AmpG is involved in the Regulation of L1 and L2 Penicillin resistant genes in

***Stenotrophomonas maltophilia* Oak Ridge Strain 02**

By

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Signature

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Abstract

Stenotrophomonas maltophilia Oak Ridge strain 02 (*S. maltophilia* 02) was isolated from East Fork Poplar Creek, a heavy metal contaminated stream in Oak Ridge, Tennessee. In addition to being resistant to metal salts of mercury, cadmium, zinc, copper, gold, chromium and arsenic, it is also resistant to the antibiotic, ampicillin. Transposon mutagenesis generated an ampicillin sensitive mutant with an interruption in the *ampG* gene. Previous work showed that the AmpG protein is a transmembrane permease found in *S. maltophilia* bacteria and moves the byproducts of murein sacculus breakdown from the periplasm into the cytosol. Transportation of degraded products by AmpG triggers expression of L1 and L2 β-lactamases. To determine if AmpG is involved in expressing L1 and L2 in *S. maltophilia* 02, the *S. maltophilia* 02 mutant and wild type strain were exposed to ampicillin during mid-log phase. RNA samples were collected before adding ampicillin and 90 minutes after adding ampicillin, with a 30-minute interval between each sample. The samples were purified and converted to cDNA. Results from RT-PCR revealed that the transcription level of *ampG* were reduced in the mutant strain compared to the wild type which in turn decreased the expression level of L1 and L2 penicillin resistant genes in the mutant. The mutation in the *ampG* gene decreased the expression levels of both ampicillin resistance genes in the mutant.

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CHAPTER 1

1.0 Introduction

1.1 Y12 plant at East Fork Poplar Creek

Tennessee's Y-12 plant was established to make nuclear weapons during World War II. The facility processed uranium that was used to fabricate nuclear weapons. As a result of the rising demand for weapons and threats from the USSR, the facility switched to processing lithium isotopes to create hydrogen bombs. The processing of lithium isotopes produced approximately 920,000 kg of mercury waste which was spilled into the nearby East Fork Poplar Creek and the surrounding environment (Brooks & Southworth, 2011).

Four unlined S-3 ponds were used to store waste from the Y-12 plant, and the adjoining East Fork Poplar Creek eventually became contaminated. In addition to acidic uranium nitrate waste from the Y-12 plant, the ponds also received waste from other sources such as East Tennessee Technology Park, and X-10 sites in Oak Ridge, Savannah River site, Idaho National Engineering Lab (Brooks & Southworth, 2011).

These sites contaminated East Fork Poplar Creek with aluminum, fluorine, potassium, sulfate, technetium-99 and plutonium-239. In 1983, dumping of waste in ponds was discontinued and the S-3 ponds were treated with neutralization and bio-denitrification processes. The ponds were capped and serve as parking lots.

Different sources have evaluated waste effects, levels, and clean-up efforts in East Fork Poplar Creek to assess the risk to wildlife and people living in the surrounding areas. One study has

concluded that there is no public risk of uranium exposure through the contaminated site (Moore et al., 1999).

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

S. maltophilia Oak Ridge strain 02 (*S. maltophilia* 02) was isolated from the Y12 plant at East Fork Poplar Creek. It is a gram-negative soil bacterium that grows well at 30°C. The multi-metal resistant bacterium grows in the presence of toxic levels of mercury, lead, copper, zinc, platinum, gold, arsenic, chromium and selenium salts (Holmes et al., 2009). The strain consists of MerR family proteins that regulate the expression to metal salts resistance (Baya et al., 2021). The resistance-mechanism used by some bacteria is detoxifying the salts and converting them to insoluble precipitates (Gupta and Diwan, 2017).

1.2.1 *Stenotrophomonas maltophilia*

The bacterium, *S. maltophilia*, was discovered in 1958 and given the name *Pseudomonas maltophilia*. The genus of this bacterium was questioned by subsequent research, and it was then reclassified as *Xanthomonas maltophilia* until a new genus type, *Stenotrophomonas*, was designated to correctly identify the bacterium as *S. maltophilia* (Palleroni and Bradbury, 1993).

S. maltophilia is a gram-negative, bacillus with cells that can be straight or slightly curved and range in length from 0.5 to 1.5 μm. It is a motile, obligate aerobe that cannot grow at temperatures below 5°C or above 40°C, and it has a few polar flagella. The optimal temperature for growth is 35°C. Methionine or cysteine is required for growth for almost all strains. Although new data suggests that some isolates may be oxidase positive, the organism is oxidase negative. It differs from *Pseudomonas aeruginosa*, which produces acid from glucose, in that it produces acid from maltose instead of glucose (Carmody et al., 2011).

The bacterium inhabits plant rhizomes, animals and water sources. It has also been found in biofilms on fractured surfaces in aquifers, and it has the ability to adhere to plastics and form biofilms (Brooke, 2012). It can develop biofilms in showerheads and potable water distribution systems, putting immunocompromised individuals at risk of infection (Feazel et al., 2009; Hoefel et al., 2005). It can also create biofilms on surfaces like Teflon, glass, and host tissues. About 65% of nosocomial illnesses have been associated with a biofilm formation. Adhesion and biofilm development utilize fimbriae (De Oliveira-Garcia et al., 2003)

To isolate *S. maltophilia*, several selective mediums have been devised. One of these is *Xanthomonas maltophilia* selective medium, which was developed to keep it isolated from habitats like soil and the rhizosphere (Juhnke ME., et. al, 1989). This medium comprises two antifungal medications; nystatin and cycloheximide, six antibacterial agents; cephalexin, bacitracin, penicillin G, novobiocin, neomycin, and tobramycin, as well as maltose and bromothymol blue.

S. maltophilia inhibits the growth of plant pathogens (Berg et al., 1996). These factors led to its usage in the development of biopesticides, and it is currently being researched for biological plant pathogen control. It has the ability to digest xenobiotic substances and metabolize high molecular weight polycyclic aromatic hydrocarbons, making it a viable tool for soil decontamination (Brooke, 2021).

S. maltophilia is frequently associated with pneumonia, acute and chronic obstructive pulmonary disease, bacteremia, biliary sepsis, endophthalmitis, endocarditis, meningitis, and obstructive lung cancer. It has been found in soft tissue infections as well as infections of the eyes, urinary tract, bones and joints (Brooke, 2012; Falagas et

al., 2009; Looney at al., 2009). The most prevalent illnesses caused by *S. maltophilia* are bacteremia, which is typically caused by an indwelling catheter, and pneumonia, which is caused by a ventilator. The severity of bacterial infections in people with cystic fibrosis increases roughly by one-third in people who have *S. maltophilia* in their respiratory tract. Patients are more susceptible to *S. maltophilia* infection if they have an underlying malignancy, a catheter, chronic respiratory disease, a weak immune system, prior use of antibiotics or a long-term hospital stay (Looney et al., 2009)

Studies have shown that *S. maltophilia* is the cause of a wide range of nosocomial disease (Brooke, 2014)These bacterial infections are challenging to treat due to the elevated mutation rate and multi-drug resistance (MDR) characteristics of *S. maltophilia*. Trimethoprim-sulfamethoxazole was once a successful treatment for *S. maltophilia* infections, but antibiotic resistance is growing. The rise in fatality rates from these infections alone suggests that nosocomial strains are becoming more resistant to conventional treatments.

Antimicrobial agents that have combined therapeutic aspects, novel agents and aerosol-form have a higher chance of reducing *S. maltophilia* multi-drug resistance (Chang et al., 2015)

1.3 Antibiotics

A wide range of antibiotic classes, such as penicillins, and cephalosporins, are intrinsically ineffective against *S. maltophilia*. Penicillin antibiotics have a high therapeutic index and focus on the bioactivity of a bacterium's cell wall production in gram negative bacteria (Fisher and Mobashery, 2020). The majority of penicillins, including penicillin G, benzylpenicillin, ampicillin and carbenicillin are 6-aminopenicillanic acid derivatives that vary from one another depending on the side chain joined to the amino group.

The most widely used antibiotics are β -lactams. There are 4 classes of β -lactam antibiotics which include: penicillins, cephalosporins, carbapenems and monobactams (Tooke. et. al, 2019). The molecule of β-lactam ring prevents cross-linking of peptides in the cell wall of bacteria. Penicillinases, also known as β-lactamases, are enzymes that are produced by a large number of penicillin-resistant bacteria and that inactivate the antibiotic by hydrolyzing a bond in the β-lactam ring (Kong et al., 2010).

1.3.1 Ampicillin

Ampicillin is a semi-synthetic β-lactam antibiotic that belongs to Penicillin. This antibiotic is active against Gram-negative and Gram-positive bacteria. An amino substituent group is connected at the α-carbon atom of its structure which adjoins the β - lactam ring (Waxman and Strominger, 1980). Ampicillin is acid stable and prevents the formation of bacterial cell walls while bacteria are actively growing. β -lactamase produced by some bacteria cleave the β -lactam ring thus inactivating the function of ampicillin. Adjustments have been made to β -lactam antibiotics by introducing β - lactamase inhibitors (Kim D., et. al 2023; Penwell et al., 2015).
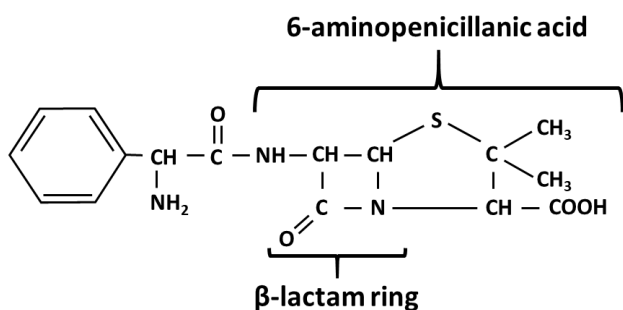
Figure 1. Structure of ampicillin.

1.3.2 Carbenicillin

Carbenicillin also belongs to penicillin and has a broad spectrum of activity on Gram-negative and Gram-positive bacteria (Butler et al., 1970). The special feature of its structure is the carboxylic-acid substituent on the α-carbon atom which renders it acid stable.

6-aminopenicillanic acid
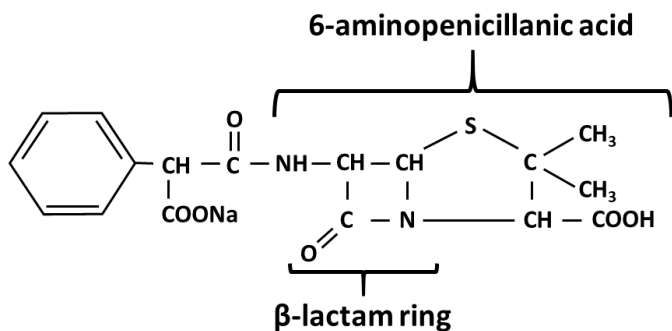
β-lactam ring

Figure 2. Structure of carbenicillin

1.3.3 Cefoxitin

Cefoxitin is a second-generation cephalosporin that contains a β-lactam ring similar to that in penicillin (Birnbaum et al., 1978). Its usefulness is based on the specific 7α-methoxy modification of their β-lactam ring, which makes them fairly resistant to the action of most β-lactamases. However, the class C molecular type β-lactamases are capable of readily hydrolyzing cefoxitin (Philippon et al., 2002).

7-aminocephalosporanic acid

β-lactam ring
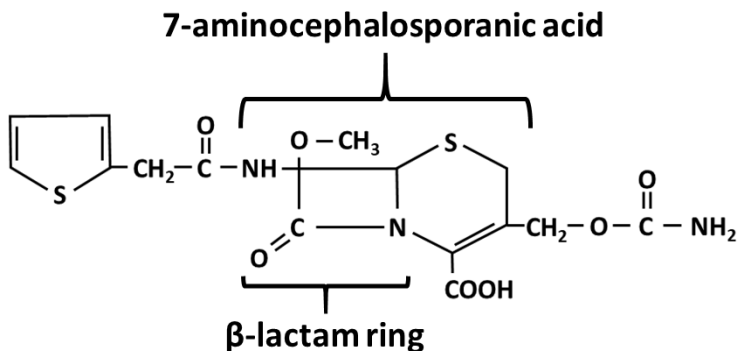
Figure 3. Structure of cefoxitin.

1.4 L1 and L2 B-lactamase in *S. maltophilia*

β-lactamases are enzymes that hydrolyze β-lactam antibiotics, with over 2000 β-lactamases identified (Bonomo, 2017). They are classified according to protein sequences into class A, B, C and D (Bush et al., 1995). Class A, C and D are serine-dependent for β-lactam hydrolysis and class B are zinc dependent metalloenzymes. These lactamases are further classified into 3 groups. Group 1 are class C cephalosporinases, group 2 consist of class A and D broad-spectrum, inhibitor-resistant and extended-spectrum β-lactamases and serine carbapenemases. Group 3 are class B metalloenzymes (Bush and Jacoby, 2010).

It has been proposed that *S. maltophilia* uses its metabolic system to detoxify and break down dangerous substances like antibiotics, which might then be utilized as a source of nutrients. Plasmids, transposons, integrons, integron-like elements, insertion element common region elements, and biofilms are examples of horizontally transferred molecular processes that contribute to its resistance (Partridge, S. R., et.al 2018).

There are two types of β-lactamase genes in *S.maltophilia* that confer resistance to β-lactam antibiotics. These are L1 and L2 β-lactamases. L1 is a holoenzyme which belongs to class B β-lactamases. It is a Zn^{2+}-dependent metalloenzyme consisting of tetraemer of four equal subunits (Palzkill, 2013). It hydrolyzes all of the β-lactam classes (penicillins, cephalosporins and carbapenems) except monobactams (Bahr et al., 2021). It has a high efficiency to hydrolyze ampicillin (Yamada, K. et.al 2024) and is not susceptible to β-lactamase inhibitors like clavulanate but it is inhibited by

aztreonam. L2 exists as a dimer and contains a class A serine active-site and clavulanic acid-sensitive cephalosporinase. It also hydrolyzes aztreonam and it is susceptible to clavulanate, a β-lactamase inhibitor (Bush K. 2018, Huang et al., 2018).

A complete *ampN-ampG* operon is required for the expression of L1 and L2 β-lactamaes. The operon is contranscibed by the promoter P$_{ampN}$ and operates independently of β-lactam inducers. When the *ampN* gene is disrupted, it causes a polar effect on the expression of *ampG,* thereby affecting the regulation of *ampG* (Huang, Y et al 2010). The *ampG* gene is located downstream *ampN* and encodes a transmembrane protein that transports degraded cell wall components from the periplasm into the cytoplasm for recycling. The activation of L1 and L2 genes is hypothesized to be controlled by the *ampR-ampDI-ampN-ampG* network, although this pathway is not well understood (Huang et al., 2010). AmpR is induced by AmpD, an amidase which hydrolizes the anhMurNAc-peptide for further recycling. The gene for AmpR is found upstream of L2 as part of an *ampR*-L2 module. The LysR-type *ampR* regulator is located adjacent to L2 which regulates its gene expression. L1 is an *ampR*-unlinked class B β-lactamase gene. The basal-level expression of L1 requires AmpR and it is also needed for the induced expression of both L1 and L2. The AmpR-L2-β-lactamase module has a divergently transcribed control unit which is where the AmpR protein regulates the β-lactamase gene expression. The regulator represses L2 expression in the absence of a β-lactam inducer and activates both L1 and L2 in the presence of the inducer (Avison et al., 2002; Okazaki et al., 2008; Lin et al., 2009).

In Citrobacter freundii, muropeptide recycling induces *ampR-ampC* type system (Lindberg et al., 1985). AmpC which belong to class C β-lactamase is controlled by other genes including *ampG, ampD, ampR* and PBP4. AmpR, a transcriptional regulator of *ampC*

expression is regulated by two cell wall products. When AmpR is bound with anhMurNAc-peptide, AmpC expression is activated. Conversely, when AmpR is bound with UDP-*N*-acetylmuramic acid-pentapeptide, the expression of AmpC is repressed.

1.4 Transposon Mutagenesis

The EZ-Tn*5*™ <*R6Kγori*/KAN-2>Tnp Transposome™ is a DNA/transposon protein complex (Hoffman et al., 2000; Goryshin et al., 2000; Metcalf et al., 1994). It contains a gene for kanamycin resistance, an *R6Kγ* replication origin and two mosaic ends which are DNA sequences for transposase binding. When introduced into *S. maltophilia* 02 by electroporation, the transposome incorporates itself randomly into the bacterium's genome.

Transposon mutagenesis was used to isolate an ampicillin sensitive *S. maltophilia* 02 strain, AJ22. MIC experiments showed that AJ22 was sensitive to ampicillin, carbenicillin and cefoxitin. The genome of *S. maltophilia* 02 was sequenced (accession number CP056088) and Basic Local Alignment Search Tool (BLAST) analysis revealed that the gene interrupted the *ampG* gene (Fig. 4) (Richiutti, 2016), which encodes a putative AmpG protein and is involved in the transportation of a degraded cell wall components (Kong et al., 2010).
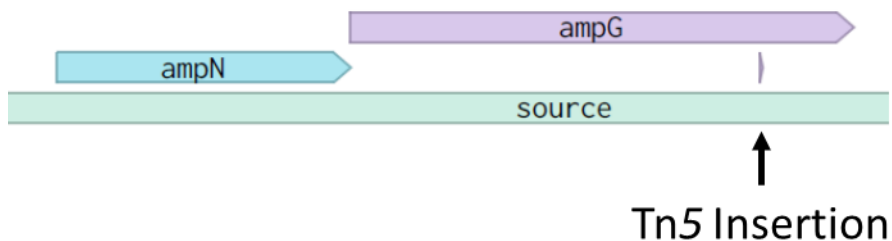
Figure 4. Picture of the *ampN/ampG* genes in the chromosome of *S. maltophilia* 02 AJ22 mutant. The arrow shows where the Tn*5*-transposon inserted itself into the *ampG* gene.

1.5 Hypothesis

In this study, the wild type *S. maltophilia* 02 strain and the AJ22 mutant were grown overnight in the absence and presence of ampicillin. Cultures were incubated at 30˚C in a roller drum while taking NanoDrop reading at 600 nm for 30 minutes. Cells were harvested using RNA protect bacterial reagent 90 minutes before adding ampicillin and 120, 150 and 180 minutes after adding ampicillin. RNA samples were purified using a Qiagen RNeasy Minikit.

The RNA samples were converted to complementary DNA (cDNA) using reverse transcriptase from the New England Labs Protoscript II kit. PCR primers designed for the L1, L2, *ampG* and GAPDH genes were used in polymerase chain reactions (PCR) to detect expression in the wild type and mutant strains grown in the presence and absence of the ampicillin. If AmpG is involved in the expression of the L1 and L2 β -lactamase antibiotic resistance genes, PCR products from the wild type strain will have a higher expression level compared to the mutant strain.

1.6 Objectives

The objective of the study was to use the AJ22 mutant to see if the AmpG protein regulates the expression of the L1 and L2 genes.

Objective/Aims

1)   Generate growth curves for the mutant wild type strain in the presence and absence of ampicillin.

2)   Purify RNA from the wild type and mutant strains that have been grown in cultures containing and lacking ampicillin.

3)   Perform RT-PCR using primers specific for the L1 and L2 penicillin-resistance

genes.

CHAPTER 2

2.0 Materials and Methods

2.1 Bacterial strains

The S. maltophilia 02 strain was obtained from the American Type Culture Collection (ATCC #53510, Manassas, VA) and stored in a freezer at -80 °C. An ampicillin-sensitive strain of *S. maltophilia* 02 (AJ22) was created in the laboratory using transposon mutagenesis (Ricchiuti 2016, unpublished data).

2.2 Growth medium

LB broth (Lennox) was made according to the protocols provided by the Hardy Diagnostics manufacturer's manual (Anderson et al., 2005). Lysogeny broth (LB) broth contained 10 g/l tryptone, 5 g/l yeast extract, and 5 g/l sodium chloride. 16 g agar bacteriological AMRESCO (Solon, OH) was added to LB broth, supplemented with 50 µg/ml kanamycin AMRESCO (Solon, OH) when required.

2.3 Overnight culture/Test for AJ22 phenotype

A single colony of *S. maltophilia* 02 and AJ22 strains were suspended in 3 ml of LB broth. The AJ22 mutant was grown in LB supplemented with 800 µg/ml kanamycin sulfate (Ricchiuti 2016, unpublished data). The strains were grown overnight in a roller drum at 30°C.

2.4 Minimal inhibitory concentration (MIC)

50 µl *S. maltophilia* 02 and AJ22 strains from an overnight culture were suspended in 5 ml LB broth to make a cell dilution ratio of 1:100. Both strains were tested for antibiotic resistance in various concentrations of ampicillin as follows: 0 µg/ml, 100 µg/ml, 200 µg/ml, 1000 µg/ml and 2000 µg/ml. The cell cultures were grown overnight in a roller drum at 30°C.

Growth was measured as a function of turbidity using a cuvette in a NanoDrop spectrophotometer 2000 (Thermo Scientific) to determining the absorbance of the culture at a wavelength of 600 nm before and after 24 hours.

2.5 Bacterial growth curves

50 µl of the bacterial cells were suspended in four 5 ml LB broth. The cells were incubated in a roller drum at 30°C for 300 minutes. A volume of 100mg/ml of ampicillin was added to experimental samples at 90 minutes, and an equal volume of water was added to the control samples. 200 µl of *S. maltophilia* 02 and 350 µl of AJ22 bacterial cell samples were taken for RNA extraction at 90 minutes, immediately before adding ampicillin. Three additional samples were taken 30 minutes, 60 minutes and 90 minutes after ampicillin was added. 1 ml of cell samples was taken after 300 minutes for DNA extraction. Turbidity was determined by measuring absorbance at 600 nm in a Nanodrop spectrophotometer every 30 minutes. The procedure was repeated 15 times.

2.6 Student T-Test statistical analysis for growth curves

Microsoft Excel was used to calculate the average turbidity at 600 nm at each time point and to plot the average turbidity VS time for each growth curve. A T-test formula for two-tailed distribution was used to calculate the standard error using the following formula:

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

Where:

N is number of observation of cell counts

CI is the confidence interval

d.f is the degrees of freedom

stdDev is the standard deviation

Error is represented as error bars for each time point

2.7 Genomics preparations

The wild-type and mutant DNA was extracted using Promega's Wizard® Genomic Purification System (Madison, WI). 1 ml of bacteria was pelleted for 2 minutes at 14,000 rpm in a centrifuge. The cells were resuspended in 600 µl of nuclei lysis solution immediately by gently pipetting the solution up and down. The sample was heated at 80°C for 5 minutes and cooled to room temperature. 3 µl of RNase solution was added, and the lysate was incubated at 37°C for 15 minutes. The lysate was mixed with 200 µl of protein precipitation solution and vortexed for 20 seconds at high speed. The lysate was incubated for 5 minutes on ice and centrifuged for 3 minutes at 14,000 rpm. The supernatant was poured into a new 1.5 ml microcentrifuge tube containing 600 µl of isopropanol, gently mixed by inversion and centrifuged for 2 minutes at 14,000 rpm. The supernatant was poured off, and the tube was drained on a paper towel. 600 µl of 70% ethanol was added and centrifuged for 2 minutes at 14,000 rpm. The supernatant was discarded and the tube was air dried for 10-15 minutes at room temperature. The purified DNA was resuspended in 100 µl of rehydration solution and further dissolved in the refrigerator at 4°C for 24 hours.

2.8 Gel Electrophoresis

One sachet of TBE buffer, 10x Ready-Pack^{TM} (Amresco, Solon, OH), was added to 1000 ml of water and stirred to mix the solution. The 10X TBE solution was diluted to make a 1X solution which contained 0.089 M Tris base, 0.089 M Borate, and 0.002 M EDTA. 1% agarose was made by adding 2.5 g of BioExcell® Agarose LE (Worldwide Medical Products, Bristol, PA) to 250 ml of 1X TBE buffer. The solution was heated in a microwave and stirred after every minute until the solution boiled uniformly to produce bubbles. 25 µl of GelGreen dye (Embi Tec, San Diego, CA) was added to the agarose gel and stirred. The gel was poured into a RunOneElectrophoresis tray, and a comb was inserted into the warm gel to create wells.

3 µl of PCR samples were loaded on a gel powered by the RunOneElectrophoresis Cell and separated at 100 volts for 25 minutes. The gel was removed from the electrophoresis cell and observed using UV light.

2.9 RNA extraction Protocol

RNA was extracted using RNAprotect® Bacterial Reagent (Qiagen, Germantown, MD, USA) 200 µl of the *S. maltophilia* 02 cells and 350 µl of AJ22 cells were mixed with 400 µl and 700 µl of Qiagen RNAprotect® Bacterial Reagent, respectively. The solution was centrifuged at low speed at 5000 Xg. The supernatant was decanted and the cells were stored at -20°C before RNA purification.

RNA purification solution was prepared by adding 100 µl of TE buffer (30 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg/ml of lysozyme (Research Products International,Company, City, State) to 10 µl of Qiagen proteinase K. Thawed bacterial

cells were resuspended in 110 µl of the RNA purification solution by pipetting up and down. The mix was incubated at 25°C and vortexed for 10 seconds every 2 minutes.

The cells were suspended in 350 µl of buffer RLT containing 10 µl of β-mercaptoethanol and centrifuged ≥10,000 rpm for 2 minutes. The supernatant was transferred to a new 1.5 ml micro-centrifuge tube and mixed with 250 µl of 100% ethanol before being loaded onto a spin column. RNA was bound to the column by centrifugation for 15 seconds at ≥10,000 rpm. 700 µl of RW1 buffer was added to the RNeasy spin column and centrifuged for 15 seconds at ≥10,000 rpm, and the flow through was discarded. 500 µl of RTE buffer was added to the spin column and centrifuged for 15 seconds at ≥10,000 rpm. This step was repeated and centrifuged for 2 minutes to wash the spin column membrane. The empty spin column was centrifuged at ≥10,000 rpm for 1 minute to remove all wash solution. The RNeasy spin column was placed in a new 1.5 ml. To elute the RNA, 30 µl of RNase-free water was added directly to the spin column and centrifuged at ≥10,000 for 1 minute. The elution process was repeated.

In DNase treatment, 6 µl of 10X TURBO DNase buffer and 1 µl of TURBO DNase were added to the eluted RNA. The mixture was gently flicked to ensure proper mixing and then incubated at 37°C for 20 minutes. 7 µl of DNase inactivation reagent was added and mixed by pipetting. The reaction was left to incubate for an additional 5 minutes at room temperature, after which it was centrifuged for 1.5 minutes. The resulting supernatant, containing the purified RNA, was carefully transferred to a new RNase-free tube. The RNA concentration was subsequently measured using a NanoDrop spectrophotometer. The purified RNA was then stored at -80°C.

2.10 cDNA synthesis

RNA was converted to cDNA using an NEB ProtoScript® II First Strand cDNA Synthesis Kit (Ipswich, MA). RNA samples were thawed. The concentration was standardized by taking 100 ng/µl of each sample. 2 µl of Random primer mix was added to the RNA samples and topped up the remaining volume with nuclease free water to make a total of 8 µl. The mixture of RNA and random primer mix was heated for 5 minutes at 65°C and placed on ice. 10 µl of ProtoScript II Reaction Mix and 2 µl of ProtoScript II Enzyme Mix were added to the samples. The control tube contained 2 µl of Random primer mix, 6 µl of nuclease free water, 10 µl of ProtoScript II Reaction Mix and 2 µl of nuclease free water. The mixture was run on a thermocycler for 1 cycle of 25°C for 5 minutes and 42°C for 60 minutes.

2.11 GoTaq Polymerase Chain Reaction (PCR)

L1, L2 and *ampG* Primers were ordered from Integrated DNA Technologies and diluted with TE buffer to 100 µM. 4 µl of diluted primer was added to 96 µl of nuclease free water to make the primer solution to 10 µM. The PCR reaction mix contained 10 µl of 2X Go Taq, 2.5 µl of 4 µM forward primer, 2.5 µl of 4 µM reverse primer, 4 µl of nuclease free water and 1 µl of extracted DNA and cDNA from *S. maltophilia* 02 and AJ22.

The PCR reactions were incubated in a thermocycler for 30 cycles. The PCR program was set to a denaturing temperature of 95°C for one minute, annealing temperature of 60°C for one minute and elongation temperature at 72°C for 30 seconds.

Table 1. List of primers

Primer	Sequence	Purpose
L1_RT-PCR_F	5'-GGT AGC AAC GAC CTG CAC T-3'	PCR and RT-PCR amplification
L1_RT-PCR_R	5'-GGT ATG CCC TGG CAT GAA GT-3'	PCR and RT-PCR amplification
L2_RT-PCR_F	5'-ACG ATC ATC ACC AGC GAC AA-3'	PCR and RT-PCR amplification
L2_RT-PCR_R	5'-TGT TCA GTT CCA GG-3'	PCR and RT-PCR amplification
AmpG_RT-PCR_F1	5'-CAG CAC ATT GTC GTG TTC GG-3'	PCR and RT-PCR amplification
AmpG_RT-PCR_R1	5'-ATG AGG TCA GCA GTC CAA GC-3'	PCR and RT-PCR amplification
AmpG_RT-PCR_F2	5'-ATA CAC CGC AAC CCA GTA CG-3'	PCR and RT-PCR amplification
AmpG_RT-PCR_R2	5'-AAG AAC AGC GCG TAG CCT T-3'	PCR and RT-PCR amplification
S02_GAPDH_F	5'-AAACCGCGCAGAAGCACATCGA-3'	PCR and RT-PCR amplification detection of controls
S02_GAPDH_R	3'-GCCGGCGTAGGTCTTGTCGTTC-5'	PCR and RT-PCR amplification detection of controls

CHAPTER 3

3.0 Results

3.1 Growth curve of *S. maltophilia* 02 and AJ22

     (Ricchiuti, 2016) showed that the AJ22 mutant of *S. maltophilia* 02 is sensitive to ampicillin. Growth curves of the wild type and AJ22 strains in the presence of 100 µg/ml ampicillin further defined this sensitive phenotype (Fig. 5). Single colonies of the wild-type control and mutant strains were grown in LB broth for 18 hours at 30°C. Kanamycin was added to the AJ22 mutant to maintain the transposon insertion in the mutant, while distilled water was added to the wild strain. A 1:100 dilution of each overnight culture was diluted into fresh LB medium, and the cells were grown for 300 minutes at 30°C. Optical density at 600 nm was measured at 30 minute intervals. After 90 minutes of growth, a volume of 100mg/ml ampicillin was added to the control tubes of the wild type and mutant strain. An equal volume of water was added to the no ampicillin controls of each strain. Figure 1 plots the turbidity vs. time. The wild-type strain in the absence of ampicillin started with an average initial turbidity of 0.05 at time 0 and demonstrated exponential growth to a turbidity of 0.8 at 270 minutes. The AJ22 mutant in the absence of ampicillin started with an initial turbidity of 0.02 and also demonstrated exponential growth to a turbidity of 0.69. The AJ22 mutant in the presence of ampicillin demonstrated a curve that was statistically similar to the untreated culture until 120 minutes of growth. Then, the curve flattened to an O.D. of 0.1.
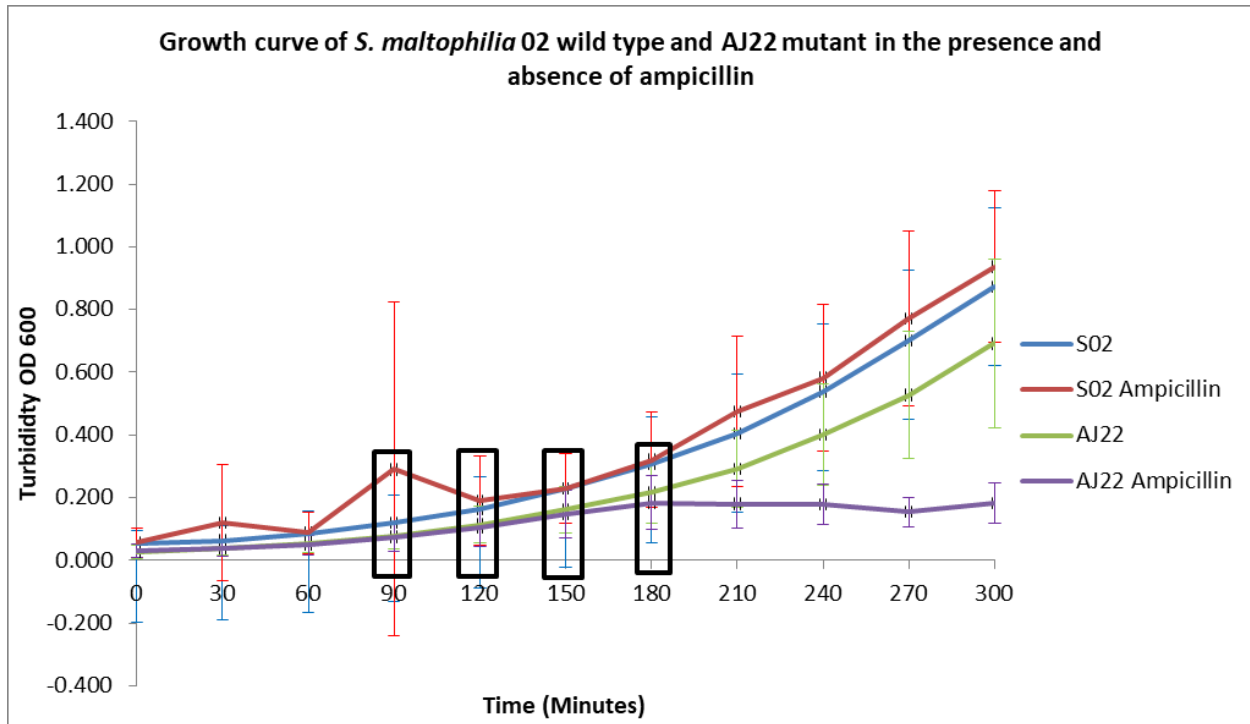
Figure 5. Growth curve of *S. maltophilia* 02 wild type and AJ22 mutant in the presence and absence of ampicillin. Blue curve: *S. maltophilia* 02 without ampicillin, Dark red curve: *S. maltophilia* 02 with ampicillin, light green line: AJ22 without ampicillin, purple line: AJ22 with ampicillin. Samples for RNA purification were taken at times 90 minutes, 120 minutes, 150 and 180 minutes as shown by the black boxes.

To see if the AJ22 mutant still expressed the L1 and L2 ampicillin resistance genes, RT-PCR was performed on samples taken from all cultures before ampicillin was added at 90 minutes, and at 120 minutes, 150 minutes and 180 minutes or 30, 60 and 90 minutes after ampicillin was added. RNA was purified from each culture and used in PCR reactions with GAPDH primers to show that there was no DNA contamination, Separation of the PCR products on a 1% agarose gel showed that the PCR reactions that used purified RNA as a

template contained no products. Thus, there was no chromosomal DNA contamination in the RNA preparations.

- L-Ladder
- 1 to 4 is L1 primer in RNA samples collected at 90, 120, 150 and 180 minutes
- 5 to 8 is L2 primers in RNA samples collected at 90, 120, 150 and 180 minutes
- 9 to 12 is AmpG1 primers in RNA samples collected at 90, 120, 150 and 180 minutes
- 13 to 16 is AmpG2 in RNA samples collected at 90, 120, 150 and 180 minutes
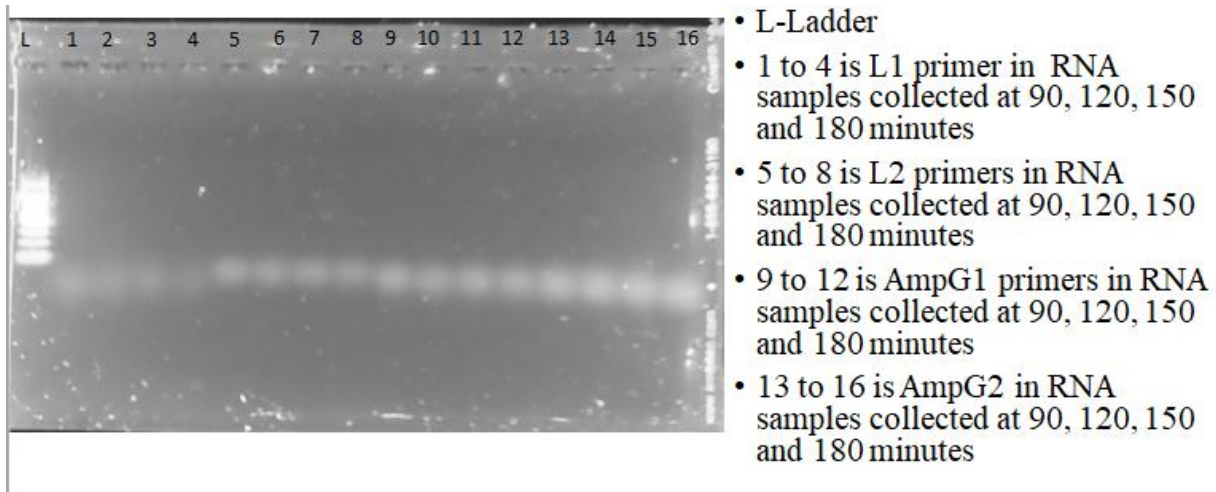
Figure 6A. Gel image displaying RNA samples collected at 90, 120, 150 and 180 minutes, tested with primers for L1, L2, ampG1 and ampG2. L is a ladder. The ladder (L) serves as a size reference. Column 1 to 4 are RNA samples collected at 90, 120, 150 and 180 minutes containing L1 primer; column 5 to 8 are RNA samples collected at 90, 120, 150 and 180 minutes containing L2 primer; column 9 to 12 are RNA samples collected at 90, 120, 150 and 180 minutes containing ampG1 primer and lastly, column 13 to 16 are RNA samples collected at 90, 120, 150 and 180 minutes containing ampG2 primer.

- L-Ladder
- 1 to 4 is GAPDH primer in RNA samples collected at 90, 120, 150 and 180 minutes
- C1 to C4 are Control water samples in RNA samples collected at 90, 120, 150 and 180 minutes
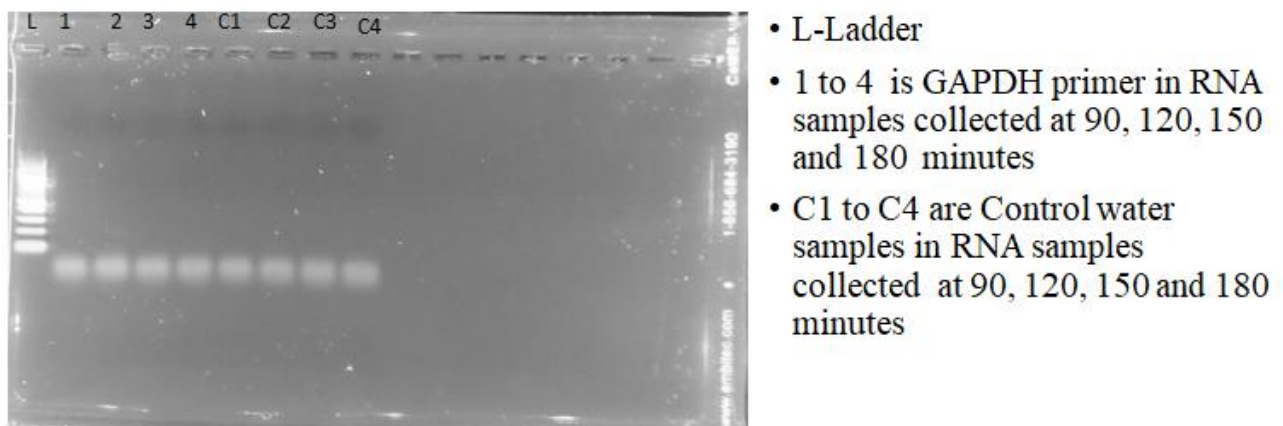
Figure 6B. Gel image showing PCR results for RNA samples collected at 90, 120, 150 and 180 minutes containing GAPDH primer and control water samples. Column 1 to 4 are RNA samples collected at 90, 120, 150 and 180 minutes tested with GAPDH primer and column C1 to C4 are RNA samples collected at 90, 120, 150 and 180 minutes containing nuclease free water

The size of the ladder and primers is 1kb. The absence of PCR products across all columns indicates that RNA samples are free from contamination by DNA. Bands observed below the 1kb marker are likely primer dimers which could be minimized by adjusting the PCR cycles to fewer than 30 but more than 20, or by redesigning the primers. 100 ng of RNA was used in cDNA synthesis reactions to normalize the sample results. The cDNA was then used as a template in PCR reactions containing primers for glyceraldeyde-3-phosphate dehydrogenase (GAPDH), *ampG*, L1 and L2. The *ampG*1 primers annealed to the 5' end of *ampG* gene on one side of the transposon insert, and *ampG*2 primers annealed to the 3' end of *ampG* on the other side of the transposon insert.

A) *S. maltophilia*						
Primers	90	120	150	180	DNA	C
GAPDH-Water						
GAPDH-Ampicillin						
ampG1-Water						
ampG1-Ampicillin						

Primers						
ampG2-Water						
ampG2-Ampicillin						
L1-Water						
L1-Ampicillin						
L2-Water						
L2-Ampicillin						

B) AJ22 mutant						
Primers	90	120	150	180	DNA	C
GAPDH-Water						
GAPDH-Ampicillin						
ampG1-Water						
ampG1-Ampicillin						
ampG2-Water						
ampG2-Ampicillin						
L1-Water						
L1-Ampicillin						

L2-Water						
L2-Ampicillin						

Figure 7. RT-PCR results for A) *S. maltophilia* 02 and B) the AJ22 mutant. Culture samples for the wild type and mutant were collected at 90 minutes, before adding ampicillin, for RNA purification during the growth curves. 100 μg/ml of ampicillin was added to experimental cell samples at 90 minutes in a volume ratio of 1:1000, and an equal volume of water was added to control samples. Additional samples were collected for RNA purification at 120, 150 and 180 minutes. The RNA samples were converted to cDNA which served as a template for PCR reactions with primers for GAPDH, ampG, L1and L2.

. Genomic DNA and nuclease free water sample were used as positive and negative controls in the reaction. All PCR reactions were separated on 1% agarose gels.

The gene for GAPDH was used as a normalization control because it is a housekeeping gene involved in glycolysis. In figure 7A for *S. maltophilia* 02, the bands for GAPDH appear to be of equal intensity at all time points in the presence and absence of ampicillin, indicating that the expression was constant at all time points. Likewise, in figure 7B for AJ22, the expression level for GAPDH appears constant in different time points but slightly higher compared to *S. maltophilia* 02. These results confirm that equal amounts of RNA were used for each sample and validate observed differences in expression levels for other genes.

Two sets of primers were used for *ampG* to test for expression levels on the 3' region of *ampG* on one side of the transposon insert and the 5' region of *ampG* on the other side of the insert, In figure 7A for *S. maltophilia* 02, the *ampG*1 primers show that *ampG* is expressed at equal levels at all time points in the presence and absence of ampicillin. The ampG2 primers

also show that ampG is expressed equally in the presence and absence of ampicillin, confirming that *ampG* expression is constitutive. Surprisingly, in figure 7B for AJ22, both sets of *ampG* primers show that transcripts for *ampG* on both sides of the transposon insert are present in equal amounts for all time points, suggesting constitutive expression. However, the expression level of *ampG* appears to lower in the mutant than in the wild type.

In figure 7A, the expression level of L1 in *S. maltophilia* 02 remains uniform in all time points in the presence and absence of ampicillin. Similarly, in figure 7B, AJ22 shows a consistent expression of L1 in water and ampicillin. This suggests that L1 is expressed constitutively. However, the expression level of L1 in AJ22 is lower compared to the wild type, showing that the mutation in *ampG* gene affects expression level of L1 gene.

Figure 7A in *S. maltophila* 02 indicates that the L2 gene produced similar intensity of bands in all time points, both in the absence and presence of ampicillin. This suggests that the L2 gene in *S. maltophilia* 02 is constitutively expressed. In Figure 7B L2 expression level in AJ22 is consistent at various time points in the absence and presence of ampicillin. However, a lower expression of the genes is evident in the experimental and control samples in the mutant compared to the wild type. The lower expression levels of theL1 and L2 genes in the mutant compared to the wild type explains the MIC differences in response to ampicillin. Additional trials of the RT-PCR experiment should be performed to confirm these results.

3.2 Trans-membrane Analysis

>AmpG

VTEAAKPRRPWQQVVSNLSQRKVLAMLLLGFSSGLPIYLVGNTLGFWMRKEGIELSTIG

FLSWVGLAYTMKFLWAPIVDKTDVPLFGRFGRRRGWMLLSQLVVVVGLVGMALVQP

KGGQIQFLGIAWQHIVVFGVMAVIVAFASATQDIVIDAWRIESADNSEQLGLLTSSSALG

YRTALLVTDALILIIAARVGWQVSYEIMAVLMALGVVAVVMAREPAREVAAVQAQATS

LWTPRGLFDAVAGPFIAFVREHRSGAILILVAISVYRMADFVMGPMANPFYVDLGLDED

TVGAVRGSVGLVATFVGIAAAGLVSVRWGVLVALMVGAVLGPASNLAFAWLAYSGPD

TTHFAVAMAIDNFASGFAGTALIAYMSSLTSIGYTATQYALLSSFYAMPGKALKGLSGW

SVQTLAQGRTLLEGYALFFVGTALVAIPVVILCALLILQQRRRQAASAT

Figure 8. The 457 AmpG amino acid residues from *S. maltophilia* 02

Deep TMHMM 2.0 software predicts the localization of proteins in microorganisms. Previous studies in strain similar to *S. maltophilia* and in organisms having a close evolutionary relationship revealed that AmpG is a trans-membrane permease that transports waste products from the periplasm into the cytoplasm. Deep TMHMM search results of *S. maltophilia* 02 AmpG amino acid residues revealed that it is a transmembrane permease with 12 transmembrane helices.

The transposon inserted between helices 10 and 11, and figure 7B shows that there is decreased expression of L1 and L2. This observation suggest that AmpG may still have activity but at a reduced level in the absence of helices 11 and 12.
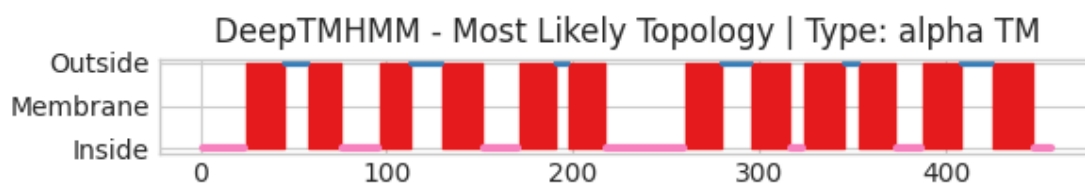
DeepTMHMM - Most Likely Topology | Type: alpha TM

Outside
Membrane
Inside

0   100   200   300   400

Figure 9. A diagram showing predicted topology of AmpG protein by DeepTMHMM 2.0 bioinformatics software. Pink color represents intracellular region of membrane and blue color extracellular region. Red color represents the transmembrane region.

TMR 1   TMR 2

VTEAAKPRRPWQQVVSNLSQRK VLAMLLLGFSSGLPIYLVGNT LGFWMRKEGIEL ST IGFLSWVGLAYTMKF
IIIIIIIIIIIIIIIIIIIIII MMMMMMMMMMMMMMMMMMMM OOOOOOOOOOOO MMMMMMMMMMMMMMMMM

TMR 3   TMR 4

L WAPIVDKTDVPLFGRFGRRRG WMLLSQLVVVVGLVGMAL VQPKGGQIQFLGIAW QHIVVFGVMAVIVAFASAT
M IIIIIIIIIIIIIIIIIIIIII MMMMMMMMMMMMMMMMM OOOOOOOOOOOOOOOOOOO MMMMMMMMMMMMMMMMMMM

TMR 5   TMR 6

QDI VIDAWRIESADNSEQLGLL TSSSALGYRTALLVTDALILI IAARVG WQVSYEIMAVLMALGVVAVV MAREP
MMM IIIIIIIIIIIIIIIIIIII MMMMMMMMMMMMMMMMMMMM OOOOOO MMMMMMMMMMMMMMMMMMMM IIIII

AREVAAVQAQATSLWTPRGLFDAVAGPFIAFVREHRS GAILILVAISVYRMADFVMGP MANPFYVDLGLDEDT V
IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII MMMMMMMMMMMMMMMMMMMMM OOOOOOOOOOOOOOO M

TMR 8   TMR 9   TMR 10

GAVRGSVGLVATFVGIAAAG LVSVRWG VLVALMVGAVLGPASNLAFAWL AYSGPDT THFAVAMAIDNFASGFAG
MMMMMMMMMMMMMMMMMMMM IIIIIII MMMMMMMMMMMMMMMMMMMMMM OOOOOOO MMMMMMMMMMMMMMMMMMM

Transposon insertion   TMR 11   TMR 12

TAL AYM SSLTSIGYTA TQYALLSSFYAMPGKALKGLS GWSVQTLAQGRTLLEG YALFFVGTALVAIPVVILCA
MMM IIII IIIIIIIIII MMMMMMMMMMMMMMMMMMMMM OOOOOOOOOOOOOOOO MMMMMMMMMMMMMMMMMMMM

LL ILQQRRRQAASAT
MM IIIIIIIIIIII

Figure 10. Amino acid residues of AmpG protein and predicted topology of the sequences. The amino acid residues marked red are amino acid residues that code for

transmembrane helices. The region marked purple represents the location of transposon insertion on AmpG protein.
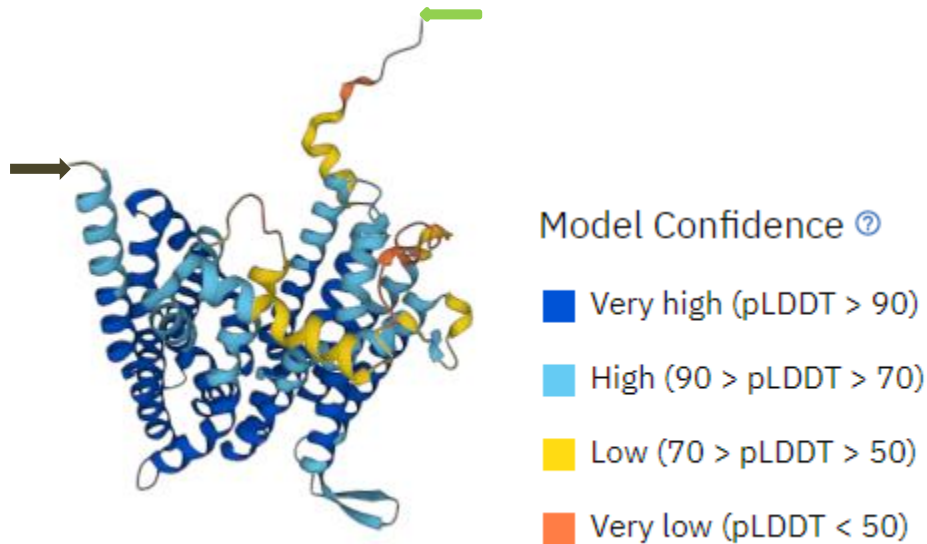

Figure 11. 3D viewer of AmpG protein by AlphaFold prediction software. AlphaFold produces per-residue model confidence score (pLDDT) ranging from 0 to 100. Some regions below 50 pLDDT may be unstructured in isolation. In the model, deep blue color indicates residues with very high pLDDT above 90, light blue color indicates high pLDDT between 90 and 70, yellow color indicates low pLDDT between 70 and 50 and red color indicates very low pLDDT below 50. The green arrow signals the start point of the structure and black arrow the end point.

AlphaFold software predicts the 3D structure of a protein from amino acid residues. Prediction results displayed AmpG protein model (AlphaFold ID, A0A2J0T3J2) belonging to the Major Facilitator Superfamily (MSF) transporter in *S. maltophilia* (organism_tax ID: 40324).

The model outlines the orientation of the 457 amino acid residues of AmpG. The first residue, methionine, and residues up to 19, have a low confidence score. High confidence score

is observed for amino acid residues 20 to 83, followed by a region of low confidence from residues 84 to 89, then high confidence from residues 90 to 116. Residue 117 has a low confidence, while residues 118 to 218 have high confidence followed by low confidence for residues 219 to 248. Residues 249 to 378 have high confidence, with a transposon insertion at residues 373 to 375. A low confidence region follows from residues 379 to 390, with high confidence from residues 391 to 455. The last residue, threonine, along with penultimate residue, also display low confidence.

CHAPTER 4

4.0 Discussion

AmpG is a transmembrane protein located in the periplasm of gram-negative bacteria. It facilitates the transportation of cell wall degradation products into the cytoplasm. These transported muropeptides subsequently induce the expression of L1 and L2 β-lactamases in *S. maltophilia* 02. Evidence from this research demonstrates that a mutation in the ampG gene reduces the expression of the L1 and L2 penicillin-resistance genes. However, to ensure the validity and reliability of the findings, it is recommended that this research study be repeated two additional times.

Gel electrophoresis analysis shows the transcription of mutated ampG; however, the mutant phenotype exhibits growth inhibition with 100 μl/ml ampicillin concentration. Predictive modeling using TMHMM 2.0 identifies AmpG as a transmembrane protein with 12 transmembrane helices and a transposon disrupting the region between transmembrane helices 10 and 11. Previous studies suggest that a truncated protein may still be produced if the essential functional domain remains unaffected. Despite the transcriptional activity of ampG, the production of AmpG protein has not been confirmed in this study. Western blotting and RNA-seq of the PCR products can provide evidence of protein synthesis.

The experimental procedure for western blotting involves purifying proteins and injecting them into mice to produce antibodies against them. The antibodies are then purified. Proteins from each time points are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane which is probed by the mouse for each protein. SDS-PAGE is thus used to confirm the expression levels of the AmpG, L1 and L2 proteins.

Alternatively, RNA sequencing (RNA-Seq) can quantify ampG, L1, and L2 expression levels. This technique involves creation of a cDNA library, sequencing on an Illumina platform, and aligning reads to quantify gene expression levels.

4.1 Future work

Future research should focus on the ampG-ampD-ampR regulatory system of the L1 and L2 β-lactamases in *S. maltophilia* 02. Studying an *ampG* knockout mutant and coupling with techniques like western blotting or RNA-Seq to analyze penicillin resistance gene expression could clarify the role of AmpG in resistance mechanism. If L1 and L2 expression persist despite ampG knockout, another protein may facilitate muropeptide transport into the cytoplasm which activates ampD-ampR regulatory system.

Understanding AmpG is crucial for antibiotic resistance research, as targeting this protein could potentially inhibit β-lactamase production and restore the efficacy of β-lactam antibiotics. Further studies should explore its molecular structure, role in resistance mechanisms, and potential inhibitors that can block its function without causing off-target effects.

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