator (4°C) overnight. After the incubation period the cells were frozen at -80°C.

3.5 Transposon Mutagenesis

The EZ-Tn5™ <R6K*γori*/Kan-2>Tnp Transposome (Epicentre; Madison, WI) was randomly incorporated into the genome of *Stenotrophomonas maltophilia* (*S. maltophilia*) OR02 by electroporation, resulting in the generation of a selenite sensitive mutant. In a sterile 1.7 ml microcentrifuge tube, 40 µl of electrocompetent cells were mixed with 0.5 µl of the EZ-Tn5™ Transposome. The control was supplemented with sterile water in place of the transposome. An electroporation cuvette with a 2 mm gap was cooled at -20°C and loaded with a cell/transposome mixture. Once the mixture reached the bottom of the cuvette (by tapping on the bench top), the cells were pulsed at 25 µF, 200 ohms, and 2.5 kV. To prevent cell death, 960 µl of SOC medium was added to the cuvette then mixed by pipetting. The SOC medium was added directly to the control and was not subjected to electroporation. The SOC medium consisted of 0.5% (w/v) yeast extract (AMRESCO; Solon, OH), 2% (w/v) tryptone (AMRESCO), 10 mM NaCl (AMRESCO), 2.5 mM KCl

(AMRESCO), 10 mM $MgCl_2$, 20 mM $MgSO_4$ (Thermo Fisher Scientific; Waltham, MA), and 20 mM Glucose (AMRESCO). The cells were then transferred to a 1.7 ml tube and incubated at 30°C for 45-60 minutes with agitation (120 rpm). After the incubation period, 100 µl of the cell mixture was spread onto LB plates supplemented with 800 µg/ml of kanamycin and incubated overnight at 30°C. The colonies were picked with sterile toothpicks and gridded onto new LB plates supplemented with 800 µg/ml of kanamycin and incubated overnight at 30°C. The gridded plates were replica plated onto plates containing 800 µg/ml of kanamycin (LB), 0.1 mM selenite (R3A), 1 mM selenite (R3A), 10 mM selenite (R3A), and M-9 minimal medium supplemented with casamino acids. A selenite sensitive mutant was identified by its ability to grow on the LB kanamycin plates, and its failure to grow on selenite plates.

3.6 Determination of the Minimal Inhibitory Concentration

The Minimal Inhibitory Concentration (MIC) of selenite for the mutant (AX55) and the wildtype (S02) were determined by liquid culture experiments. Overnight cultures of were prepared by picking a single colony from a streak plate and placing it into 5 ml of LB. The mutant culture was supplemented with 80 µl of kanamycin. The cultures were grown at 30°C for 18 hours in a roller drum (Fisher Scientific; Waltham, MA). Following the incubation period, the cultures were diluted into fresh LB in a 1:50 ratio. For each strain of bacteria, 5 ml of the dilution was transferred into six sterile test tubes. Sodium selenite (MP Biomedicals; Solon, OH) was added to each tube in the following concentrations: 0 mM, 1 mM, 10 mM, 20 mM, 30 mM, and 40 mM. A Klett Colorimeter (Fisher Scientific; Waltham, MA) was used to measure the turbidity of the cells. Then, the cultures were incubated at 30°C for 18 hours in a roller drum. Turbidity was measured again following

the incubation and the minimal inhibitory concentration of selenite was determined for AX55 and S02. The MICs were calculated by first taking the average of the turbidity measurements at each concentration of selenite. In Microsoft excel, the average measurements were used to calculate the standard deviation and the standard error. The standard error was calculated using the equation below [38]. The TINV function was used to calculate the inverse of the two-tailed T Distribution.

$$\textbf{Error} = \frac{t_{(95\%\text{CI},(N-1)\text{d.f.})} * StdDev}{\sqrt{N-1}}$$

3.7 Genomic DNA Purification

The genomic DNA of the mutant, AX55, was purified by picking a single colony and growing it overnight in 20 ml of LB supplemented with 800 µg/ml of kanamycin at 30°C with agitation (120 rpm). 10 ml of the overnight culture was pelleted by centrifugation at 5,000 X g for 5 minutes at 25°C. The supernatant was poured off and the cells were resuspended in 2 ml of TE buffer (10 mM tris-HCl (pH 8.0) and 1 mM EDTA). Once the cells were resuspended, 5 µl of RNase A (AMRESCO; Solon, OH) and 5.5 ml of the genomic prep lysing solution containing 50 mM tris-HCl and 3% SDS) were added and mixed by inversion. The genomic prep lysing solution was prepared using 9.8 ml of nuclease free water, 0.75 ml of 1 M tris-HCl (pH 8.0), and 4.5 ml of 10% SDS. After incubating the mixture for 30-45 minutes at 65°C, 2 ml of warm 5 M NaCl were added to the mixture. The mixture was vortexed for 20 seconds (until the tube became cloudy) and immediately placed on ice for 15 minutes. Next, the mixture was pelleted by centrifugation

at 10,000 X g for 10 minutes at 25°C. The supernatant was poured into a 50 ml sterile tube and the DNA was precipitated using 6 ml of isopropanol and mixed by inversion. Once the DNA began to clump, a glass rod was used to spool the DNA. The DNA was transferred into a 1.7 ml microcentrifuge tube containing 500 µl of 70% ethanol. The DNA was pelleted by centrifugation at 13,000 X g for 1 minute at 25°C. The supernatant was poured off, and the DNA was resuspended in 500 µl of TE buffer and stored at 4°C.

3.8 Genomic and Plasmid DNA Digestions

Four separate reactions were set up with the following enzymes: *Eco*RI, *Pvu*II, *Nco*I, and *Sac*I. Each digestion reaction contained 2 µl of 10X CutSmart buffer (New England BioLabs; Ipswich, MA), 5 µl of nuclease free water, 12 µl of the purified genomic DNA, and 1 µl of enzyme. The reactions were then incubated for 60 minutes at 37°C.

The purified plasmid DNA was cut using the same restriction endonucleases that were used in the genomic DNA digestions: *Eco*RI, *Pvu*II, *Nco*I, and *Sac*I. Each digestion reaction contained 2 µl of 10X CutSmart buffer (New England BioLabs; Ipswich, MA), 5 µl of nuclease free water, 12 µl of the purified plasmid, and 1 µl of enzyme. The reactions were incubated for 60 minutes at 37°C. The size of the plasmid was estimated by comparing the digested DNA bands to the DNA bands in the 1 Kb ladder on a 1% agarose gel.

3.9 Agarose Gel Electrophoresis

A 1% agarose gel was prepared by adding 1.3 g of BioExcell Agarose LE (WorldWide Medical Products; Bristol, PA) to 130 ml of 1X TBE buffer (AMRESCO; Solon, OH). The 1X TBE buffer was made by diluting 10X TBE buffer (0.089 M Tris Base, 0.089 M Borate, and 0.002 M EDTA) with water. The mix was placed in a

microwave until the agarose dissolved, then 13 µl of GelGreen Nucleic Acid Stain (Embi Tec; San Diego, CA) was added and mixed using a stir bar and stir plate. Once mixed, the contents were poured into a gel tray and combs were inserted to form wells. After the gel hardened the combs were removed. The gel was transferred to a RunOne Electrophoresis System and covered with 1X TBE buffer.

The wells were then loaded by mixing 2 µl of Agarose Gel Loading Dye 6X (AMRESCO) with either 3 µl of DNA MW Marker-1 Kb ladder (AMRESCO), 3 µl of the genomic DNA digestion reaction, or 10 µl of the plasmid DNA digestion reaction. S02 genomic DNA and the undigested plasmids were used as positive controls for the genomic DNA digestions and plasmid DNA digestions respectively. The DNA was then separated at 100 V and the migration of the DNA was tracked using the indigo band (2^{nd} band), which migrates at ~600bp. When the bromophenol blue was close to reaching the end of the gel, the current was removed and the gel was visualized using a PrepOne Sapphire illuminator with an image catcher (Embi Tec).

3.10 Southern Blot

A southern blot was used to determine the number of transposon inserts in the genomic DNA of the mutant, AX55.

3.10.1 Probe Preparation

A biotinylated EZ-Tn5™ transposon probe needed to be generated for a southern blot. This was accomplished using the PCR to amplify a region of the transposon. A 50 µl reaction was prepared with 2.5 µl of biotin-11-dUTP (PromoKine; Heidelberg, Germany), 2.5 µl of biotin-11-dCTP (PromoKine; Heidelberg, Germany), 25 µl of 2X GoTaq DNA polymerase (Promega; Madison, WI), 6.25 µl of Kan Probe F (*Table 1*), 6.25 µl of Kan

Probe R (*Table 1*), 6.5 µl of nuclease free water, and 1 µl of plasmid DNA that contains a transposon insert. The control reaction contained 25 µl of 2X GoTaq DNA polymerase, 6.25 µl of 4 µM Kan Probe F (*Table 1*), 6.25 µl of 4 µM Kan Probe R (*Table 1*), 11.5 µl of nuclease free water, and 1 µl of plasmid DNA. The reaction mixture was placed in a thermocycler on the following program: 95°C for 2 minutes, then 35 cycles of 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. After the completion of 35 cycles the reaction was incubated at 72°C for 10 minutes, then it was held at 10°C.

3.10.2 PCR Purification

The PCR reactions were purified using a QIAquick PCR Purification Kit (Qiagen; Hilden, Germany). First, 5 volumes of Buffer PB was added to 1 volume of the PCR reaction and it was mixed by inversion. The sample was then transferred to QIAquick spin column that was placed in a 2 ml collection tube prior to the addition of the sample. The assembly was centrifuged at 13,000 rpm for 1 minute at 25°C to bind the DNA to the spin column. The spin column was removed, the flow-through was discarded, and the spin column was placed back into the collection tube. The spin column was washed by the addition 750 µl of Buffer PE and centrifugation at 13,000 rpm for 1 minute at 25°C. The spin column was removed, the flow-through was discarded, and the spin column reinserted into the collection tube. The assembly was centrifuged at 13,000 rpm for 1 minute at 25°C to remove excess ethanol. Then, 35 µl of the 60°C EB Buffer was added to the center of the membrane in the spin column which was incubated for 1 minute at room temperature. The DNA was eluted at 13,000 rpm for 1 minute at 25°C. The spin column was discarded.

3.10.3 Southern Blot

A southern blot was used to determine if the EZ-Tn5 transposon was inserted at only one site of the AX55 genome, or if multiple inserts were present. Digestions were performed on the genomic DNA of the wildtype (*S. maltophilia*) and the mutant (AX55) using the restriction endonucleases *Pvu*II and *Eco*RI. The digestion reactions consisted of the following: 4 µl of 10X CutSmart buffer, 19 µl of nuclease free water, 15 µl of the purified genomic DNA, and 2 µl of enzyme. The reactions were then incubated for 60 minutes at 37°C. A 1% agarose gel was loaded with the following samples after they were mixed with 2 µl of Agarose Gel Loading Dye 6X (AMRESCO; Solon, OH): 5 µl of biotinylated ladder, 2 µl of biotinylated-EZ-Tn5 probe (diluted to 1:50), 3 µl of 1 Kb DNA ladder, and 10 µl of each digestion reaction. After gel electrophoresis was performed, the gel was incubated in ethidium bromide for 30 minutes and the gel was visualized using an UltraCam Digital Imaging System (Ultra-Lum, Inc.; Claremont, CA).

The gel was then rinsed with distilled water, placed in a plastic tray, covered with 50 ml of depurination solution (AMRESCO; Solon, OH), and was incubated at room temperature for 30 minutes with gentle shaking (VWR S-500 Orbital Shaker). The depurination solution contained 0.25 M HCl. The depurination solution was poured off and the gel was rinsed with distilled water. 50 ml of denaturation solution (AMRESCO) was then added to the tray containing the gel and this was incubated at room temperature for 20 minutes with gentle shaking. The denaturation solution contained 0.5 M NaOH and 1.5 M NaCl. The denaturation solution was poured off and the gel was rinsed with distilled water. Then, 50 ml of neutralization solution (AMRESCO) was added. This was incubated at room temperature for 20 minutes with gentle shaking. The neutralization solution

contained 1 M Tris (pH 7) and 1.5 M NaCl. The neutralization solution was poured off, then another 50 ml of neutralization solution was added. Again, this was incubated at room temperature for 20 minutes with gentle shaking.

The southern blot was performed via an upward capillary transfer method. A plastic reservoir was filled with 20X Saline-Sodium Citrate (SSC) Buffer containing 3 M NaCl and 300 mM sodium citrate (AMRESCO) and a solid support that the gel would sit on was placed in the reservoir. A wick was prepared by cutting a strip of 3MM filter paper (Fisher Scientific; Pittsburgh, PA) to a width that was just wider than the gel. The wick was saturated in the buffer and placed on top of the solid support with each end submerged in the in the buffer. The gel was then placed on top of the wick with the wells facing down. Air bubbles were removed by gently rolling a sterile pipette tip over the gel. Next, a piece of Biodyne® B Pre-Cut Modified Nylon Membrane (0.45 μm) (Thermo Fisher Scientific; Waltham, MA) was cut to the same size of the gel. The membrane was submerged in the 20X SSC buffer, then placed on top of the gel. Three strips of 3MM filter paper were placed on top of the membrane, then six inches (when compressed) of paper towels that were cut to the size of the gel were placed on top of the filter paper. The stack of paper towels and filter paper were weighted down and incubated overnight. The following day the assembly was taken down and position of the wells were scratched into the membrane using a pen before the membrane was removed from the gel. The membrane was baked at 80°C for 30 minutes in a Mini Hybridization Oven (Bellco Glass, Inc.; Vineland, NJ).

3.10.4 Hybridization and Probe Detection

The dried membrane was transferred to a 50 ml glass tube with a lid and a North2South Chemiluminescent Hybridization and Detection Kit (Thermo Fisher

Scientific; Waltham, MA) was used to detect the biotinylated probe. The North2South Hybridization Buffer was set out until it warmed to room temperature, then 5 ml was added to the glass tube containing the membrane. Pre-hybridization was carried out by rotating the membrane in a Mini Hybridization Oven at 55°C for 30 minutes. During the pre-hybridization step, 3 µl of the biotinylated DNA probe was diluted with 147 µl of TE buffer. The diluted probe was placed in a thermocycler and incubated at 98°C for 10 minutes to denature the DNA, and then it was placed on ice for 5 minutes. After the pre-hybridization step, 50 µl of the probe was added to the container. The membrane was incubated at 55°C overnight while being rotated.

The hybridization mix from the previous day was poured off. Then, the membrane was subjected to three stringency washes. 10 ml of 1X stringency wash buffer (2X SSC/0.1% SDS) was added to the tube and it was incubated at 55°C for 15 minutes while rotating. The buffer was poured off and the wash step was repeated two more times. 20 ml of 1X Blocking Buffer was added to the tray and the membrane was incubated at room temperature for 15 minutes with gently shaking. During this incubation step, 66 µl of streptavidin-HRP was mixed with 10 ml of 1X Blocking Buffer. The 20 ml of 1X Blocking Buffer was poured off and the blocking buffer-streptavidin mix was poured over the blot. This was incubated at room temperature for 15 minutes with gentle shaking. The membrane was transferred to a sterile tray and was covered with 25 ml of 1X Wash Buffer. The membrane was incubated at room temperature for 5 minutes with gentle shaking. Then, the solution was poured off. This wash step was repeated three more times. Then, the membrane was transferred to clean tray and was covered with 20 ml of Substrate Equilibrium Buffer. The membrane was incubated at room temperature for 5 minutes with

gentle shaking. Next, a Chemiluminescent Substrate Working Solution was prepared using 3 ml of Luminol/Enhancer and 3 ml of Stable Peroxide Solution. The membrane was transferred to clean tray and the Substrate Working Solution was poured over the membrane. The membrane was left to incubate at room temperature for 5 minutes. Then, the solution was poured off. The membrane was transferred to a sheet of plastic wrap and the air bubbles were removed. The ChemiDoc MP Imaging System (Bio-Rad Laboratories; Hercules, CA) was used to detect the probe and take a picture of the membrane.

3.11 T4 DNA Ligation

To generate circularized DNA (plasmids) from the DNA fragments, the digestion reactions were first incubated in a thermocycler for 20 minutes at 80°C to inactivate the restriction endonucleases. The ligation reactions were carried out in 1.7 ml microcentrifuge tubes containing 10 µl of 10X T4 DNA ligase buffer, 73 µl of nuclease free water, 2 µl of T4 DNA ligase (New England BioLabs; Ipswich, MA), and 15 µl of digested DNA. After the DNA was added to the ligation mix it was incubated overnight at 4°C.

3.12 Calcium Chloride Transformation

A chemical transformation technique was used to transform the ligated DNA into the competent *Escherichia coli* EC100D *pir*-116 cells. After the ligation reactions were incubated at 4°C, the ligated DNA was precipitated by the addition of 10 µl of 3 M sodium acetate (pH 5.2) and 200 µl of 95% ethanol to each reaction which were subsequently incubated for 10 minutes at -20°C. The mixture was pelleted via centrifugation at 25°C and 14,000 rpm for ten minutes; the supernatant was discarded. The pellet was washed with 200 µl of 70% ethanol and mixed gently by inversion. The mixture was then centrifuged at 25°C and 14,000 rpm for five minutes. The supernatant was poured off, the tube was

blotted onto a paper towel to remove excess liquid, and the pellet was dried using a CentriVap DNA concentrator (Labconco; Kansas City, MO) for 10 minutes at 37°C. The DNA was resuspended in 10 µl of nuclease free water while the competent *pir*-116 cells were thawed on ice. Once thawed, 100 µl of the cells were transferred to the 1.7 ml tube containing the resuspended DNA which was then incubated on ice for 30 minutes. The control reaction contained 100 µl of cells without the resuspended DNA. The DNA was heat shocked into the cells by placing the mix into a water bath at 42°C for 50 seconds, then immediately placing the cells on ice. 900 µl of LB medium was added to the cells, and they were incubated at 37°C for 45 minutes with agitation (120 rpm). The cells were pelleted by centrifugation at 25°C and 14,000 rpm for two minutes. 100 µl of the supernatant was saved to resuspend the cells and the rest was discarded. All the cells were plated onto LB agar plates supplemented with 50 µg/ml of kanamycin and incubated overnight at 37°C. Only the cells containing a plasmid with the kanamycin resistance gene of the transposon will grow; these are called transformants.

3.13 Plasmid Purification

A single transformant was picked and grown overnight at 30°C in 5 ml of LB supplemented with 50 µg/ml of kanamycin. The bacterial cells were then pelleted using a centrifuge at 6,000 X g and 4°C for six minutes. The supernatant was poured off and any excess liquid was blotted onto a paper towel. The plasmid DNA was then purified using the Wizard Plus SV Minipreps DNA Purification System (Promega; Madison, WI). The pellet was resuspended in 250 µl of Cell Resuspension Solution. 250 µl of Cell Lysis Solution was added, and it was mixed by inverting the tube four times. The cells were incubated at room temperature for 1-5 minutes until the solution cleared, then 10 µl of

Alkaline Protease Solution was added and mixed by inverting the tube four times. The cells were incubated again at room temperature for 1-5 minutes, and 350 µl of Neutralization Solution was added and mixed by inverting the tube four times. The mixture was pelleted using a microcentrifuge at 14,000 rpm and 25°C for ten minutes. The cleared lysate was decanted into a spin column that was inserted into a collection tube. The assembly was placed in a microcentrifuge at 14,000 rpm and 25°C for one minute, the spin column was removed, the flow-through was discarded, and the spin column was placed back into the collection tube. 750 µl of Column Wash Solution was added to the spin column, and it was centrifuged at 14,000 rpm and 25°C for one minute. The spin column was removed, the flow-through was discarded, and the spin column was placed back into the collection tube. 250 µl of Column Wash Solution was added to the spin column, and it was centrifuged at 14,000 rpm and 25°C for one minute. The spin column was removed, the flow-through was discarded, and the spin column was placed back into the collection tube. The assembly was centrifuged at 14,000 rpm and 25°C for two minutes, and the spin column was transferred to a sterile 1.7 ml microcentrifuge tube. The plasmid was eluted from the spin column using 100 µl of nuclease free water and centrifuging at 14,000 rpm and 25°C for one minute. The spin column was discarded and the purified plasmid was stored at -20°C.

3.14 DNA Sequencing

The Sanger method was used to determine the DNA sequence of the purified plasmid. Sequencing was carried out with the GenomeLab Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter; Fullerton, CA) and the Beckman Coulter CEQ 2000XL DNA analysis system (Beckman Coulter; Fullerton, CA).

3.14.1 Sequencing Prep

First, a NanoDrop Spectrophotometer (Thermo Fisher Scientific; Waltham, MA) was used to determine the concentration of the DNA that would be used in each sequencing reaction. The NanoDrop was blanked with 2 µl of nuclease free water. Then, 2 µl of DNA was placed on the pedestal for measurements. The nucleic acid concentration, the measurements at the wavelengths of 260 nm (A_{260}) and 280 nm (A_{280}), and the ratio of the measurements at the wavelengths (A_{260}/A_{280}) were recorded. The volume of DNA needed for each sequencing reaction was calculated by the amount of ng required for 50 fmol divided by the plasmid concentration. The amount of ng required to obtain 50 fmol of plasmid in the sequencing reaction was determined by comparing the estimated size of the plasmid that was found upon digesting the plasmid to a table provided by the sequencing kit. The DNA was then mixed with nuclease free water to bring the total volume of the reaction to 10 µl.

Two reactions were carried out for each plasmid: a forward and a reverse. A 0.2 ml PCR tube (GeneMate; Lodi, CA) was filled with the calculated amount of DNA and water for a specific plasmid. The tubes were then incubated at 96°C in a thermocycler for 1 minute and cooled to room temperature. Next, 8 µl of the DTCS quick start mix was added to every tube. 2 µl of a 1.6 µM primer (*Table 1*), either the forward or reverse primer, was then added to the corresponding tubes. The mixtures were then placed into a thermocycler and sequencing reaction was carried out with 30 cycles at 96°C for 20 seconds (denatures the DNA), 50°C for 20 seconds (primer annealing), 60°C for 4 minutes (elongation via DNA polymerase). The reactions were then held at 4°C.

3.14.2 Sequencing Clean-up

Once the PCR was completed, 5 μl of stop solution was added to each sequencing reaction. The stop solution contained 1.2 M sodium acetate, 40 mM EDTA, and 1.6 mg/ml glycogen. All 25 μl of the sequencing reaction-stop solution mix was transferred to a 0.65 ml microcentrifuge tube containing 60 μl of ice cold 95% ethanol. The tubes were centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatant was discarded. The pellet was then washed with 200 μl of ice cold 70% ethanol and centrifuged at 14,000 rpm for two minutes at 4°C; the supernatant was discarded. Again, the pellet was washed with 200 μl of ice cold 70% ethanol and centrifuged at 14,000 rpm for two minutes at 4°C. The supernatant was removed using a pipette and was discarded. The pellet was then dried in a CentriVap DNA concentrator for 10 minutes at 37°C. The pellet was resuspended by the addition of 40 μl of the Sample Loading Solution to each tube. The DNA sequences were resolved by the Beckman Coulter CEQ 2000XL DNA analysis system (Beckman Coulter; Fullerton, CA).

3.15 Sequence Analysis

GeneStudio Professional was used to convert the raw data from the sequencer into the FastA and plain text formats. The nucleotides of the transposon were removed by copying the sequences into Microsoft Word and searching for the nucleotides at the 3' end of the transposon: 5' – GAGACAG – 3'. All of the nucleotides beginning at the 3' G through the 5' end of the transposon insert were deleted. The remaining nucleotides belonged to the interrupted gene. Each nucleotide sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) [39], specifically a nucleotide BLAST (BLASTn) to determine the gene(s) that each encoded. A blastn search will compare the query

sequence to other sequences in the National Center for Biotechnology Information (NCBI) database to find similar sequences that are ranked by how identical the query is to the found sequences. Next, the contig program in GeneStudio Professional was used to align multiple overlapping sequences to obtain a consensus sequence. The consensus sequence was analyzed using BLASTn and BLASTx to translate and identify coding sequences. The coding nucleotide sequence with the highest similarity to the consensus sequence was found and downloaded. The downloaded sequence was entered into GeneStudio Professional to be used as a reference in correcting ambiguities and forming a better consensus sequence. The consensus sequence was uploaded to Genome Compiler for further analysis.

A multiple sequence alignment was generated to determine the homology of the protein coding region of the identified gene. First, the coding DNA (cDNA) sequence of the mutant was translated in Genome Compiler to the amino acid sequence. Then, the amino acid sequence was analyzed using BLASTP. The amino acid sequences of nineteen homologs that shared at least 90% identity were selected (Appendix A). The amino acid sequence of *E. coli* K12 was obtained using the Swiss Institute of Bioinformatics (SIB) Expert Protein Analysis System (ExPASy) [40] and the Universal Protein Resource (UniProt) [41]. The sequences were aligned using the MUSCLE function in UGENE [42] and analyzed further using the UniProt and InterPro databases [43]. For phylogenetic analysis, the alignment was exported from UGENE and imported into MEGA7 [44–46]. The sequences were aligned once more using the MUSCLE on MEGA7. A phylogenetic tree for maximum likelihood was constructed using the Jones-Taylor-Thornton (JTT) model (Appendix B) and the Bootstrap method for 1000 replicates (Appendix C).

Primer	Application	Nucleotide Sequence	Manufacture
AX55 F2	DNA Sequencing	5' – ACG CGG TTG GCC GTA TC – 3'	IDT-DNA, Coralville, IA
AX55 F3	DNA Sequencing	5' – GTT GCG AAG TGC AGG TTT C – 3'	IDT-DNA, Coralville, IA
AX55 F4	DNA Sequencing	5' – AGC CGA AGG AAT TCA GCT AC – 3'	IDT-DNA, Coralville, IA
AX55 R2	DNA Sequencing	5' – GGC CAA GAT CCG CAA GAA – 3'	IDT-DNA, Coralville, IA
AX55 R3	DNA Sequencing	5' – CGT TGA GGT AGC TGA ATT CCT T – 3'	IDT-DNA, Coralville, IA
AX55 R4	DNA Sequencing	5' – CTG ATC GTC GCC AAC CAC – 3'	IDT-DNA, Coralville, IA
KAN-2 FP-1	DNA Sequencing	5' – ACC TAC AAC AAA GCT CTC ATC AAC C – 3'	Epicentre, Madison, WI
R6KAN-2 RP-1	DNA Sequencing	5' – CTA CCC TGT GGA ACA CCT ACA TCT – 3'	Epicentre, Madison, WI
Kan Probe F	Southern blot	5' – GGT ATA AAT GGG CTC GCG ATA A – 3'	IDT-DNA, Coralville, IA
Kan Probe R	Southern blot	5' – CCG ACT CGT CCA ACA TCA ATA C – 3'	IDT-DNA, Coralville, IA

***Table 1*:** Primers.

Chapter IV: Results

4.1 Transposon Mutagenesis

A selenite-sensitive mutant was generated via transposon mutagenesis using an EZ-Tn5™ transposome. The transposon was inserted into the genome of *Stenotrophomonas maltophilia* OR02 (S02) by electroporation. Transformants were selected by growing the cells on LB agar plates supplemented with 800 µg/ml of kanamycin. Then, 880 transformants were gridded onto LB agar plates supplemented with 800 µg/ml of kanamycin for replica plating onto agar plates containing: 800 µg/ml of kanamycin (LB), 0.1 mM selenite (R3A), 1 mM selenite (R3A), 10 mM selenite (R3A), and M-9 minimal medium supplemented with casamino acids. The selenite sensitive mutant (AX55) was identified by its ability to grow on the LB kanamycin plates (*Figure 7*.A), and its failure to grow in the presence of 10 mM selenite (*Figure 7*.B). AX55 also failed to grow on the minimal media plates.

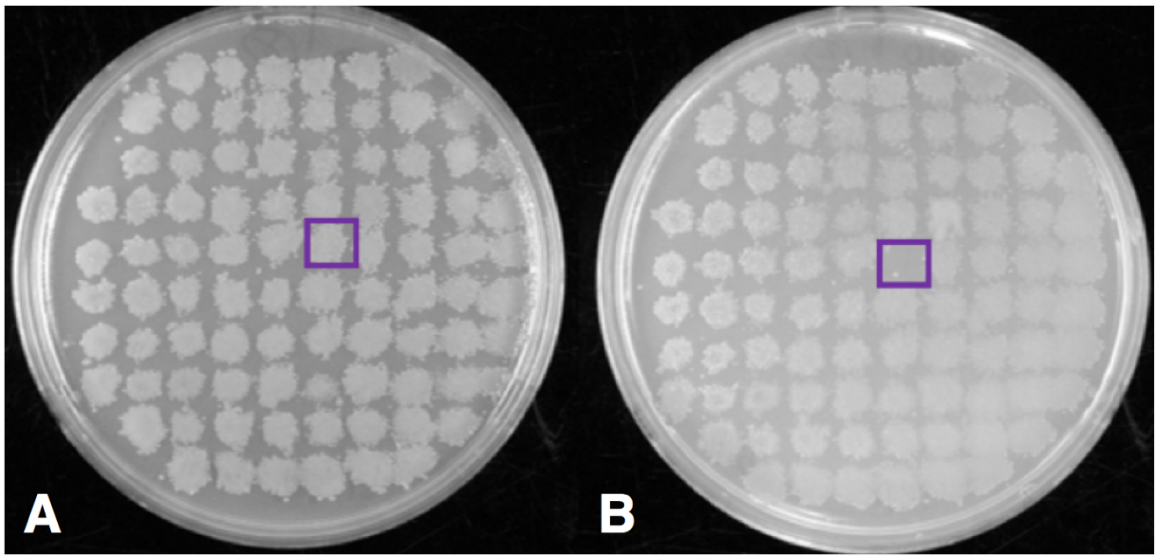

***Figure 7*:** Identification of a Selenite Sensitive Mutant. Replica plating of S02 onto **(A)** LB agar plates supplemented with 800 µg/ml of kanamycin and **(B)** R3A agar plates supplemented with 10 mM selenite identified the selenite-sensitive mutant, AX55.

4.2 Determination of the Minimum Inhibitory Concentration

To determine the mutant's (AX55) sensitivity for selenite, 5 mL overnight cultures of AX55 and S02 were prepared. The following day each culture was diluted 1:50 into fresh LB, then split amongst six sterile test tubes. Selenite (1 mM, 10 mM, 20 mM, 30 mM, and 40 mM) was added to five of the test tubes and water was added to the positive control. The turbidity (Klett units) for each condition was measured using a Klett colorimeter, then the cultures were incubated in a roller drum at 30°C for 18 hours. The turbidity of each was measured again and recorded. Once the experiment was conducted three additional times, the MIC was calculated by the subtracting the initial measurement (0 hour) from the final measurement (18 hours), then averaging the four measurements at each concentration. The standard error was calculated using a t-test with a 95% confidence interval. To account for the variation in growth and SeNP production the results were normalized by dividing the calculated average at each concentration by the respective positive control.

The positive controls (no selenite) for S02 and AX55 had an average turbidity measurement of 316.5 ± 13.87 Klett units and 145 ± 13.99 Klett units respectively. Due to the poor growth of the mutant the results were normalized as described above. The wildtype deposited SeNPs into the growth media at each concentration (*Figure 8.A*). There was also a presence of a garlic-like odor, suggesting that a volatile selenide was also produced. AX55 showed a significant increase in selenite sensitivity by only producing SeNPs at 1 mM selenite (*Figure 8.B*). In addition, AX55 did not exhibit the garlic-like odor of the wildtype. The MIC was then determined by the first instance of less than 50% growth. Thus, the MICs for S02 and AX55 were 30 mM and 10 mM respectively (*Figure 8.C*).

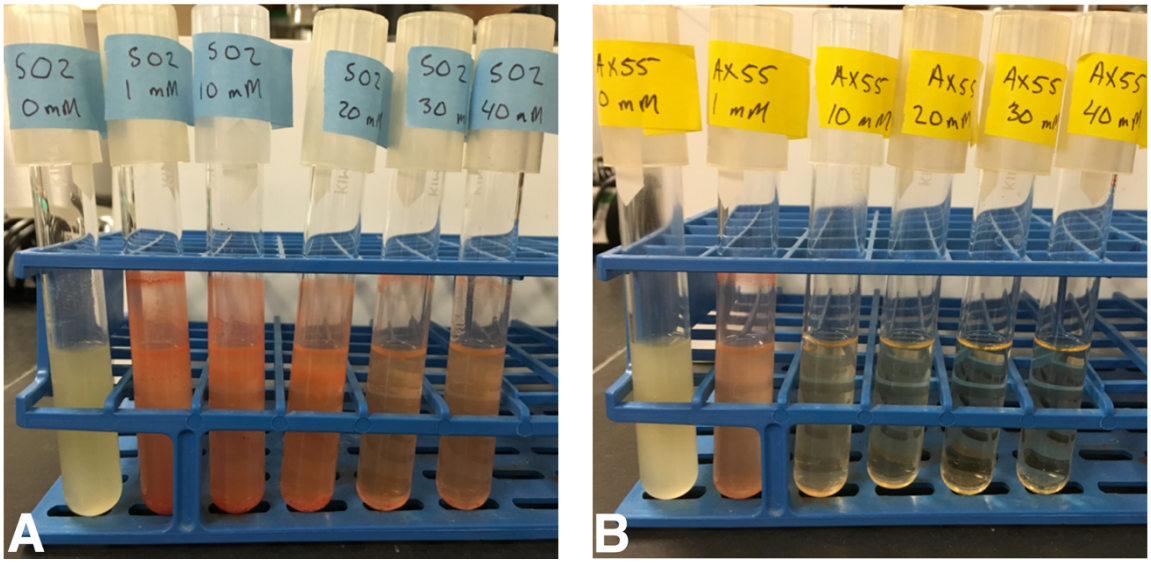

Figure 8.A & B: Selenite Supplementation of LB Cultures. Overnight cultures (18 hours) of S02 and AX55 were diluted into fresh growth media (1:50), sodium selenite was added, and the cultures were incubated an additional night. Cultures after the second incubation show the production of reduced, red elemental selenium in the media containing S02 **(A)** at each concentration. **(B)** AX55 only produced SeNPs at 1 mM, thereby showing an increased sensitivity to selenite.

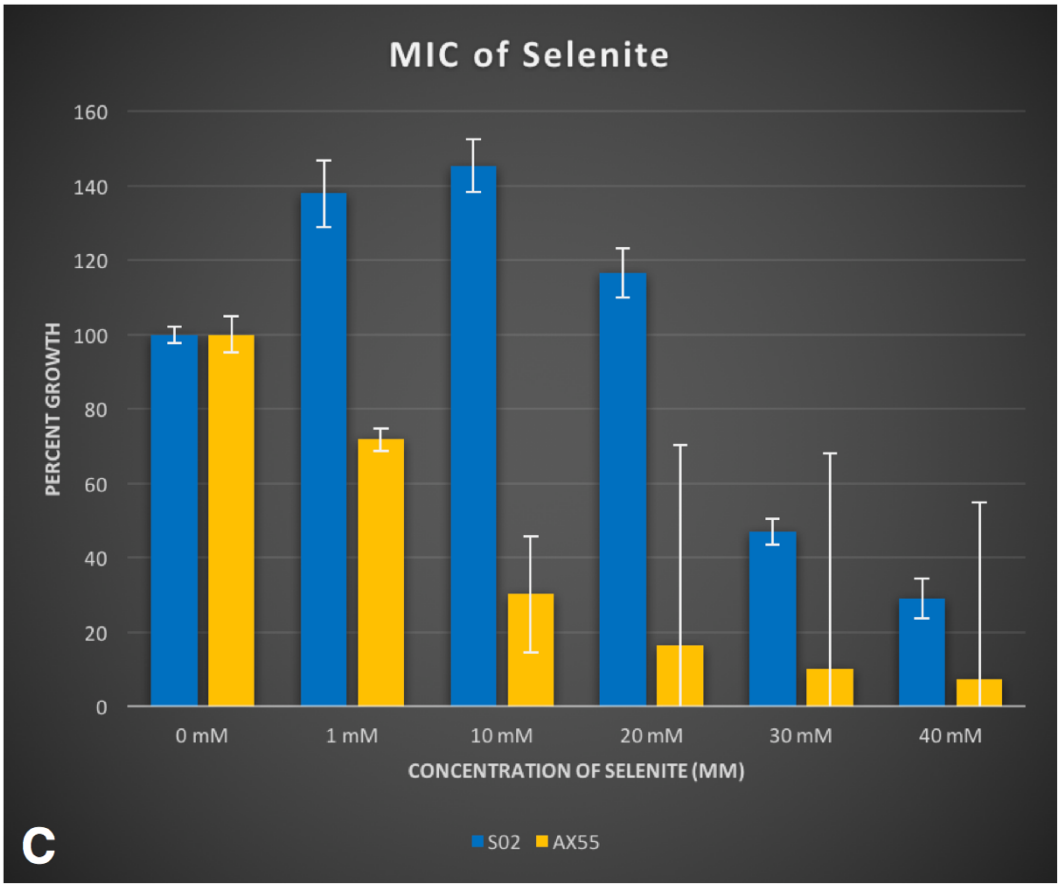

Figure 8.C: Determination of the MIC. Overnight cultures (18 hours) of S02 and AX55 were diluted into fresh growth media (1:50), sodium selenite was added, and the cultures were incubated an additional night. The average of the turbidity measurements at each concentration were normalized as percent growth. The MIC for S02 (30 mM) and AX55 (10 mM) were determined by the first instance of less than 50% growth.

4.3 Southern Blot

A southern blot was used to determine the number of transposon inserts that led to the selenite sensitivity of AX55. Genomic DNA of S02 and AX55 were digested with restriction enzymes (*Pvu*II and *Eco*RI) to generate fragments, and a biotinylated EZ-Tn5 probe was used as a positive control. The appearance of a single band in the lanes that contained the genomic DNA of the mutant (lanes 4 and 6) confirmed the presence of only one transposon insert in the genome.

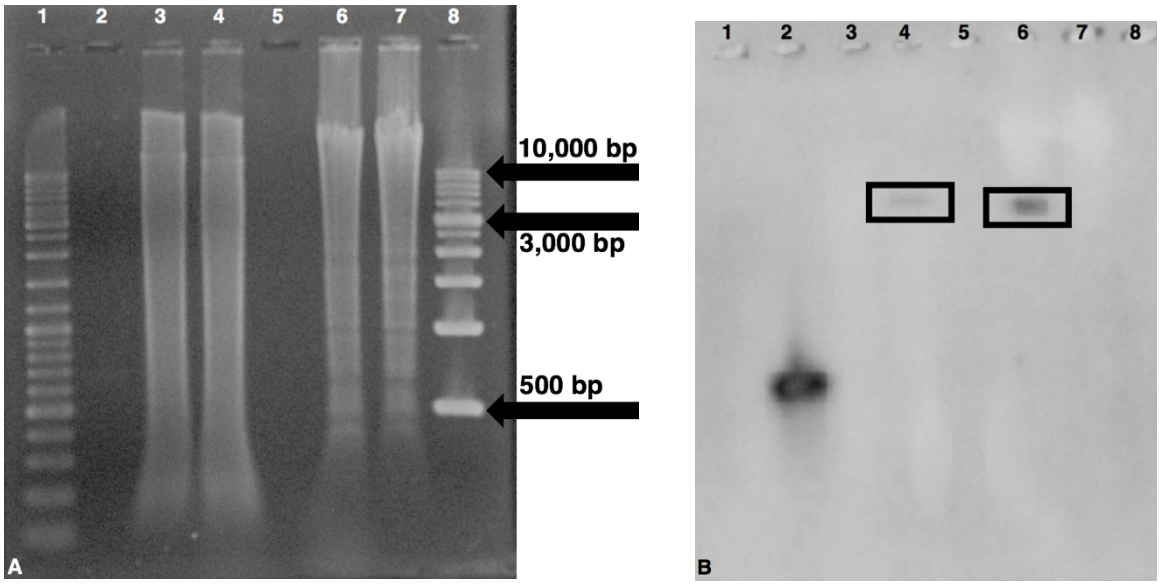

***Figure 9*:** Southern Blot. 1% agarose gel electrophoresis **(A)** and southern blot **(B)** of S02 and AX55 genomic DNA. Biotinylated ladder (lane 1), TN5 probe (lane 2), S02-*Pvu*II (lane 3), AX55-*Pvu*II (lane 4), AX55-*Eco*RI (lane 6), S02-*Eco*RI (lane 7), and 1 Kb ladder (lane 8).

4.4 Genomic DNA Digestions

The genomic DNA was digested with restriction enzymes to generate fragments containing the transposon interrupted gene. Agarose gel electrophoresis was used to determine if the digestion reactions were successful. The digestion of DNA at multiple restriction sites will appear as a smear on the gel. The smears observed in lanes 3, 4, 6, and 7, confirm the multi-site digestion of genomic DNA by *Eco*RI, Pv*u*II, *Nco*I, and *Sac*I (*Figure 10*).

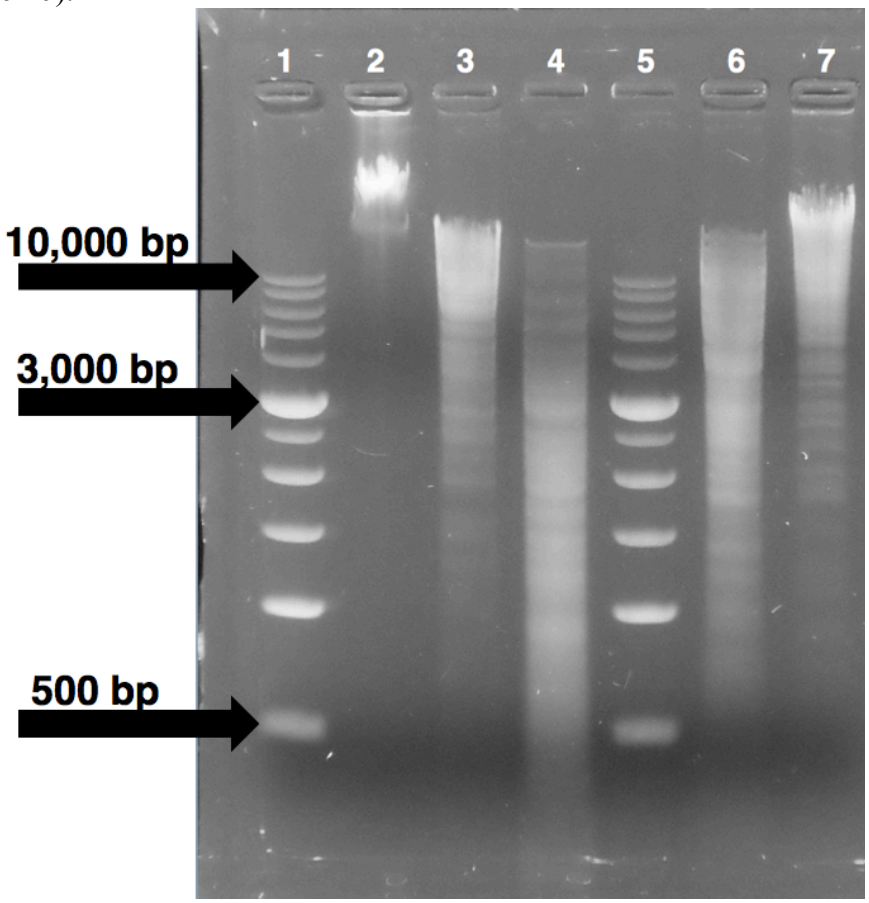

***Figure 10*:** Genomic DNA Digestions. 1% agarose gel electrophoresis of AX55 genomic DNA. 1 Kb ladder (lanes 1 & 5), undigested gDNA (lane 2), *Eco*RI (lane 3), *Pvu*II (lane 4), *Nco*I (lane 6), and *Sac*I (lane 7). 1% agarose gel electrophoresis of AX55 genomic DNA. 1 Kb ladder (lanes 1 & 5), undigested gDNA (lane 2), *Eco*RI (lane 3), *Pvu*II (lane 4), *Nco*I (lane 6), and *Sac*I (lane 7).

4.5 Plasmid DNA Digestions

A T4 ligation was carried out on the digested genomic DNA to generate recombinant plasmids containing the transposon flanked by segments of the interrupted DNA sequence. The ligated DNA was transformed into *E. coli* ECD100Dpir116 competent cells. Then, the plasmids were purified from the transformants. These plasmids were then digested with the same enzyme they were formed from via the genomic DNA digestion and separated on a 1% agarose gel. This was done to estimate the size of the plasmid to indicate the volume of DNA required for sequencing reactions. The estimated size of each plasmid was: *Eco*RI (~ 3 Kb), *Pvu*II (~ 5 Kb), and *Nco*I (~ 6 Kb).

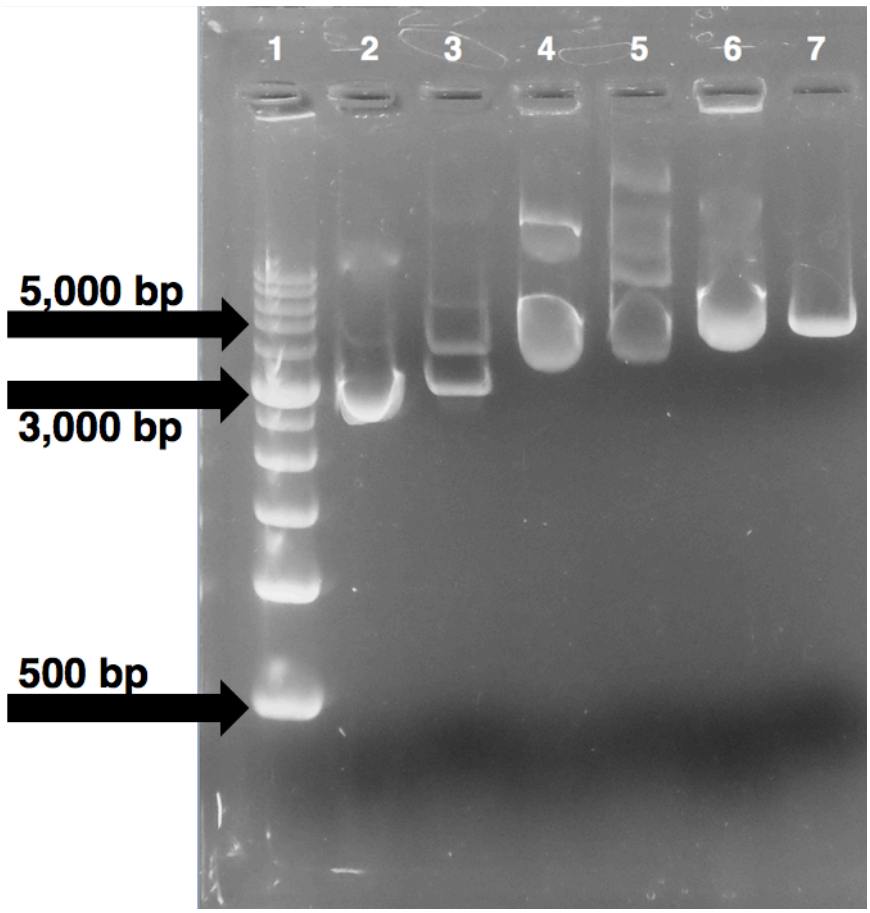

***Figure 11*:** Plasmid DNA Digestions. 1% agarose gel electrophoresis of AX55 plasmids. Undigested plasmids have 'u' before enzyme. 1 Kb ladder (lane 1), u*Eco*RI (lane 2), *Eco*RI (lane 3), u*Pvu*II (lane 4), *Pvu*II (lane 5), u*Nco*I (lane 6), and *Nco*I (lane 7).

4.6 Sequence Analysis

The first round of DNA sequencing was carried out using primers that identify the transposon insert within the DNA sequence: KAN-2 FP-1 and R6KAN-2 RP-1 (*Table 1*). After converting the raw sequences to a plain text file, the nucleotides encoding the transposon were removed, and each nucleotide sequence was analyzed via BLASTn [39]. The BLAST identified the interrupted sequence as a putative *metK* gene, which encodes S-adenosyl methionine synthetase. Subsequent sequencing reactions were carried out using the remaining primers (*Table 1*) to generate a consensus sequence that was analyzed using BLASTn (*Figure 12*) and BLASTx (*Figure 13*) to identify the coding DNA sequence of the protein. The nucleotide sequence with the highest similarity was used as a reference to generate a better consensus sequence, which was uploaded to Genome Compiler for further analysis (*Figure 14*).

Stenotrophomonas maltophilia JV3, complete genome

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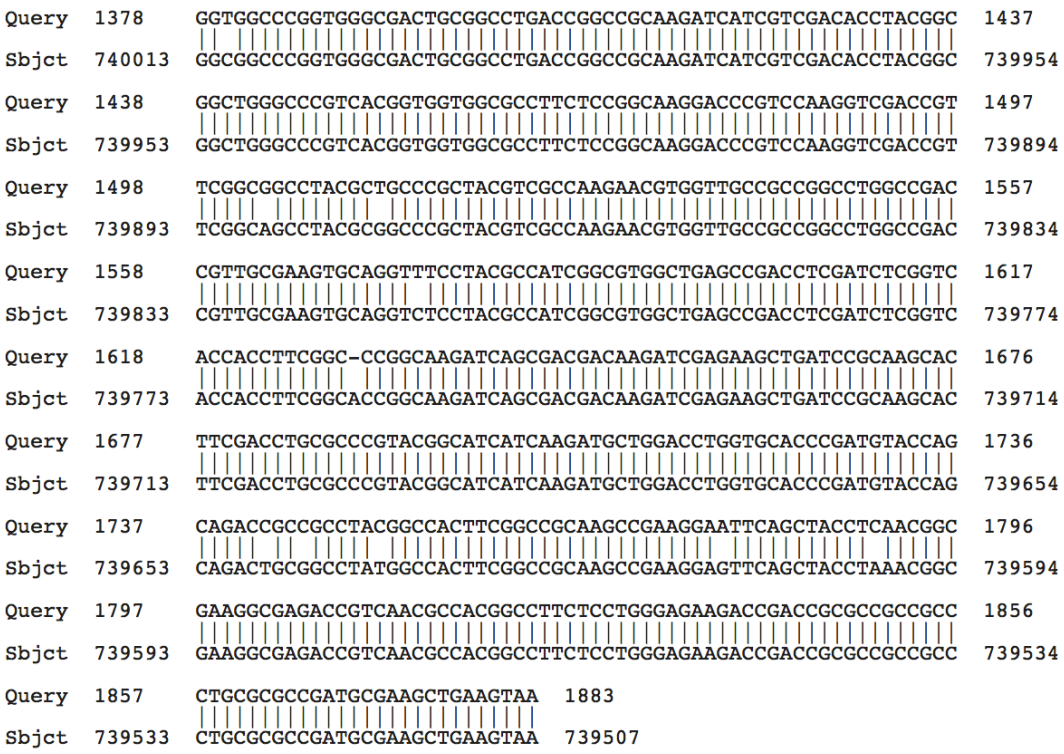
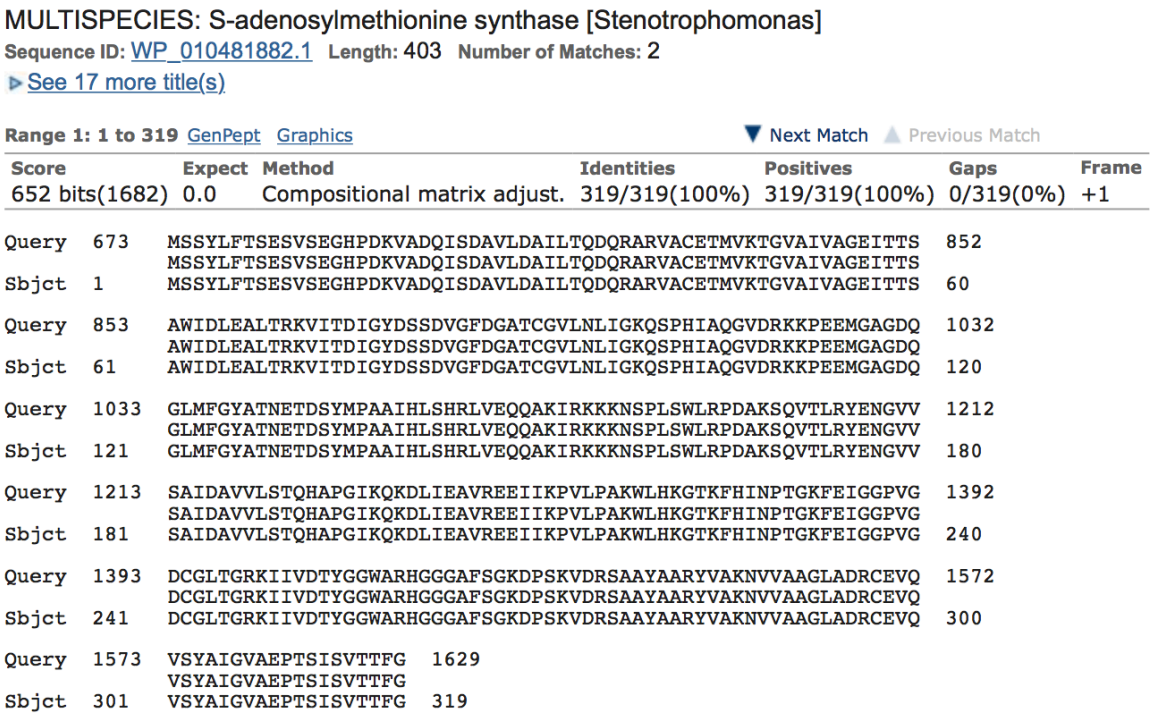


***Figure 12*:** BLASTn Analysis [39]. The consensus sequence generated via DNA sequencing was identified as a putative *metK* gene. The consensus sequence (Sbjct) was found to have a 97% identity to the *metK* sequence of *Stenotrophomonas maltophilia* JV3 (Query), which would then be used as a reference sequence.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See 17 more title(s)

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***Figure 13*:** BLASTx Analysis [39]. The consensus DNA sequence was converted to the amino acid sequence of the protein that the gene encodes. The coding DNA sequence of S-adenosylmethionine synthase (synthetase) was identified in the genome of *Stenotrophomonas maltophilia* JV3.


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EagI
AX55 R4
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PstI
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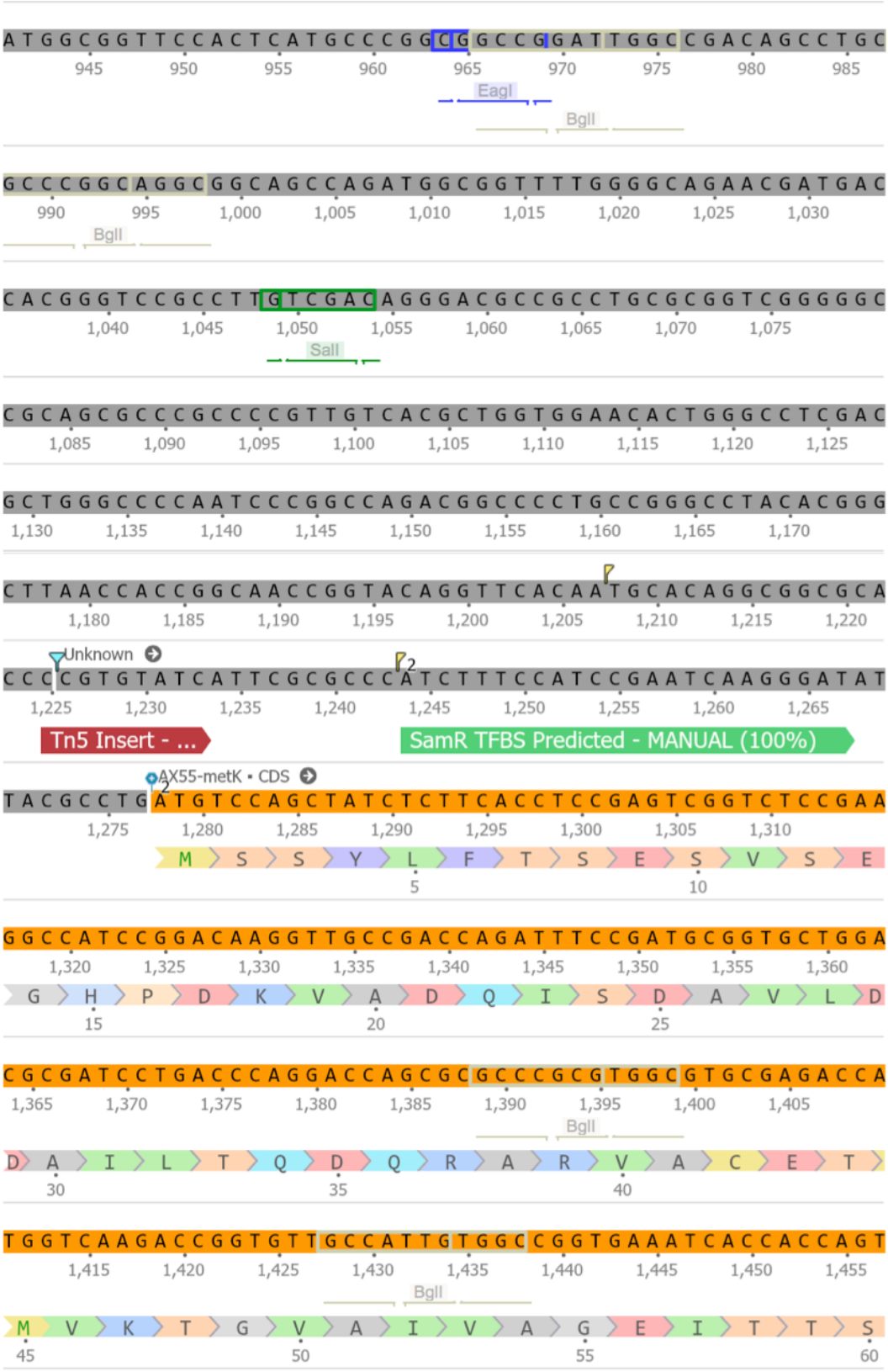
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SalI
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Tn5 Insert - ...
SamR TFBS Predicted - MANUAL (100%)
AX55-metK • CDS
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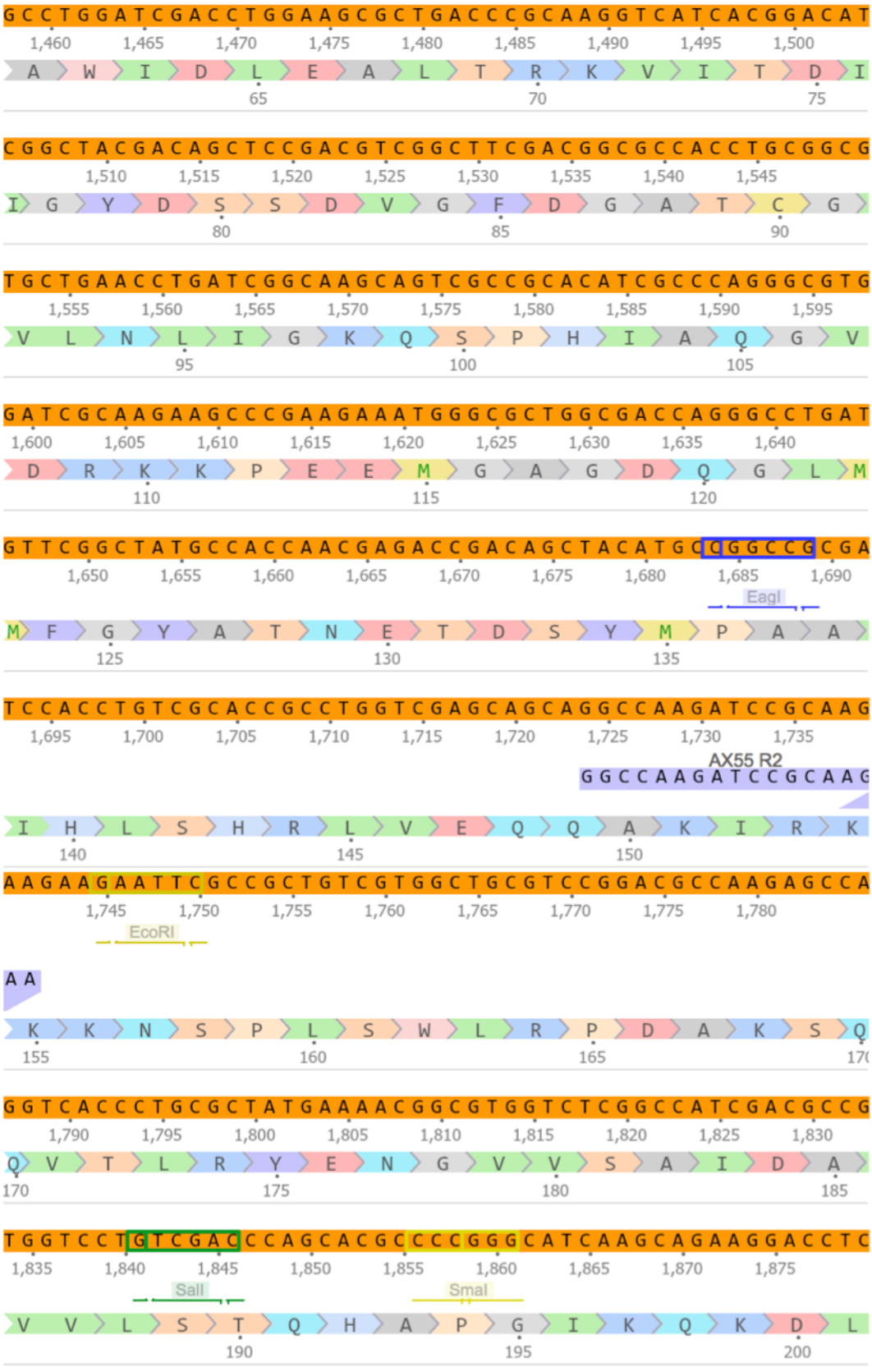
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AX55 R2
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EcoRI
AA
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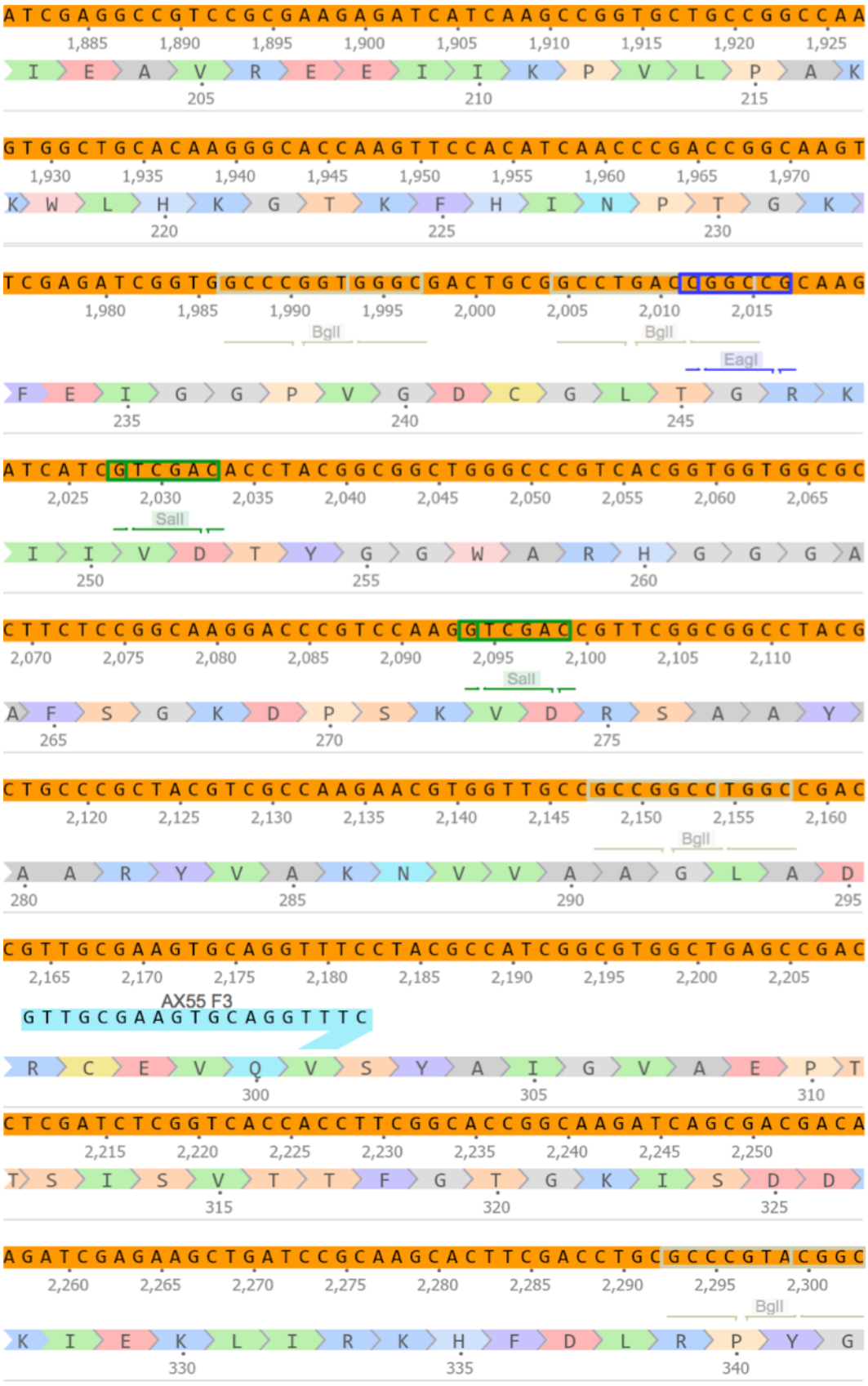

ATCGAGGCCGTCCGCGAAGAGATCATCAAGCCGGTGCTGCCGGCCAA
1,885 1,890 1,895 1,900 1,905 1,910 1,915 1,920 1,925
I E A V R E E I I K P V L P A K
205 210 215
GTGGCTGCACAAGGGCACCAAGTTCCACATCAACCCGACCGGCAAGT
1,930 1,935 1,940 1,945 1,950 1,955 1,960 1,965 1,970
K W L H K G T K F H I N P T G K
220 225 230
TCGAGATCGGTGGCCCGGTGGGCGACTGCGGCCTGACCGGCCGCAAG
1,980 1,985 1,990 1,995 2,000 2,005 2,010 2,015
BglI
BglI
EagI
F E I G G P V G D C G L T G R K
235 240 245
ATCATCGTCGACACCTACGGCGGCTGGGCCCGTCACGGTGGTGGCGC
2,025 2,030 2,035 2,040 2,045 2,050 2,055 2,060 2,065
SalI
I I V D T Y G G W A R H G G G A
250 255 260
CTTCTCCGGCAAGGACCCGTCCAAGGTCGACCGTTCGGCGGCCTACG
2,070 2,075 2,080 2,085 2,090 2,095 2,100 2,105 2,110
SalI
A F S G K D P S K V D R S A A Y
265 270 275
CTGCCCGCTACGTCGCCAAGAACGTGGTTGCCGCCGGCCTGGCCGAC
2,120 2,125 2,130 2,135 2,140 2,145 2,150 2,155 2,160
BglI
A A R Y V A K N V V A A G L A D
280 285 290 295
CGTTGCGAAGTGCAGGTTTCCTACGCCATCGGCGTGGCTGAGCCGAC
2,165 2,170 2,175 2,180 2,185 2,190 2,195 2,200 2,205
AX55 F3
GTTGCGAAGTGCAGGTTTC
R C E V Q V S Y A I G V A E P T
300 305 310
CTCGATCTCGGTCACCACCTTCGGCACCGGCAAGATCAGCGACGACA
2,215 2,220 2,225 2,230 2,235 2,240 2,245 2,250
T S I S V T T F G T G K I S D D
315 320 325
AGATCGAGAAGCTGATCCGCAAGCACTTCGACCTGCGCCCGTACGGC
2,260 2,265 2,270 2,275 2,280 2,285 2,290 2,295 2,300
BglI
K I E K L I R K H F D L R P Y G
330 335 340

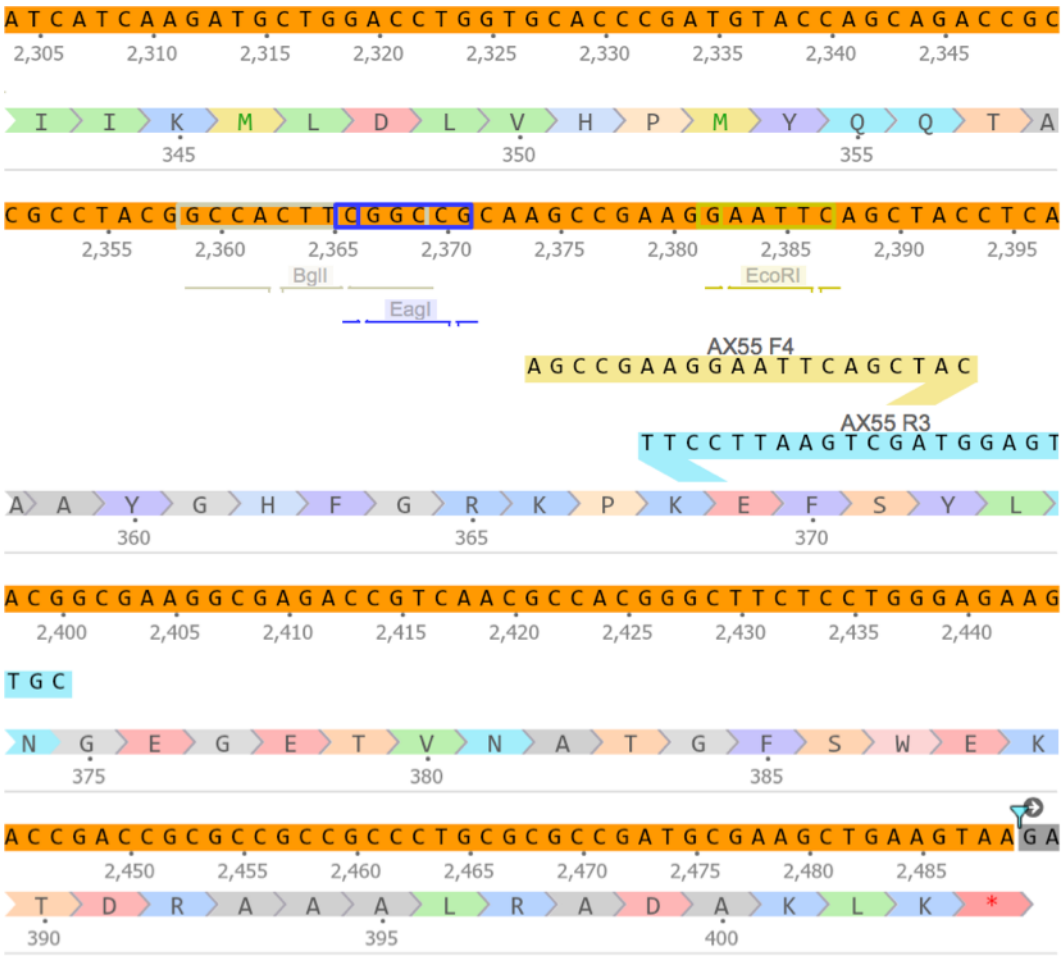
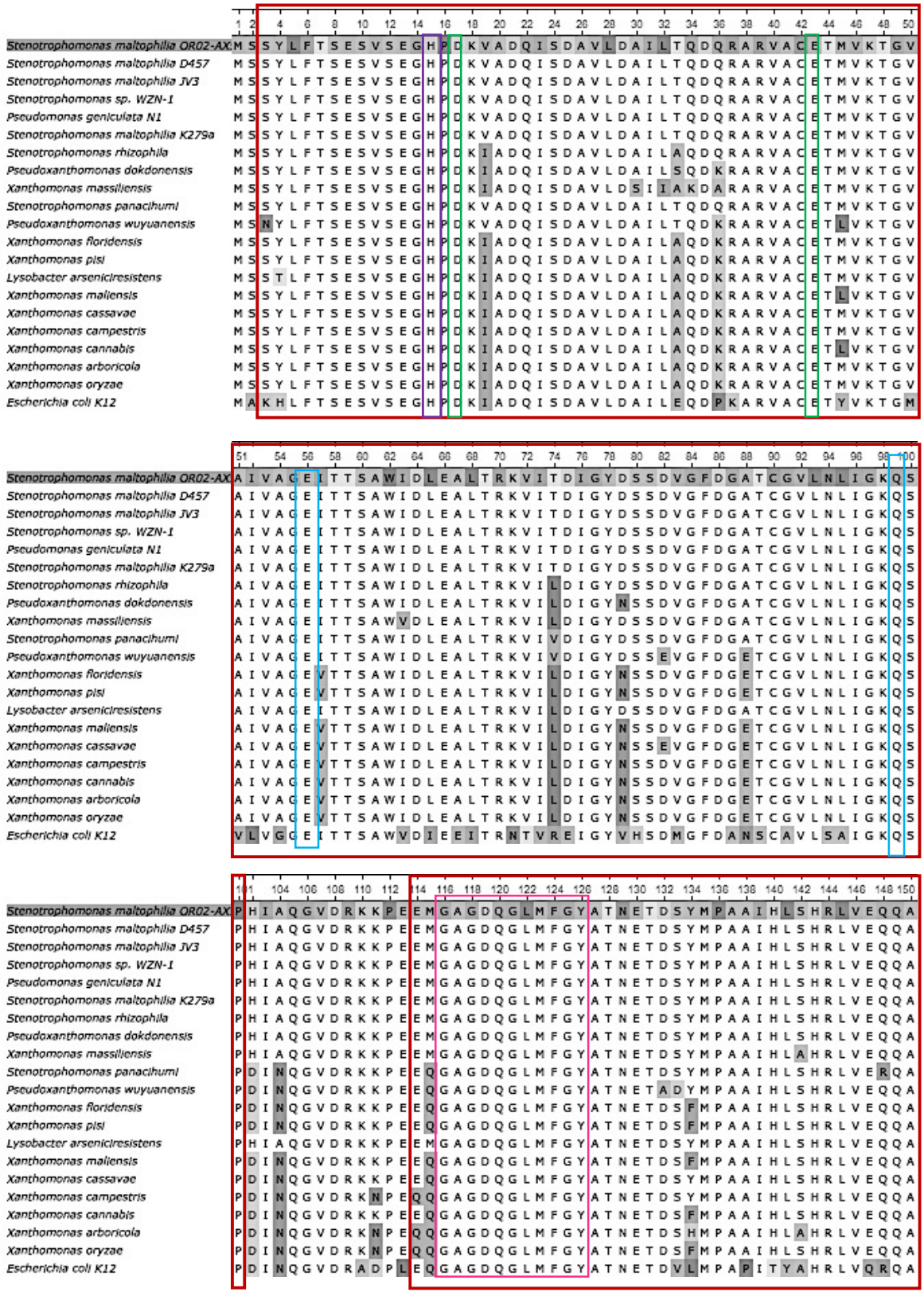
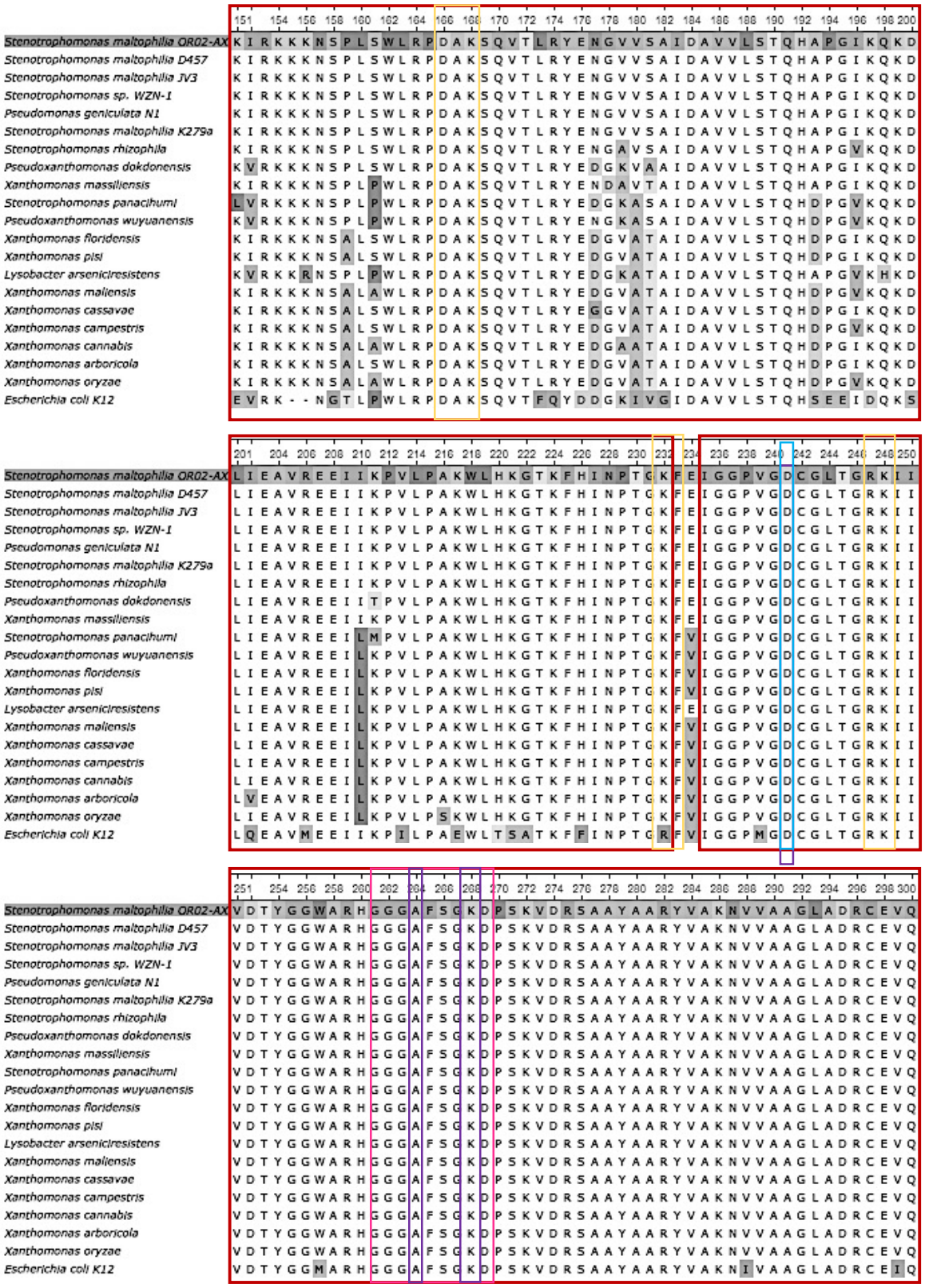


***Figure 14*:** AX55 Consensus Sequence. The *metK* coding region (orange) begins at nucleotide 1278. Less than 50 bp upstream from the +1 codon is the transposon insert, and the predicted SamR transcription factor binding site. Genome Compiler was used to visualize this sequence. The primers that were used during the sequencing reactions (*Table 1*) are also included.

4.7 Multiple Sequence Alignment

A multiple sequence alignment (*Figure 15*) was generated to compare S-adenosylmethionine synthetase from AX55 and the 20 homologous that were retrieved [39–41]. The alignment was performed using the MUCSLE function in UGENE, and amino acid (AA) residues that were not identical to AX55's sequence were shown by a colored background [42]. The sequences were aligned to AX55 using the MUSCLE function in UGENE. The 403 residues of the mutant's SAM synthetase were found to be conserved amongst *S. maltophilia* D457 (402/403 residues), *S. maltophilia* JV3 (402/403 residues), *S, maltophilia sp.* WZN-1 (402/403 identical residues), *P. geniculata* N1 (402/403 residues), and *S. maltophilia* K279a (401/403 identical residues). The conserved regions and binding sites associated with SAM synthetase are: the N-terminal domain (AA residues 3-101; red box), central domain (AA residues 114-233; red box), C-terminal domain (AA residues 235-369; red box), metal binding sites (AA residues 17 and 43; green box), ATP binding sites (AA residues 15, 241, 264, and 268; purple box), methionine binding sites (AA residues 56, 99, 241, and 272; blue box), nucleotide binding regions (AA residues 166-168 and 232-233; yellow box), conserved sequence pattern 1 (AA residues 116-126 (G/N-A/S-G-D-Q-G-x-x-x-G-F/Y/H/G); pink box), and conserved sequence pattern (AA residues 261-269 (G-G/A-G-A/S/C-F/Y-S-x-K-D/E); pink box). The lone ambiguity between AX55 and the previously mentioned five sequences occurs at AA residue 384 (orange box) [40, 41, 43].


Stenotrophomonas maltophilia OR02-AX
Stenotrophomonas maltophilia D457
Stenotrophomonas maltophilia JV3
Stenotrophomonas sp. WZN-1
Pseudomonas geniculata N1
Stenotrophomonas maltophilia K279a
Stenotrophomonas rhizophila
Pseudoxanthomonas dokdonensis
Xanthomonas massiliensis
Stenotrophomonas panacihumi
Pseudoxanthomonas wuyuanensis
Xanthomonas floridensis
Xanthomonas pisi
Lysobacter arseniciresistens
Xanthomonas maliensis
Xanthomonas cassavae
Xanthomonas campestris
Xanthomonas cannabis
Xanthomonas arboricola
Xanthomonas oryzae
Escherichia coli K12


Stenotrophomonas maltophilia OR02-AX
Stenotrophomonas maltophilia D457
Stenotrophomonas maltophilia JV3
Stenotrophomonas sp. WZN-1
Pseudomonas geniculata N1
Stenotrophomonas maltophilia K279a
Stenotrophomonas rhizophila
Pseudoxanthomonas dokdonensis
Xanthomonas massiliensis
Stenotrophomonas panacihumi
Pseudoxanthomonas wuyuanensis
Xanthomonas floridensis
Xanthomonas pisi
Lysobacter arseniciresistens
Xanthomonas maliensis
Xanthomonas cassavae
Xanthomonas campestris
Xanthomonas cannabis
Xanthomonas arboricola
Xanthomonas oryzae
Escherichia coli K12

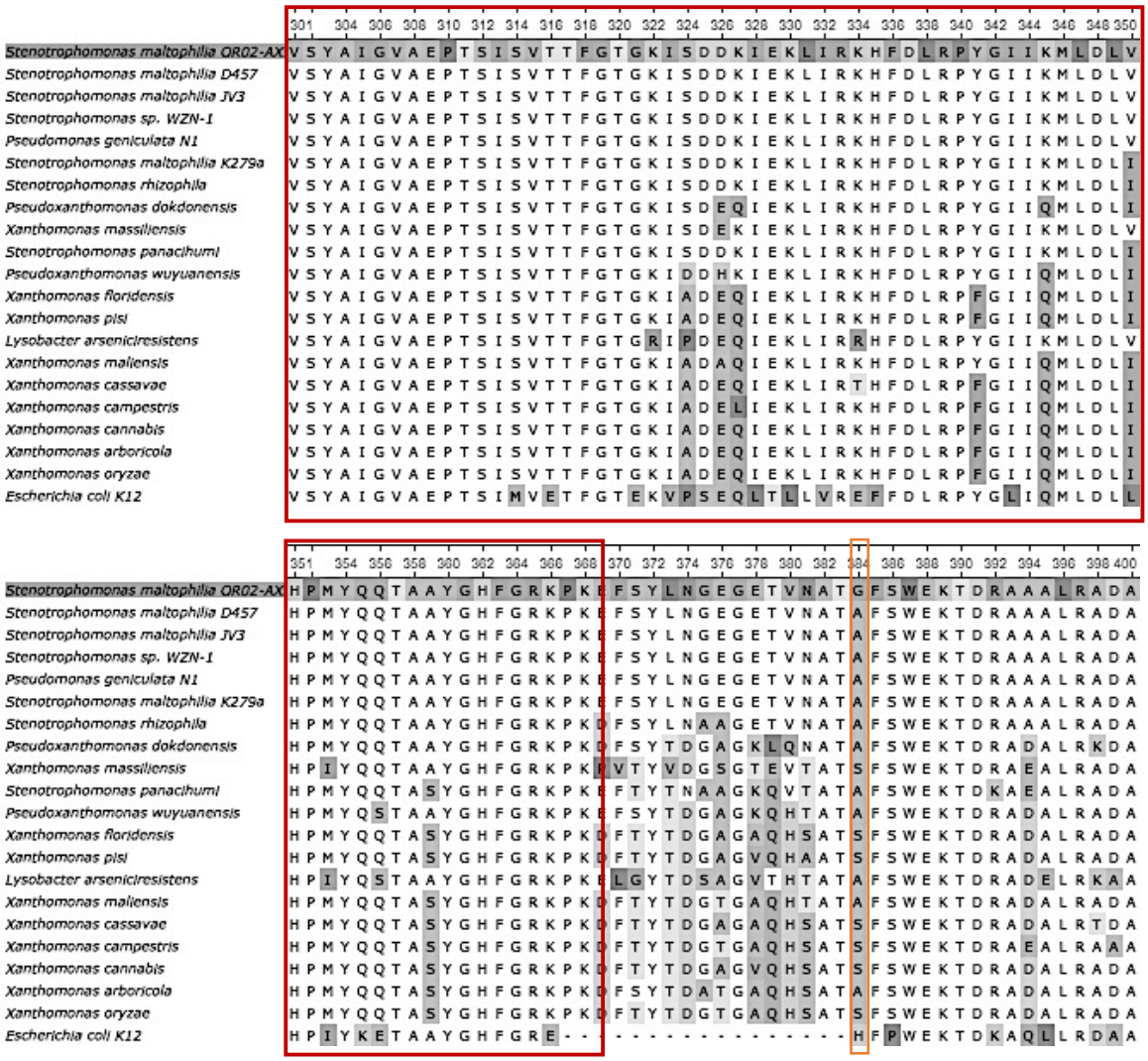


***Figure 15*:** Multiple Sequence Alignment [42].

The conserved regions and binding sites associated with SAM synthetase are: the N-terminal domain (red box), central domain (red box), C-terminal domain (red box), metal binding sites (green box), ATP binding sites (purple box), methionine binding sites (blue box), nucleotide binding regions (yellow box), conserved sequence pattern 1 (pink box), and conserved sequence pattern (pink box). The lone ambiguity between AX55 and the five sequences directly below is the substituted residue at 384 (orange box).

4.8 Phylogenetic Analysis

A phylogenetic tree (*Figure 16*) was constructed to compare the evolutionary relationship of SAM synthetase from AX55 with its homologs. The sequences were imported into MEGA7 and realigned by MUSCLE once again [44–46]. The maximum likelihood of the evolutionary history for each taxa was analyzed from 1000 bootstrap replicates. The percentages that each clade appeared in the 1000 bootstrap replicates is included. In all, the tree shows that the *S. maltophilia* 02 mutant is closely related to other *S. maltophilia* species.

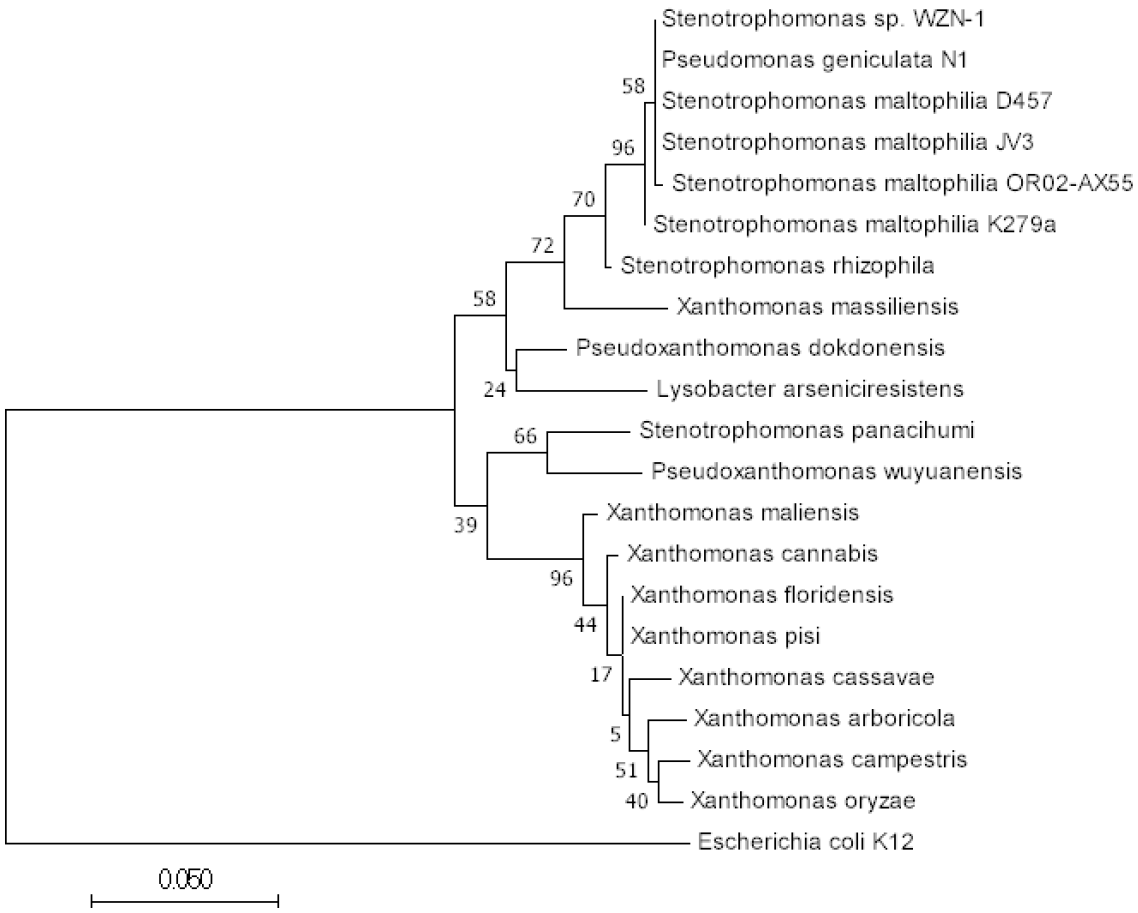


***Figure 16*:** Phylogenetic Analysis. The phylogenetic tree shows the evolutionary relationship of AX55 S-adenosylmethionine synthetase with closely related homologs. The tree is drawn to school and the branch lengths are measured by the number of amino acid substitutions per site. The bootstrap value is also included, showing the percentage each clade appeared out of the 1000 replicates [44–46].

Chapter V: Discussion

The detoxification of selenite in *Stenotrophomonas maltophilia* was previously shown to incorporate mechanisms of enzymatic reduction and methylation in the production of SeNPs (reduction only), DMSe, DMDSe, and DMSeS. Therefore, a selenite-sensitive mutant could be generated by interrupting a gene involved in the (1) GSH/GR reduction mechanism or (2) the proposed methylation by a thiopurine methyltransferase. The generated *S. maltophilia* OR02 mutant, AX55, was found to exhibit a lower MIC for selenite compared to the wildtype (*Figure 8.C*). Through the use of DNA sequencing and bioinformatic analysis, the transposon insert was located in the 5' untranslated region (UTR) of a putative *metK* gene. *metK* encodes S-Adenosyl-L-Methionine (SAM) synthetase, the enzyme that catalyzes the formation SAM.

In microbial detoxification mechanisms SAM serves as a methyl-donating substrate in the of volatilization of heavy metals. It should be noted that methyl-selenides exhibit a distinct garlic-like odor, which was not observed when AX55 was supplemented with sodium selenite. The presence of SeNPs at 1mM selenite without the production of methyl-selenide(s), thereby supports the methylation pathway (*Figure 5*) that requires the reduction of selenite to elemental selenium before a methyl-group is added. However, the inability of the mutant to produce SeNPs at each concentration that was tested suggests that SAM also functions in selenite reduction.

5.1 *S. maltophilia* OR02 may require SAM for GSH biosynthesis

The enzymatic reduction of selenite is largely dependent on the intracellular concentration of GSH and the production of oxidative stress proteins [9, 12, 19, 47–49]. Once selenite is transported into the cytoplasm the initial reduction by GSH is nearly

spontaneous. As hypothesized by Kessi and Hanselmann, subsequent reductions were found to result in an accumulation of superoxide anions that will initiate the cell's response to oxidative damage, thus corroborating the role of super oxide dismutase (SOD) in selenite reduction. The thiol containing proteins GSH and GR have an important role in protecting the cell from oxidative stress by reducing selenite and regenerating the reduced form of GSH. Further production of GSH is dependent on cysteine, the rate limiting substrate in the first reaction of GSH biosynthesis. If needed, SAM can be shuttled through the trans-sulfuration pathway for the production of cysteine, a precursor for GSH and glutathione reductase [17, 33–35].

In *Rhodobacter sphaeroides* (*R. sphaeroides*), the transformation of SAM to GSH was found to be essential for selenite resistance [50]. The mutant was generated by a point mutation within the coding region of *metK* that resulted in a 70% decrease of the intracellular SAM concentration. Furthermore, the selenite-sensitive mutant was also found to overexpress cysteine synthase (*cysK*) in response to inadequate amounts of cysteine and GSH within the cell. The overexpression of *cysK* has previously been associated with selenite resistance in *E. coli* [47] and *Enterobacter sp. YSU* [15]; however, selenite resistance is thought to occur from excess cysteine blocking the import of selenite into the cell. Following the introduction of a functional *metK* through genetic complementation in trans, the wildtype phenotype was restored in the mutant [50]. These results substantiate the claim that *metK* was the only gene affected by the transposon interruption of S02. However, unlike the *R. sphaeroides* mutant, the amino acid sequence of AX55 did not show any variations within the conserved regions of the protein (*Figure 15*). Therefore, *metK* may have been effected on the transcriptional level.

5.2 *metK*

The synthesis of SAM is strictly regulated at the level of transcription. In addition to feedback inhibition by SAH, transcription factors (TFs) and riboswitches that are associated with methionine metabolism control the genes of the SAM cycle. These regulatory elements vary not only between α, β, and γ-classes of proteobacteria, but bacterial species within each class as well [33–35, 51, 52]. In *S. maltophilia* (*Xanthomonadaceae* family), genes involved in the SAM cycle and the biosynthesis of homocysteine and methionine are controlled by the SAH-riboswitch, SamR (TF), and MetR (TF). The SahR-like homolog, SamR, is a metabolite-sensing repressor that was identified experimentally through comparative genomics. When regulating the SAM cycle, SamR acts as a transcriptional repressor to *metK* by binding to a region within the 5'UTR [51–53]. Because the transposon inserted into the same region as the TF binding site, the TF regulation of *metK* may have been affected. Upon locating the SamR binding motif downstream from the transposon insert, it appears to be fully intact. However, the short distance between the transposon insert and the TF binding site suggests that the transposon may have inserted ahead, or within the transcriptional promoter region. In *E. coli metK* is an essential gene for growth, and *metK* mutations have been shown to decrease methionine and SAM biosynthesis, DNA methylation activity, and cell division [30, 54–57].

5.3 Bioremediation

The characterization of microbial-based mechanisms for elemental cycling is leading towards advancements in the removal of heavy metals from the environment. In the San Joaquin Valley, there has been an increase in the total concentration of selenium in drinking water due to an agricultural drainage pond close by [58, 59]. The drainage

waters contain selenium at concentrations between 140-1400 μg/l, which is 10-100 times the concentration of natural waters. Moreover, these toxic levels of selenium are harmful to wildlife in the surrounding areas. In aquatic birds, selenite toxicity was found to vary, but was shown to have detrimental effects on ducklings beginning at concentrations as low as 20 ppm [60]. Therefore, identifying selenite-resistant microbes for the safe removal of the oxyanion from such waters is of great interest. Currently, the combined bioremediation of selenite with mercury and/or nitrate are being studied by multi-metal resistant species [61–64].

5.4 Future Work

The current project has identified a putative gene, *metK*, that is involved in the detoxification of selenite by *Stenotrophomonas maltophilia* OR02. The eventual *metK* gene product, SAM, is an importer substrate for selenite methylation and GSH biosynthesis. Since the transposon inserted into the 5' UTR of the gene, and not in the coding region, the mutant's sensitivity for selenite cannot definitively be attributed to an interruption of *metK*. RT-qPCR should be conducted to determine if *metK* is being transcribed, and to measure the level of transcription in response to selenite. Additionally, the protein expression can be analyzed using SDS-PAGE and tandem mass spectrometry (MS/MS). Lastly, functional *metK* could be transformed into AX55 to determine if the wildtype phenotype is restored. However, due to the multi-metal and –antibiotic resistances of *S. maltophilia* 02, a competent plasmid specific for this strain must be developed.

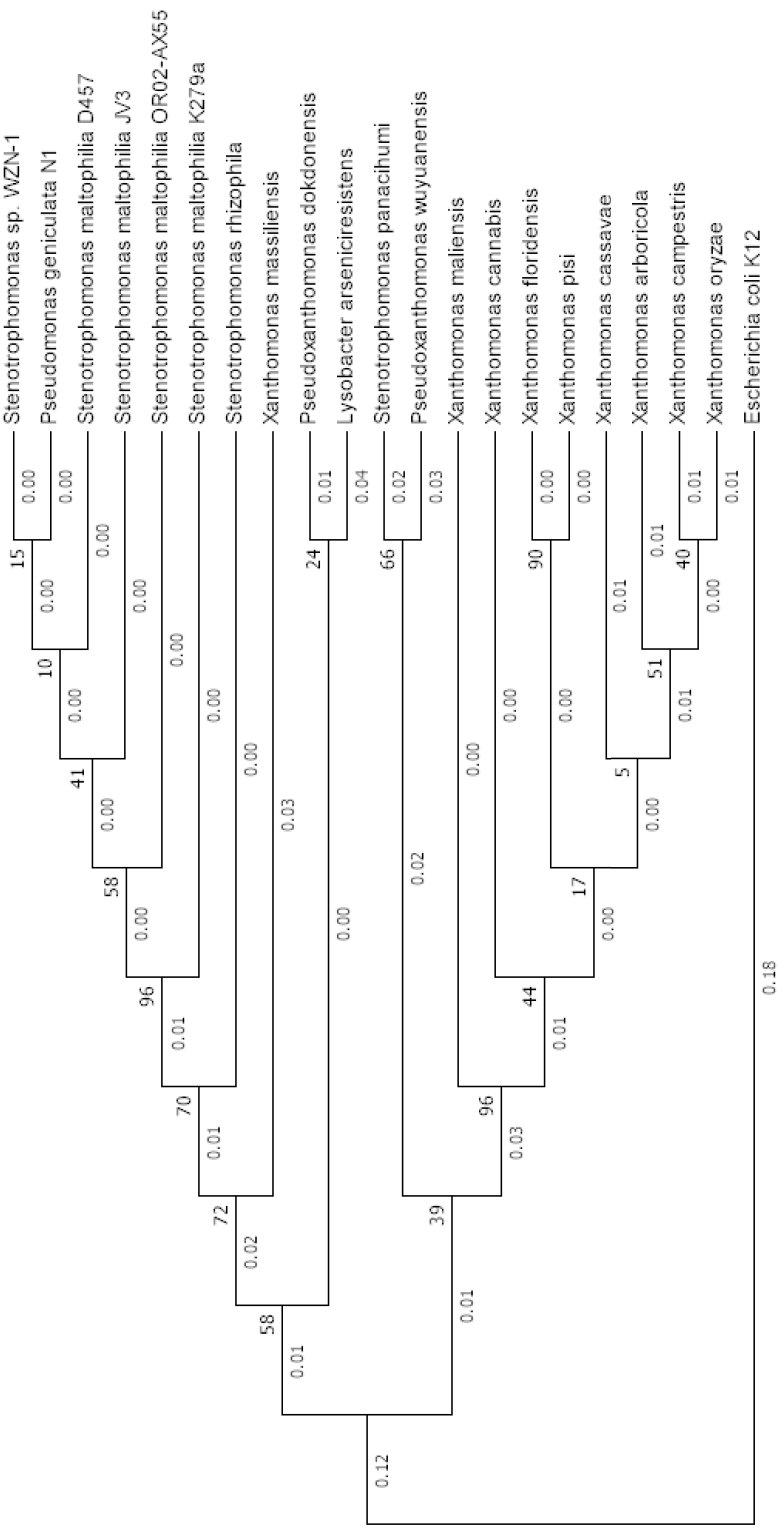
Chapter VI: Appendices

Appendix A: Accession Numbers of Bacteria for Phylogenetic Analysis.

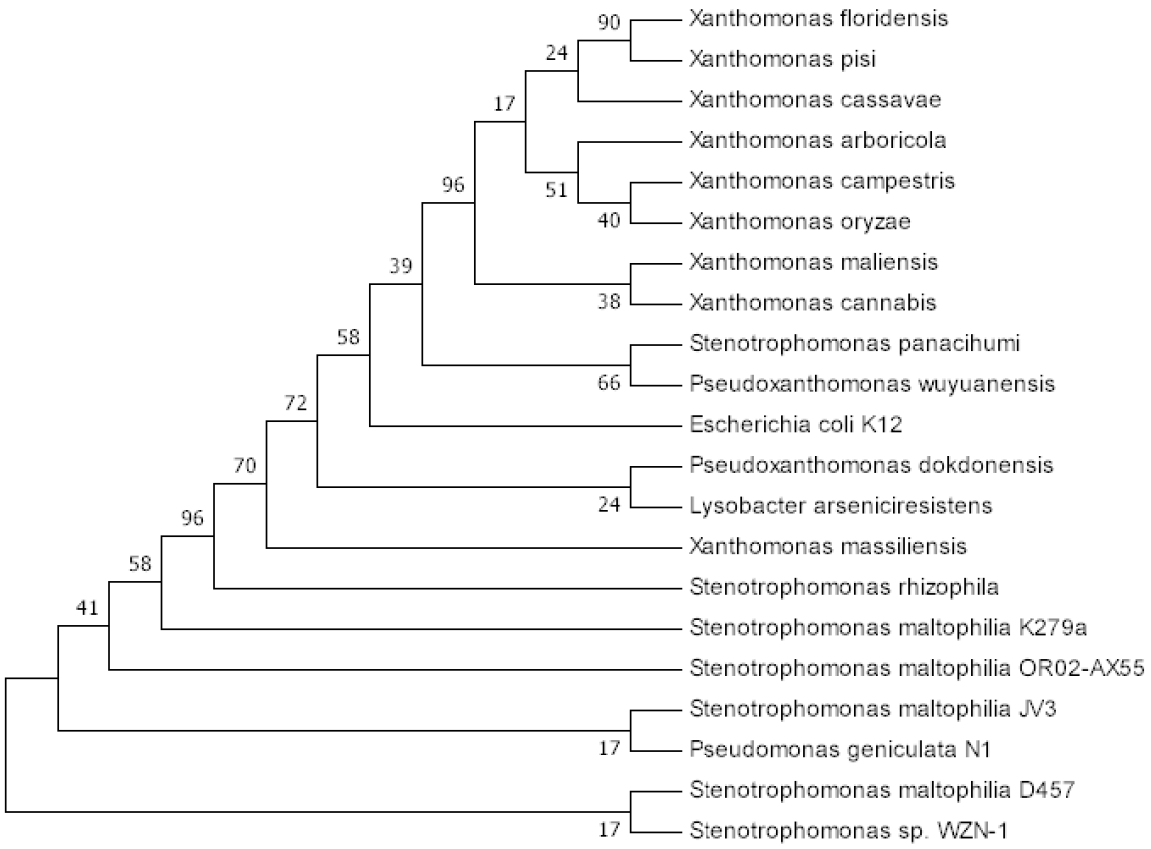
Species	**Accession Number**
Escherichia coli K12	CQR82379.1
Lysobacter arseniciresistens	WP_036207560.1
Pseudomonas geniculate N1	KOE97976.1
Pseudoxanthomonas dokdonensis	KRG70110.1
Pseudoxanthomonas wuyuanensis	SOD53373.1
Stenotrophomonas maltophilia D457	CCH11253.1
Stenotrophomonas maltophilia JV3	AEM49979.1
Stenotrophomonas maltophilia K279a	CAQ44356.1
Stenotrophomonas maltophilia OR02	Not Submitted
Stenotrophomonas panacihumi	KRG39248.1
Stenotrophomonas rhizophila	AHY60275.1
Stenotrophomonas sp. WZN-1	ARZ73286.1
Xanthomonas arboricola	WP_039530648.1
Xanthomonas campestris	WP_011269998.1
Xanthomonas cannabis	WP_047694400.1

Xanthomonas cassavae	WP_029219094.1
Xanthomonas floridensis	OAG67912.1
Xanthomonas maliensis	WP_022973174.1
Xanthomonas massiliensis	WP_066097824.1
Xanthomonas oryzae	WP_014502025.1
Xanthomonas pisi	WP_046962641.1

Appendix B: Phylogenetic tree expanded [44–46].



Appendix C: Phylogenetic Tree with Bootstrap Replicates [44–46].



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