Identification of an L2 ß-lactamase gene from *Stenotrophomonas maltophilia* OR02

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Jamielynn Doyle

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Jamielynn Doyle

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Jamielynn Doyle, Student Date

Approvals:

Dr. Jonathan Caguiat, Thesis Advisor Date

Dr. David Asch, Committee Member Date

Dr. Xiangjia Min, Committee Member Date

Dr. Salvatore A. Sanders, Dean of Graduate Studies Date

Abstract

Stenotrophomonas maltophilia Oak Ridge strain 02 (*S. maltophilia* 02) was isolated from a heavy metal contaminated stream, East Fork Poplar Creek in Oak Ridge, Tennessee. This strain has high levels of resistance to both metals and antibiotics. All strains of *S. maltophilia* are gram-negative bacilli. Some strains are opportunistic pathogens that are resistant to high levels of metals and to antibiotics such as cephalosporins, quinolones, carbapenems, penicillins, and β-lactam/β-lactamase inhibitor combinations. *S. maltophilia* 02 contains two β-lactamase genes, L1 and L2. In this study, the L2 β-lactamase and its regulator, LysR, were cloned and sequenced. MICs were performed to test for antibiotic resistance against ampicillin, showing resistance even at 1500 μg/ml. In addition, L2 was also found to confer resistance to the antibiotic carbenicillin, but not to cefoxitin. By studying penicillin resistance mechanisms, it may be possible to develop strategies to combat drug resistance in pathogenic strains of *S. maltophilia.*

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Chapter I: Introduction

1.1 Oak Ridge Y-12 plant

The Y-12 plant in Oak Ridge, Tennessee was responsible for nuclear weapons production during and after World War II and the Cold War. The plant, which was one of three sites on the Oak Ridge Reservation, produced fuel for atomic bombs by enriching uranium and separating lithium-isotope, processes that lead to the contamination of nearby water sources (Campbell, Ford, & Levine, 1998).

Waste from the Y-12 plant, including mercury, polychlorinated biphenyls (PCBs), radionuclides, and other heavy metals, as well as waste from secondary sites was disposed into several impoundments known as S-3 ponds at the base of East Fork Poplar creek (Campbell et al., 1998; Moore, Sample, Suter, Parkhurst, & Teed, 1999; Ts- et al., 2000). Wastes in the ponds were treated by denitrification and removed in 1983. Then, the ponds were capped and repurposed to serve as a parking lot (Brooks, 2001).

During production at the Y-12 plant, contamination leaked into East Fork Poplar creek through the unlined S-3 ponds and through accidental spills from the plant. Other sources of pollution included research sites and labs upstream from East Fork Poplar creek. In 1989 the area was put on the National Priority List as Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) to address the contamination levels (Campbell et al., 1998). Different sources have evaluated waste effects, levels, and clean-up efforts to assess the risk to wildlife and people living in the surrounding areas, although one study has concluded that there is no public risk of uranium exposure through the contaminated site (Moore et al., 1999; Oak, Reservation, Comment, & April, 2003; Ts- et al., 2000). Studies found that East Fork Poplar creek

contains strains of bacteria that are multi-drug resistant (MDR) and multi-metal resistant (Holmes, Vinayak, Benton, Esbenshade, et al., 2009).

1.2 *Stenotrophomonas maltophilia* Oak Ridge strain 02

Stenotrophomonas maltophilia Oak Ridge strain 02 (*S. maltophilia* 02) was isolated from East Fork Poplar Creek in Oak Ridge, Tennessee. It showed resistances to lethal levels of several heavy metals including copper, mercury, gold, lead, and chromium (Holmes, Vinayak, Benton, Esbenshade, et al., 2009), and shows MDR to penicillin, tetracycline, chloramphenicol, kanamycin and other antibiotic treatments (unpublished data).

1.3 Stenotrophomonas maltophilia

In 1958, a strain of *S. maltophilia* was isolated and given the name *Pseudomonas maltophilia*. Studies that followed brought into question the genus of this bacterium and it was later reclassified as *Xanthomonas maltophilia* until a new genus type, *Stenotrophomonas*, was created in order to accurately classify the bacterium as *S. maltophilia* (Denton & Kerr, 1998).

Characteristically, *S. maltophilia* is a gram-negative bacillus bacterium that moves by way of polar flagella that range about 0.5 to 1.5 μm in length. It has been classified as a nonsporulating obligate aerobe and frequently displays multiple drug resistance (MDR) (Denton & Kerr, 1998). Isolated strains of *S. maltophilia* grow ideally at 35°C and require methionine or cysteine for growth (Denton & Kerr, 1998).

Studies have also centered on *S. maltophilia* in relation to fungal pathogens (Berg, Roskot, & Smalla, 1999). Some strains of this bacterium inhibit fungal growth,

making it a potential resource for biodegradation or biological control (Denton & Kerr, 1998).

Research on both environmental and clinical strains of *S. maltophilia* showed that it is a common and resilient bacterium capable of adhering to different surface types and displaying resistances to both antibiotics and metals (Corlouer, Lamy, Desroches, Ramos-vivas, & Mehiri-zghal, 2017). Environmentally, it is frequently found in water sources and plant rhizospheres. *S. maltophilia* is believed to be beneficial for plant growth as it can be harmful to plant pathogens and assist in soil decontamination. *S. maltophilia* is also capable of forming biofilms in showerheads, pools, bottled water, and host tissue, making it a relevant risk for immunocompromised individuals (Crossman et al., 2008). It adheres to different forms of plastic medical equipment in clinical settings, increasing its importance in nosocomial infection prevention (Denton & Kerr, 1998; Nicodemo & Paez, 2007).

1.3.1 Clinical vs environmental

Nosocomial strains of *S. maltophilia* are most commonly found in catheters and ventilators and affect immunocompromised individuals, but show prevalence in patients with cystic fibrosis (Crossman et al., 2008; Mojica et al., 2017). These strains are opportunistic MDR pathogens showing increased mutation rates in comparison with environmental strains. Since ventilators are a common reservoir for these strains, respiratory infections are the highest reported infections associated with *S. maltophilia* (Mojica et al., 2017). Other common infections due to *S. maltophilia* include bacteremia, meningitis, endocarditis, urinary tract infections, and gastrointestinal infections, all having varying degrees of severity (Denton & Kerr, 1998). Bacteremia, a bacterial blood

infection, has become as predominant as respiratory infections with mortality rising from 21% to near 69%.

The increased mutation rate along with the MDR properties of *S. maltophilia* make these bacterial infections difficult to treat. In the past, trimethoprim-sulfamethoxazole acted as an effective treatment for *S. maltophilia* infections, however resistance to these antibiotics are escalating (Bodilis et al., 2017). The increase in mortality rates of these infections alone points to increased resistance of nosocomial strains to traditional antibiotics.

1.4 Antibiotic Resistance

Intrinsic antibiotic resistance is maintained in *S. maltophilia* strains for different antibiotic types including quinolones, carbapenems, cephalosporins, and penicillins. Resistance to these antibiotics is mediated in part by low membrane permeability and multidrug efflux pumps which continue to show progressing resistance against current treatment methods (Bodilis et al., 2017; Calvopiña et al., 2016; Toleman, Bennett, Bennett, Jones, & Walsh, 2007).

Previous research speculated that *S. maltophilia* may also be capable of breaking down antibiotics and other harmful components for detoxification and nutrient use, though little research has been done to verify this (Brook, 2012).

S. maltophilia also produces proteases and elastases that lend themselves to antibiotic resistance and virulence factors (Nicodemo & Paez, 2007). Mechanisms are also acquired through horizontal gene transfer from plasmids, integrons, transposons, biofilms, and other sources (Brook, 2012). It also contains two different β-lactamases. These produced β-lactamases, L1 and L2, serve as resistance to a wide range of β-lactam

(β-lactamase inhibiting) antibiotics making them important genetic structures in the *S. maltophilia* genotype.

1.4.1 β-lactams

β-lactams work by targeting peptidoglycan synthesis. Peptidoglycan consists of a helical polysaccharide backbone made up of the alternating sugars, N-acetylglucosamine (NAG) and N- acetylmuramic acid (NAM), which are crosslinked by peptides. Penicillin Biding Proteins (PBPs) are responsible for crosslinking peptides to form the mesh-like structure of peptidoglycan. PBPs also bind to penicillins and β-lactams which inhibit their ability to crosslink the structure and results in disruption of cell wall synthesis (Vollmer, Blanot, & De Pedro, 2008).

β-lactam antibiotics include derivatives of penicillins, cephalosporins, monobactams, and carbapenems. The six β-lactam combinations commonly used are amoxicillin-clavulanic acid, ampicillin-sulbactam, cefoperazone-sulbactam, piperacillin-tazobactam, ticarcillin-clavulanic acid and sultamicillin (Page et al., 2011). While studies show significant results against *S. maltophilia* infections from several of these combinations, these groups are largely ineffective or their rate of effectiveness continues to decline, though carbapenems still show the greatest ability against infection (Papp-wallace, Endimiani, Taracila, & Bonomo, 2011).

Overall, these antibiotics work by targeting cell wall synthesis. Once the antibiotic is introduced, it prevents the cross-linking of the bacterial cell wall by blocking PBPs responsible for this part of cell wall synthesis usually leading to cell lysis (Michigan State University, 2011).

Carbapenems do this by acylating PBPs and inhibiting crosslinking, ending in lysis through weakening of the peptidoglycan and have shown the greatest potency against *S. maltophilia* infections (Papp-wallace et al., 2011). Cephalosporins and penicillins similarly bind to PBPs to interrupt formation of peptidoglycan, however most studies find *S. maltophilia* to be resistant to all cephalosporins (Livermore, 1987; Denton, 1998; Ghooi, 1995).

The L1 and L2 β-lactamases began to significantly impact clinical treatment of *S. maltophilia* in the 1960's (Yang, Huang, Hu, Huang, & Lin, 2009). Around this time, a β-lactamase inhibitor, olivanic acid, was found to be produced by the gram-positive bacteria *Streptomyces clavuligerus.* It contains a carbapenem backbone and acted as a β-lactam making it a possibility for clinical treatment as carbapenems had previously been the most successful treatment for *S. maltophilia* as a last resort drug. Further studies concluded that olivanic acid displayed poor penetration into the cell so was not further pursued as treatment against β-lactam resistance (Papp-wallace et al., 2011). Clavulanic acid, a β-lactam drug, was since discovered and is a successful L2 inhibitor and shows effectiveness when paired with penicillins.

1.4.2 L1 and L2

The L1 and L2 of *S. maltophilia* are chromosomally encoded β-lactamase genes and have shown to be effective against a broad range of β-lactam antibiotics (Yang, Huang, Hu, Huang, & Lin, 2009). They are found in both wild-type and clinical strains of *S. maltophilia*.

L1 can be characterized as a Class B β-lactamase. It is a Zinc-dependent metalloenzyme that works by hydrolyzing β-lactams (Huang et al., 2010; Nicodemo &

Paez, 2007). The L1 gene is surrounded by TonB dependent receptor and a copper resistance gene. Research has shown that L1 is not susceptible to any β-lactamase inhibitors and is found in all wild-type strains (Mojica et al., 2017). It exists as a holoenzyme containing a tetramer of equal subunits (Denton & Kerr, 1998).

L2 is similar to *amp-C* in that they are both regulated by *ampR*. It is a Class A β-lactamase that is capable of hydrolyzing penicillins, cephalosporins, and monobactams (Yang et al., 2009). L2 is adjacent to a LysR regulatory gene, *ampR*, upstream and a sodium ion channel downstream. It is an *ampR*-linked gene, meaning that *ampR* serves as the key regulator for L2 induction, similar to the *ampR-ampC* system, though *ampR* is needed for the basal expression of both L1 and L2 (Hu et al., 2008; Yang et al., 2009). L2 has also shown to be susceptible to inhibitors such as clavulanic acid (Nicodemo & Paez, 2007).

Research has shown that the SecB general secretory pathway transports the L1 β-lactamase, while the twin-arginine translocation (TAT) pathway transports the L2 β-lactamase (Pradel et al., 2009).

L1 is an unlinked *ampR* gene and remains activated in the absence of a β-lactam inducer (Lin et al. 2009). The L2 gene is located adjacent to a LysR-type *ampR* regulator, which regulates its gene expression. This regulator represses L2 expression in the absence of a β-lactam inducer and activates it in the presence of the inducer (Lin et al. 2009).

Along with AmpR, AmpC, AmpD, AmpN, and operon AmpN-AmpG are all critical for expression of the L1 and L2 genes. AmpR is a transcriptional regulator of *ampC* which encodes a protein that aids in cell wall recycling. These degraded

components are transported to the cytoplasm by AmpG and cleaved into 1,6-anhydromuramic acid and peptide by AmpD. The produced peptide contributes to the formation of UDP-N-acetylmuramic acid-pentapeptide, a monomer that is then synthesized in the cytoplasm, transported to the periplasm, and used as a component in peptidoglycan structure. AmpR activates AmpC when bound to anhydro-N-acetylmuramylpeptide from damaged peptidoglycan, and represses it when bound to the peptidoglycan monomer, UDP-N-acetylmuramic acid-pentapeptide. AmpD proteins degrade AmpR activator ligands during normal growth, repressing AmpC production. Loss of AmpD derepresses AmpC production increasing β-lactam resistance (Brook, 2012; Lin et al, 2011; Ricchiuti, 2016; Yang et al., 2009).

Studies concluded that the relationship between AmpN and AmpG are also important for β-lactamase induction; AmpN, located downstream from AmpG, has several hypothesized functions, and research has shown that the disruption of AmpN has polar effects on AmpG creating non-inducibility in L1 and L2. AmpN and AmpG form an operon that may be responsible for induction precursors (Brook, 2012; Huang et al., 2010; Ricchiuti, 2016).

Peptidoglycan recycling is also a key factor in L1 and L2 regulation through disruption of penicillin binding proteins (PBPs). The antibiotic imipenem, a carbapenem, is known to inhibit PBPs which play a role in the late stages of peptidoglycan synthesis, though resistance to imipenem continues to increase. Changes in PBP activity through β-lactamase saturation cause structural changes to peptidoglycan and cytosol components, inducing β-lactamase activity (Van Oudenhove et al., 2012).

As L1 and L2 are crucial for β-lactam resistance, targeting these genes to enhance susceptibility to β-lactams may have medical benefits for patients with *S. maltophilia* infections and for infection prevention. Previous research showed L1 exhibits resistance to cefoxitin, but L2 does not, and L2 exhibits resistance to carbenicillin, but L1 does not. Both L1 and L2 confer resistance to ampicillin (Hu et al., 2008).

Chapter II: Objectives and Hypothesis

2.1 Objectives

This study aims to clone and sequence the L2 β-lactamase genes from *S. maltophilia* and test it for ampicillin resistance in *E. coli*.

2.2 Hypothesis

It is expected that L2 will confer resistance to ampicillin when tested in *E. coli*. L2 should also confer resistance to carbenicillin but not to cefoxitin (Hu et al., 2008).

Chapter III: Methods

3.1 Bacterial Strains

S. maltophilia 02 was obtained from the American Type Culture Collection (ATTC, Manassas, VA). YSU was isolated from East Fork Poplar Creek in Oak Ridge, TN (Holmes, et al., 2009). *E. coli* strain ECD100D *pir116* was obtained from Illumina, Inc. (Madison, WI).

3.2 Growth Medium

Genomic Grade™ Culture Media LB (Lennox) Broth containing 10 g/l tryptone, 5 g/l yeast extract and 5 g/l sodium chloride was used to make LB Broth and agar. LB agar was made using 1.6% agar Amresco (Solon, Ohio). Cultures were grown in plain media. When, required, media was supplemented with 50 µg/ml kanamycin (Amresco, Solon, OH), 20 µg/ml chloramphenicol (Amresco, Solon, OH), 12 µg/ml of tetracycline (Amresco, Solon, OH), 100 µg/ml cefoxitin (Thermo Fisher Scientific, Waltham, MA), 100 µg/ml carbenicillin (Thermo Fisher Scientific, Waltham, MA) or 100 µg/ml ampicillin (Thermo Fisher Scientific, Waltham, MA), unless specified otherwise.

3.3 Genomic Prep

A 10 ml of overnight culture was pelleted by centrifugation for 5 minutes at 8,000 x g. The supernatant was discarded, and cells were resuspended in 2 ml TE Buffer (10 mM tris-HCl, pH 8.0, and 1 mM EDTA). 5 µl RNase (Amresco, Solon, OH) was added followed by 5.5 ml genomic prep lysis solution. 15 ml Genomic prep lysis solution was made using 9.8 ml Nuclease-free water, 0.75 ml 1 M tris-HCl, pH 8.0, and 4.5 ml 10% SDS. Samples were mixed by inverting and incubated at 65^{o}C for 15 minutes. 2 ml of warmed (65^{o}C) 5 M NaCl was added to samples and vortexed for 20 seconds until samples were well mixed. Samples were incubated on ice for 15 minutes, centrifuged at 10,000 X g for 15 min and the supernatant was poured into a 50 ml

conical tube. DNA was precipitated by adding 6 ml of isopropanol. A glass rod was used to spool the DNA into a 1.5 ml microcentrifuge tube containing 0.5 ml of 70% ethanol and centrifuged for 3 minutes. The Ethanol was poured off and DNA was resuspended in 500 µl TE Buffer and stored at 4^{o}C.

3.4 *Eco*R I Digestions

DNA was digested by mixing 2 µl of New England BioLabs (Ipswich, MA) 10X Cutsmart Buffer, 5 µL nuclease-free water, 12 µL DNA sample, and 1 µl of *Eco*R I. Digestions were incubated at 37^{o}C for one hour.

3.5 Polymerase Chain Reaction

PCR was performed on samples using primers found in Table 1. Primers were dissolved in TE buffer to 100 µM and further diluted to 10 µM or 4 µM in nuclease free water for PCR reactions. The PCR reaction mix was made using either 2X Q5 High Fidelity Mix (New England BioLabs, Ipswich, MA) or 2X GoTaq DNA Polymerase (Promega, Madison, WI). One reaction mix for Q5 was made with 1.25 µl of 10 µM forward and reverse primers, 12.5 µl of Q5 Mix, 9 µl of nuclease-free water, and 1 µl DNA template for a 25 µl reaction. GoTaq PCR reactions were made with 10 µl GoTaq, 4 µl nuclease-free water, 2.5 µl of 4 µM forward and reverse primers, and 1 µl of DNA template for a 20 µl reaction. Samples were run in a thermalcycler using the following programs for GoTaq and Q5 respectively: 95°C for 2 minutes, 95°C for 1 minute, 55°C for 1 minute for 34 cycles, 72^{o}C for 10 minutes, and hold at 4^{o}C or 98^{o}C for 30 seconds, 98^{o}C for 10 seconds, 65^{o}C for 15 seconds, 72^{o}C for 2 minutes for 34 cycles, and 72^{o}C for two minutes, and hold at 10^{o}C. Samples were then stored at –20^{o}C.

3.6 Gel Electrophoresis

Gels were made by microwaving 2.5 g of BioExcell® Agarose LE (Worldwide Medical Products, Bristol, PA) in 250 ml of 1X TBE (Amresco, Solon, OH) containing 0.089 M tris, 0.089 M boric acid, and 0.002 M EDTA for a 1% gel. GelGreen Nucleic Acid Stain (Embi Tec, San Diego, CA) was added to a 1:10,000 dilution (25 µl) in a plastic graduated cylinder (this reagent sticks to glass) and mixed. The gel was then poured into trays and combs were inserted to form loading wells. Gels were run using a RunOne™ Electrophoresis System (San Diego, CA). Amresco (Solon, OH) 10X TBE buffer was diluted to 1X using 0.089 M Borate and 0.002 M EDTA to run the gels. Gels were loaded by mixing 1 µl of 6X Amresco Agarose Gel Loading Dye (Amresco, Solon, OH) with 5 µl of sample and pipetted into individual wells. 5 µl of an Amresco 1 kb DNA ladder was also loaded with 2 µl of the loading dye. All gels were run at 100 volts. Gel images were taken using an Embi Tec PrepOne™ Sapphire (San Diego, CA).

3.7 DNA Purification from Gel Slabs

DNA was purified from gel slabs using a QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany). DNA fragments were excised from an agarose gel and weighed to determine the volume of the agarose. 3 volumes of Buffer QG were added to each sample for every 1 volume of gel. The samples were incubated at 50 ^{o}C for 10 minutes using a water bath and vortexed every 1 – 2 minutes until the gel was completely dissolved. 10 µl of sodium acetate, pH 5.0, was added to each sample. 1 volume of isopropanol was added to each sample and mixed by inverting. Spin columns were placed in 2 ml collection tubes, samples were added and centrifuged for 1 minute. The flow-through was discarded and the spin columns were placed back into the collection tubes. 750 µL Buffer PE was added, and the columns were centrifuged for 1 minute. The flow-through was discarded, and the spin columns were placed back into the collection tubes. Samples were centrifuged again for 2 minutes to remove residual wash, and

the columns were inserted into new 1.5 ml collection tubes. 50 µl of Buffer EB was added to the column membrane and centrifuged for 1 minute. 30 µl Buffer EB was then added similarly, incubated for 4 minutes, and centrifuged for 1 minute. Samples were stored at –20^{o}C.

3.8 PCR Purification

PCR Purifications were performed using a QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany). 5 volumes of PB Buffer were added to each sample for every 1 volume of PCR reaction. Samples were mixed and 10 µl of sodium acetate, pH 5.0, was added. Provided spin columns were inserted into 2 ml collection tubes and samples were loaded into the spin columns. Samples were centrifuged for 1 minute at room temperature. Flow-through was discarded and the Spin Columns were reinserted into the collection tubes. 750 µl PE Buffer was added to the columns which were centrifuged for 1 minute at room temperature. The flow-through was discarded and the columns were centrifuged again for 1 minute to remove any residual buffer. Each column was placed into a new 1.5 ml microcentrifuge tube and 50 µl of EB Buffer was added to the column membrane and centrifuged for 1 minute. Purified DNA was stored at –20^{o}C.

3.9 Ligation

A Strataclone PCR cloning kit (La Jolla, CA) was used to clone DNA fragments. To add 3' adenine overhangs, PCR products amplified using Q5 were cleaned up using a QIAquick® PCR Purification Kit. Then, 5 µl of cleaned up PCR product was mixed with 5 µl of 2X GoTaq mix and incubated at 72^{o}C for 10 minutes. A mix was made using 3 µl of Strataclone Cloning Buffer, 2 µl of PCR product, and 1 µl of Strataclone Vector Mix. Samples were incubated for five minutes at room temperature and then placed on ice to begin transformation.

3.10 StrataClone Transformation

Competent cells provided by the kit were placed on ice and transformed by adding 1 µl ligated sample to each. Cells were incubated on ice for 20 minutes while SOC was prewarmed to 42^{o}C. Samples were then heat shocked at 42^{o}C for 45 seconds using a water bath and incubated on ice for 2 minutes. 250 µl of pre-warmed SOC was added to each sample and samples were agitated for 1 hour at 37^{o}C. 100 µl of cells were then plated on LB-agar plates containing 100 µg/ml ampicillin and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated overnight at 37^{o}C. Selected white colonies were grown overnight in 5 ml LB Broth containing 50 µg/ml kanamycin at 37^{o}C.

3.11 Ligation and Transformation for Subcloning

8 µl of DNA was added to 2 µl T4 DNA Ligase Buffer, 1 µl T4 PNK, and 9 µl of nuclease free water for a 20 µl reaction. The reaction was incubated at 37^{o}C for 30 minutes and then heat inactivated at 65^{o}C for 20 minutes. 1 µl T4 Buffer, 3.5 µl DNA vector, 2 µl T4 Ligase, and 3.5 µl of the insert DNA were combined in a 10 µl reaction. 2 µl of this ligation reaction was added to 100 µl ECD100 *pir*116 competent cells and incubated on ice for 30 minutes. Samples were then heat shocked at 45^{o}C for 50 seconds and added to 900 µl of LB broth. Cells were agitated for 1 hour at 37^{o}C, plated on tetracycline plates, and incubated overnight at 37^{o}C.

3.12 Preparation of Competent Cells for Subcloning

100 ml of cells were grown at 37^{o}C in LB medium to an O.D. of about (600 nm) = 1.0 without passing 1.0. Cells were then cooled on ice and pelleted at 4^{o}C by centrifugation at 5,000X g for 5 minutes. Cells were resuspended in 15 ml sterile 0.15 M NaCl. Cells were pelleted again and resuspended in 1 ml of ice cold transformation buffer containing 15% glycerol (v/v),

0.1 M $CaCl_2$, 10 mM Tris HCl, pH 8, and 10 mM of $MgCl_2$. 400 µl of resuspended cells were placed into 3 to 4 different 1.5 ml tubes. Cells were incubated on ice for at least thirty minutes or overnight. Cells were frozen at -80^oC to become competent and then thawed on ice. 100 µl of cells were used per transformation reaction with 1 µl of DNA and incubated for thirty minutes. Samples were then heat shocked at 42^oC for 50 seconds and placed on ice. 900 µl of LB medium was added and cells were incubated at 37^oC for 45-60 minutes. 100 µl of cells were plated on LB agar plates supplemented with the appropriate concentration of antibiotic.

3.13 Plasmid Prep

Promega Wizard® Plus SV Minipreps DNA Purification System (Madison, WI) was used to purify plasmid DNA. A 5 ml overnight culture was pelleted at 10,000 x g for 5 minutes at room temperature and supernatant was removed. 250 µl of Cell Resuspension solution was added and the pellet was resuspended by pipetting. The resuspended samples were transferred to a 1.5 ml microcentrifuge tube. 250 µl of Cell Lysis solution was added and samples were inverted four times and incubated for 5 minutes at room temperature until clearing of lysate was observed. 10 µl of Alkaline Protease Solution was added and samples were mixed by inverting. Samples were incubated for 5 minutes at room temperature. 350 µl of Neutralization Solution was added, and samples were inverted and centrifuged at 10,000 x g for 10 minutes at room temperature.

Lysate was transferred to a provided Spin Column by decanting and centrifuged at maximum speed for 1 minute. The flow-through was discarded, and the Spin Column was reinserted into the collection tube. 750 µl of Column Wash Solution was added to the Spin Column and centrifuged at maximum speed for one minute. The flow-through was discarded, and the Spin Column was reinserted into the collection tube. 250 µl of Column Wash solution was added, and samples were centrifuged at maximum speed for 2 minutes. The Spin Column

was transferred to a new 1.5 ml microcentrifuge tube. Plasmid DNA was eluted by adding 100 µl of Nuclease-free water and centrifuging at maximum speed for 1 minute. The Spin Column was discarded, and the sample was stored at – 20^{o}C.

3.14 Sequencing Reaction

The concentrations of each DNA sample were obtained using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The DNA concentration was used to determine the volume of DNA needed for 50 fmol. Nuclease free water was added to 10 µl in a 0.2 ml microcentrifuge tube and heated at 96^{o}C for 1 minute. Then, 2 µl of 1.6 µM primer and 8 µl of the DTCS Quick Start Master Mix was added to heated DNA. The samples were run using the following program: 90°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 minutes for 30 cycles, hold at 4^{o}C.

3.15 Sequencing Reaction Cleanup

60 µl of 95% cold ethanol was added to a 0.5 ml microcentrifuge tube for each sample and placed on ice. 5 µl of Stop Solution, containing 1.2 M Sodium Acetate (pH 5.2), 40 mM Na_2-EDTA (pH 8.0) and 8 mg/mL glycogen, was added to each sample and mixed. The sample was then transferred to a previously prepared microcentrifuge tube containing cold 95% ethanol and mixed. Samples were centrifuged at 10,000 x g at 4^{o}C for 15 minutes. Supernatant was discarded and 200 µl of ice cold 70% ethanol was added and centrifuged for 1 minute. Supernatant was discarded, and the 70% ethanol wash was repeated. Supernatant was discarded, and excess ethanol was removed using a pipette. The samples were then dried for 10 minutes using a CentriVap (Labconco Corporation, Kansas City, MO) and resuspended in 40 µL Sample Loading Solution (SLS).

3.16 Sequencing Analysis

Raw sequence data was imported into the SeqVerter program from the GeneStudio™ Pro software package (www.genestudio.com) to be converted to the FASTA format. FASTA files were trimmed in NotePad and reimported into the Contig editor program from the GeneStudio software package. This program assembled the sequences using a reference sequence operon identified through the Basic Local Alignment Search Tool (BLAST) as a guide (Zhang Z., et al., 2000). Gene sequences for lysR and L2 were identified by using these sequences from the refence sequences in BLAST analysis. The sequences were aligned using Genome Compiler (Los Altos, CA) and primers were designed using the Primer3 program associated with Genome Compiler.

3.17 Minimum Inhibitory Concentrations

Overnight cultures were diluted 1/50 into fresh LB medium and mixed with 0, 10, 50, 100, 500, and 1000 µg/ml Ampicillin (Thermo Fisher Scientific, Waltham, MA). Turbidity was measured using a Klett™ Colorimeter (Fisher Scientific, Waltham, MA) and samples were incubated for 24 hours overnight at 30°C in a roller drum (Fisher Scientific, Waltham, MA) before measuring turbidity again. MICs were repeated in triplicate to obtain averages. Statistical analysis was performed using a standard 2-factor ANOVA on SPSS Software.

Table 1. Primers used for sequencing

Primer	Sequence
L2_4F	5’ – GAT CAT CAC CAG CGA CAA CA – 3’
L2_4R	5’ – CAG CAC GGC GAT GTC RTT – 3’
L2 LysR F1	5’ – CCA CCA GAA CTT CAC CCG TGC – 3’
L2 LysR R1	5’ – AGC TCG ATG TCC GGG TGC GC – 3’
L2 LysR F2	5’ – GCG GCG CTG TAT CCG GTG CT – 3’
L2 LysR R2	5’ – CGG CCA TTC GTC GCT GCG GT – 3’
L2 NaChan F1	5’ – TCC TCG CTG TTC CTG TTC GC – 3’
L2 NaChan R1	5’ – GCG CGT GCA GGT TCT CGT GC – 3’
L2 NaChan F2	5’ – GCA TGC CGC TGC TGG CCT CG – 3’
L2 NaChan R2	5’ – TCC ACC GGA TCA ACG TCA AA – 3’
L2-3_F	5’ – CAC CAG ATG CGC CAG CAG – 3’
L2-3_R	5’ – GCT TCA TCG ACC GCT TCA AG – 3’
M13_F	5’ – GTA AAA CGA CGG CCA GTG – 3’
LysR_F1	5’ – CGC GAG GTG CTG ACG CT – 3’
LysR_R1	5’ – GCT GAC ACA CAG CTC CA – 3’
L2_5R	5’ – CAT CGG TGG GTG CGT TG – 3’
L2 PCR_2	5’ – CTT TAC AGA GTC GAG CCG – 3’
LysR_F2	5’ – CCT GCA GAC CCA CAA CA – 3’
LysR_R2	5’ – CAC GGG TGA AGT TCT GGT G – 3’
LysR_F3	5’ – AAG CGT TAG GAT CAG CCA TC -3’
L2_5F	5’ – CGT GCG AGA GCA GAT CG – 3’
L2_6R	5’ – TGA CCC TGC TCG ACA CC – 3’
L2_7R	5’ – GAC AAT GAA ACC GGT GAT GC – 3’

L2-LysR_1	5’ – GGC AAG CCG TTT AGG GAT G – 3’
L2-LysR_2	5’ – TTA CGT GCC CTG CGC CTG – 3’
LysR_4F	5’ – CAG CCG TTC AGC ACC AC – 3’
L2_6F	5’ – GAC ATC GCC GTG CTG TG – 3’

Chapter IV: Results

4.1 Genomic DNA Isolation

Genomic DNA purifications were performed on *Stenotrophomonas maltophilia* OR02 strain in order to obtain DNA for PCR application. Gel electrophoresis used to visualize the DNA in lanes 2-3 show high molecular weight above 10 kb in size (Figure 1).

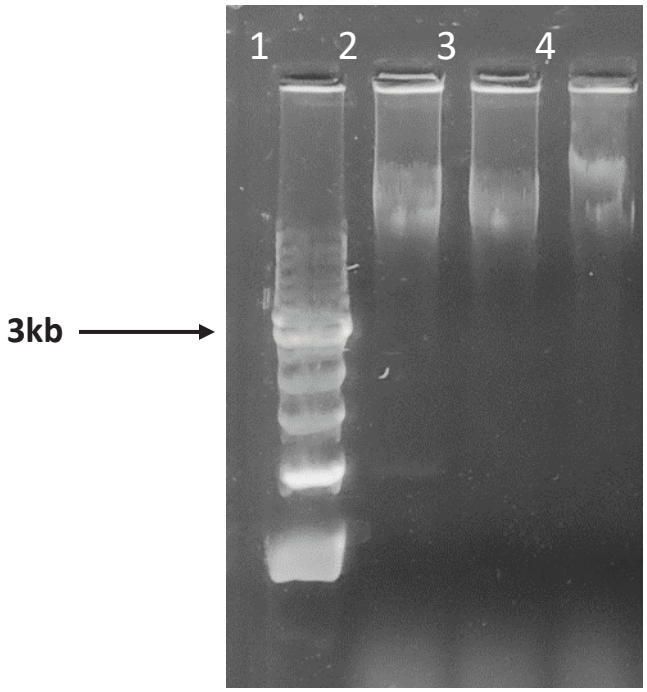


Figure 1. Gel Electrophoresis of genomic DNA. Lane 1 – 1 kb ladder, lanes 2, 3, and 4 – *S. maltophilia* genomic DNA.

4.2 Polymerase Chain Reaction

PCR was performed using L2-3 primers to amplify the L2 gene. Although gel electrophoresis showed that PCR produced multiple bands due to non-specific amplification, a band of the expected size, 3.47 kb, was observed (Figure 2). This band was excised from the gel and purified using the QIAquick® Gel Extraction Kit (Figure 3).

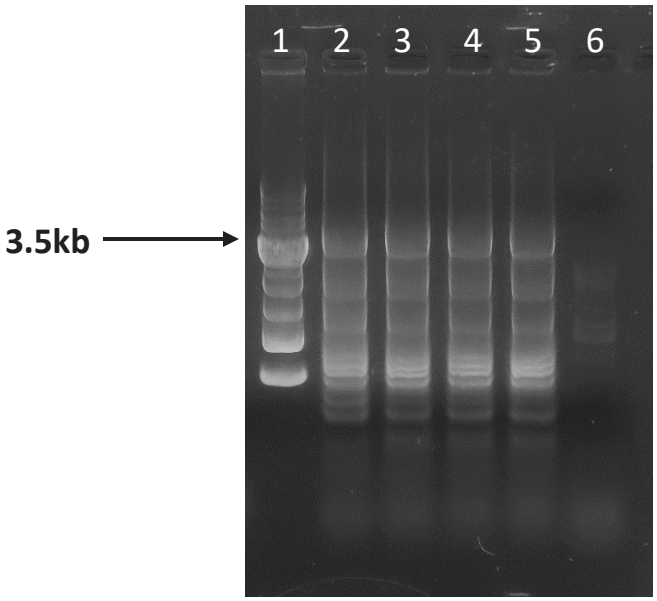


Figure 2. PCR Gel Electrophoresis on L2. Lane 6 – Negative control, lane 1 – 1 kb ladder, lanes 2, 3, 4, and 5 – L2 PCR.

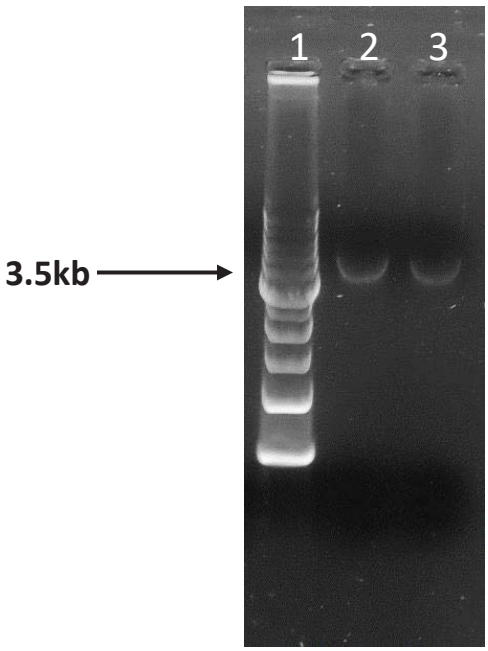


Figure 3. Gel Purification on L2. Lane 1 – 1 kb Ladder, lanes 2 and 3 – gel purified L2.

4.3 Cloning of the *lysR*/L2 PCR product

The L2 PCR product was ligated into the cloning vector using Strataclone PCR cloning kit (La Jolla, CA) and transformed into competent cells. Cells were then grown on LB agar plates overnight at 37^{o}C. Overnight cultures of LB broth and kanamycin were prepared and cells were grown overnight at 37^{o}C. Following plasmid preps, samples were digested using *Eco*R I and separated by agarose gel electrophoresis (Figure 4). Lane 3 shows the digested plasmid at the expected size of around 1.9kb. This recombinant plasmid containing the pSC-A-amp/kan vector and the *lysR*/L2 insert was named, pJD1.

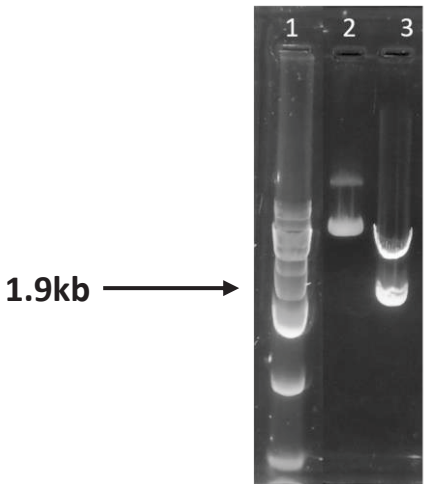
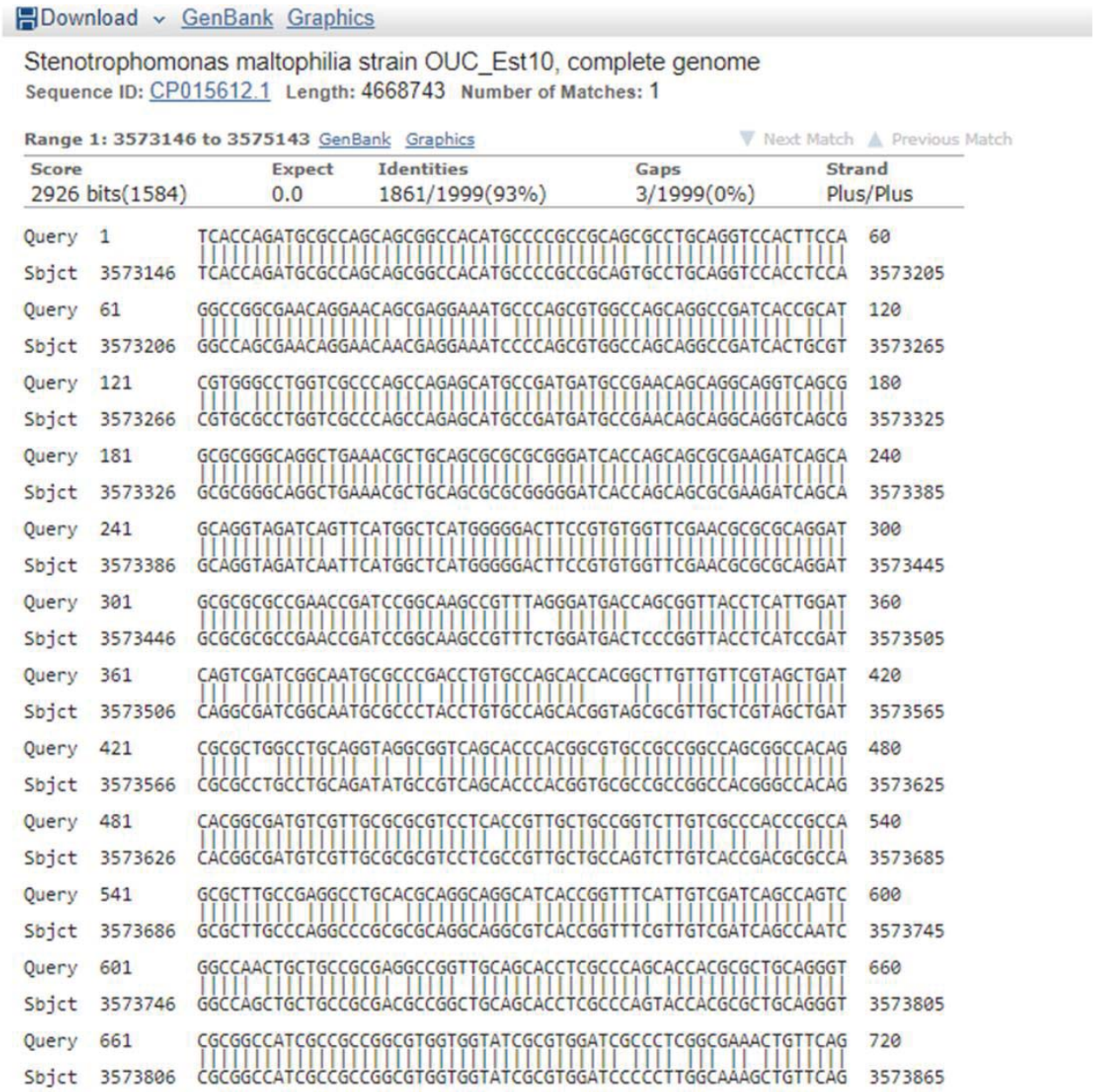


Figure 4. EcoR1 digestion on L2. EcoR1 digestions performed on purified L2 plasmid. Lane 1 – 1 kb Ladder, lane 2 – undigested lysr/L2 plasmid, lane 3 – *Eco*R1 digested plasmid.

4.4 Sequencing

Sequencing reactions were completed on all L2 samples using primers listed in Table 1. Once sequences were obtained, contigs were created using GeneStudio. The contigs were then used to perform a BLAST search and identify a reference sequence (Figures 5 and 6). Figure 6 shows that the sequence was 93% similar to a sequence from *S. maltophilia* strain OUC Est10. Clicking on the GenBank link suggested that the sequence encodes a type A beta-lactamase or

L2 type beta-lactamase and LysR proteins that may regulate beta-lactamase activity (Figure 6). The contig and reference sequence obtained from BLAST (Figure 7) were then aligned to create a consensus sequence and correct ambiguities that would be used to design new L2 primers.


Download GenBank Graphics

Stenotrophomonas maltophilia strain OUC_Est10, complete genome

Sequence ID: CP015612.1 Length: 4668743 Number of Matches: 1

Range 1: 3573146 to 3575143 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
2926 bits(1584)	0.0	1861/1999(93%)	3/1999(0%)	Plus/Plus

```
Query  1        TCACCAGATGCGCCAGCAGCGGCCACATGCCCCGCCGCAGCGCCTGCAGGTCCACTTCCA  60
Sbjct  3573146  TCACCAGATGCGCCAGCAGCGGCCACATGCCCCGCCGCAGTGCCTGCAGGTCCACCTCCA  3573205

Query  61       GGCCGGCGAACAGGAACAGCGAGGAAATGCCCAGCGTGGCCAGCAGGCCGATCACCGCAT  120
Sbjct  3573206  GGCCAGCGAACAGGAACAACGAGGAAATCCCCAGCGTGGCCAGCAGGCCGATCACTGCGT  3573265

Query  121      CGTGGGCCTGGTCGCCCAGCCAGAGCATGCCGATGATGCCGAACAGCAGGCAGGTCAGCG  180
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Query  181      GCGCGGGCAGGCTGAAACGCTGCAGCGCGCGCGGGATCACCAGCAGCGCGAAGATCAGCA  240
Sbjct  3573326  GCGCGGGCAGGCTGAAACGCTGCAGCGCGCGGGGGATCACCAGCAGCGCGAAGATCAGCA  3573385

Query  241      GCAGGTAGATCAGTTCATGGCTCATGGGGGACTTCCGTGTGGTTCGAACGCGCGCAGGAT  300
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Query  301      GCGCGCGCCGAACCGATCCGGCAAGCCGTTTAGGGATGACCAGCGGTTACCTCATTGGAT  360
Sbjct  3573446  GCGCGCGCCGAACCGATCCGGCAAGCCGTTTCTGGATGACTCCCGGTTACCTCATCCGAT  3573505

Query  361      CAGTCGATCGGCAATGCGCCCGACCTGTGCCAGCACCACGGCTTGTTGTTCGTAGCTGAT  420
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Sbjct  3573746  GGCCAGCTGCTGCCGCGACGCCGGCTGCAGCACCTCGCCCAGTACCACGCGCTGCAGGGT  3573805

Query  661      CGCGGCCATCGCCGCCGGCGTGGTGGTATCGCGTGGATCGCCCTCGGCGAAACTGTTCAG  720
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```

Figure 5. BLAST results from L2 sequencing. Nucleotide BLAST performed using obtained L2 sequences showing 93% identity with sequence found in *S. maltophilia*.

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                gene prediction method: Protein Homology."
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                gene prediction method: Protein Homology."
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```

Figure 6. BLAST results from L2 sequencing. BLAST results identify sequences obtained match L2 β-lactamase gene of *S. maltophilia*. Surrounding genes, LysR and sodium channel are also shown.

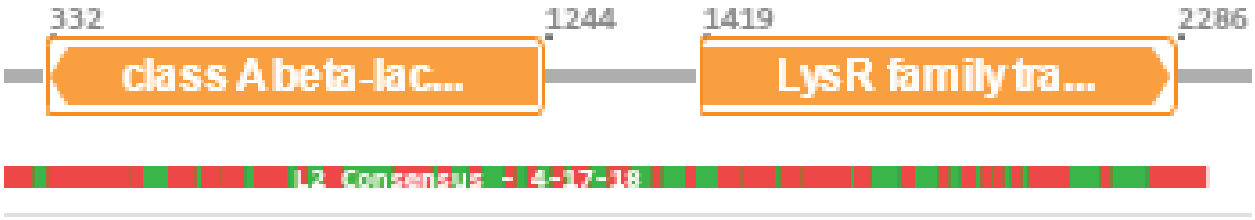

Figure 7. Constructed gene map for L2 using GenomeCompiler. Orange blocks represent genes from the *S. maltophilia* strain OUC Est10 reference sequence. The consensus sequence below the reference sequence represents the *S. maltophilia* 02 contig. Green represents matches to the reference sequence and red represents mismatches to the reference sequence.

4.5 Phylogenetic Analysis

DNA sequences for both L2 and LysR were used in a nucleotide BLAST search to identify homologous genes with over 50% identity. MEGA7 was used to construct phylogenetic trees for both L2 and LysR. These trees show the estimated evolutionary relationship between species based on sequence data.

Phylogenetic tree output for L2 in S. maltophilia (Figure 8) shows the closest evolutionary relationship with *Stenotrophomonas rhizophilia*. This group also shows strong evolutionary similarities with the *Xanthomonas* genus. The tree constructed for LysR (Figure 9) shows the closest evolutionary relationship to other species in the *Stenotrophomonas* genus.

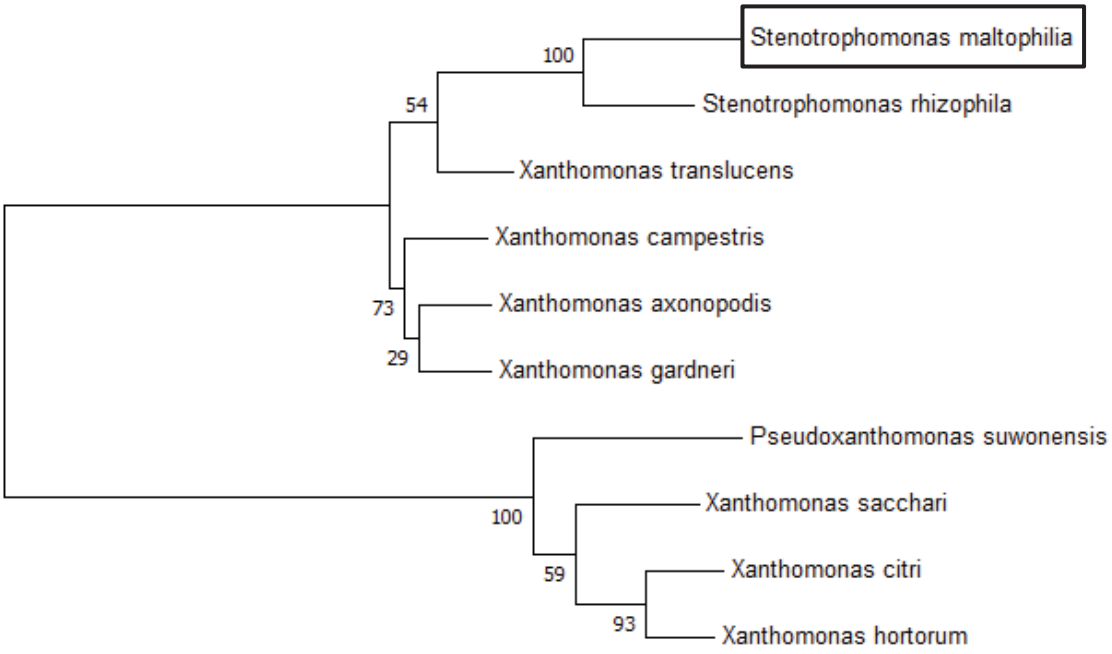

Figure 8. Phylogenetic tree showing relationship of L2. Phylogenetic tree of maximum likelihood constructed using 10 homologous Class A beta-lactamase genes.

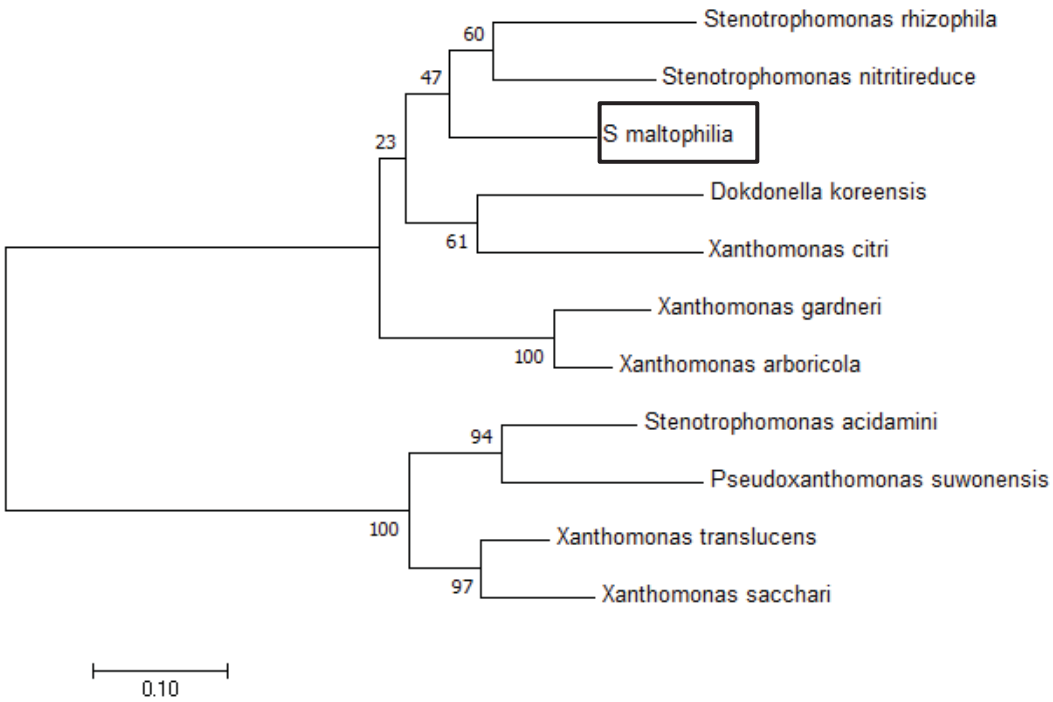

Figure 9. Phylogenetic tree showing relationship of LysR. Phylogenetic tree of maximum likelihood constructed using 10 homologous LysR genes.

4.6 Subcloning of *lysR*/L2 into pACYC184

Since the StrataClone plasmid, pSC-A-amp/kan in pJD1 already contained an ampicillin resistance gene, it was not possible to test the *lysR*/L2 insert in pJD1 for ampicillin resistance. Thus, an *Eco*R I digestion of pJD1 was separated on an agarose gel, and the *lysR*/L2 fragment was excised. After purifying the fragment using a QIAquick® Gel Extraction Kit, it was ligated into the *Eco*R I site of pACYC 184, which contains resistance genes for chloramphenicol and tetracycline but not ampicillin. The ligated DNA was transformed into ECD100D *pir116*, *E. coli* competent cells and plated on tetracycline plates. To test for ampicillin resistance, transformants were spotted on ampicillin plates. Colonies growing on ampicillin plates were grown in overnight cultures for plasmid preparations. The purified plasmids were digested with *Eco*R I and separated by agarose gel electrophoresis. Figure 10 shows that the inserts of approximately 3 kb in lanes 2, 4, 7, and 9 were the expected size for the *lysR*/L2 DNA fragment. This new recombinant plasmid was named pJD2.

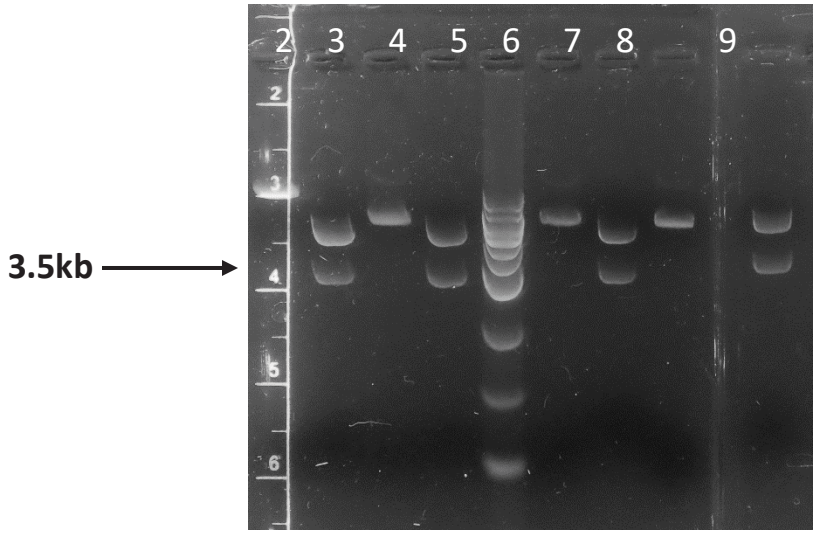

Figure 10. EcoR1 digestions on L2 Ampicillin resistant colonies. Lane 5 – 1 kb Ladder, lanes 1, 3, 6, and 8 – undigested samples, lanes 2, 4, 7, and 9 – EcoR1 digestions.

4.7 Minimum Inhibitory Concentrations

Overnight cultures of wild-type *S. maltophilia* OR02, ECD100 *pir116* (pJD2), and ECD100 *pir116* (pACYC184) were diluted into LB broth and distributed into test tubes containing six concentrations of ampicillin (0, 10, 50, 100, 500, and 1000 μg/ml). At 0 and 24 hours of incubation, turbidity was measured in Kletts units. Three replicates were performed and average turbidity differences between 0 and 24 hours were calculated and plotted vs ampicillin concentration in a bar graph (Figure 11).

Statistical analysis was performed using a two-factor ANOVA through SPSS (IBM) to test for significance between bacterial sample, concentration of ampicillin, and turbidity. Significance for all data was determined by a p-value of less than 0.05 ($p < 0.05$).

ANOVA results showed no significance in turbidity between concentrations of ampicillin ($p > 0.05$). Significance was found between the wild-type strain of *S. maltophilia* OR02 and ECD100D *pir116* (pACYC184) ($p = 0.000078$). Significance was also found between ECD100 *pir116* (pJD2) and ECD100D *pir116* (pACYC184) ($p = 0.008$). There was no significance between the wild-type strain and ECD100 *pir116* (pJD2) ($p = 0.246$).

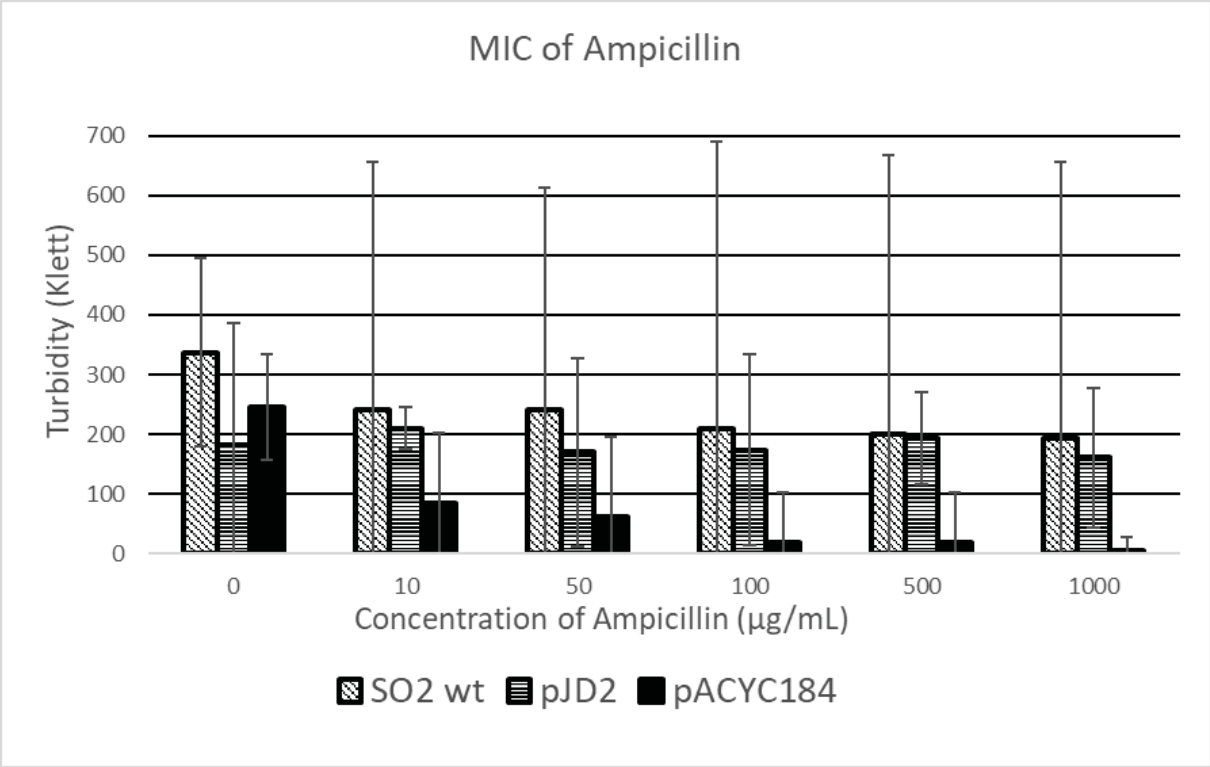

Figure 11. Minimum inhibitory concentrations for L2. The first bar at each concentration represents the wild-type strain of *S. maltophilia*, the second bar represents ECD100 *pir116* (pJD2) and the third, solid black represents ECD100 *pir116* (pACYC184).

4.8 Carbenicillin and Cefoxitin Resistance

The wild-type strain of *S. maltophilia* OR02, ECD100 *pir116* (pJD2), and ECD100D *pir116* (pACYC184) were streaked onto ampicillin, carbenicillin, and cefoxitin plates (Figure 12). As expected, all three grew on the plate lacking an antibiotic. The positive control, S. *maltophilia* 02, which contained the L1 and L2 genes, grew on all three plates that contained antibiotics, although not well on the carbenicillin plates. The negative control, ECD100D *pir116* (pACYC184), which lacked a penicillin resistance gene, failed to grow on the three plates that contained antibiotics. As hypothesized, ECD100 *pir116* (pJD2), which contained the L2 gene, grew on ampicillin and carbenicillin plates but not on the cefoxitin plate.

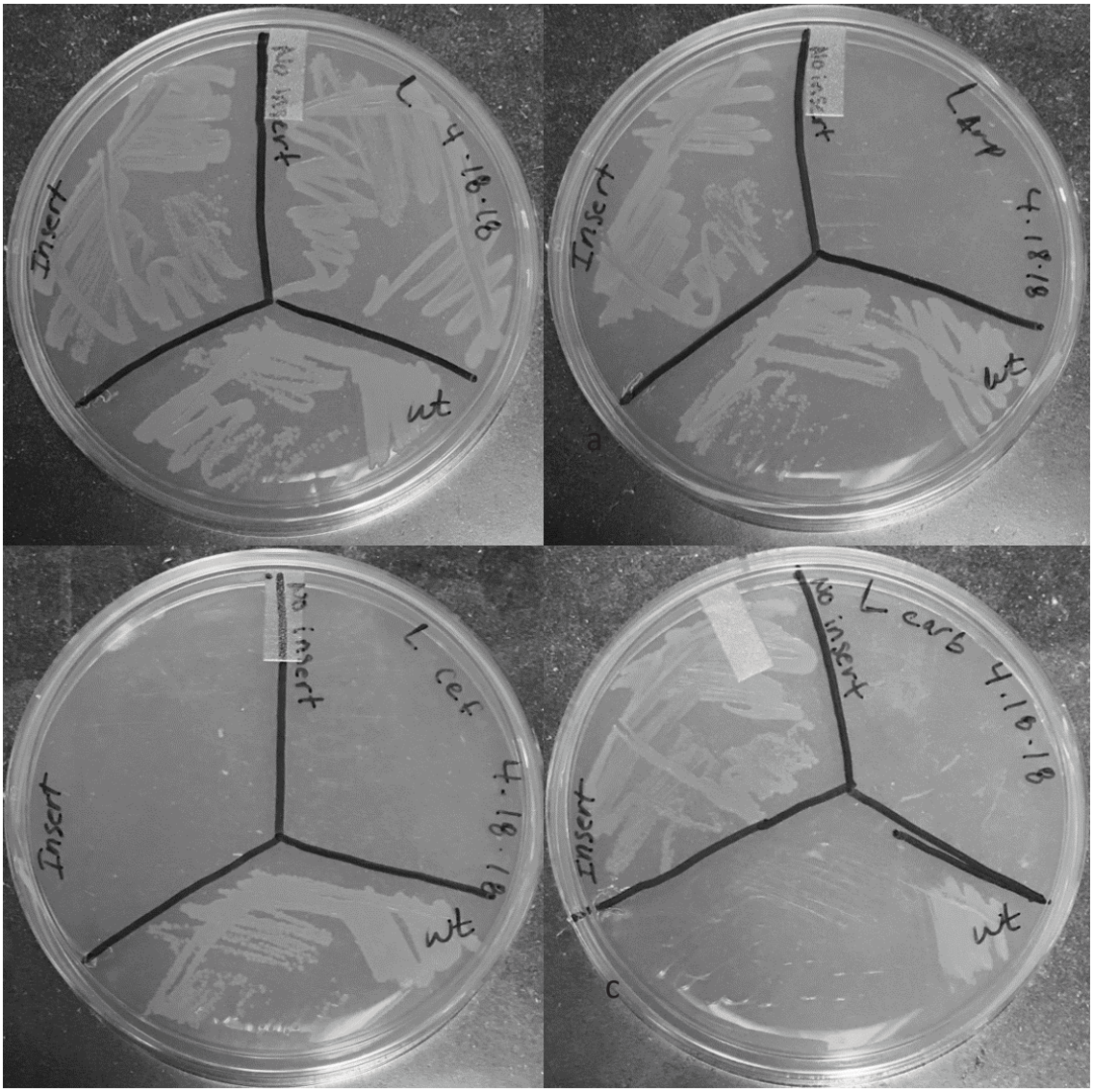

Figure 12. Spread plates testing for carbenicillin and cefoxitin resistance. Streak plates with wild-type (wt), Insert (ECD100 *pir116* (pJD2)), and no insert (ECD100D *pir116* (pACYC184)). (a) Growth on LB agar with no added antibiotic. (b) LB plates with ampicillin. (c) LB plates with cefoxitin. (d) LB plates with carbenicillin.

Chapter V: Discussion

In accordance with the hypothesis, L2 showed high levels of ampicillin resistance when transformed into *E. coli* in comparison to *E. coli* without the L2 insert. L2 also conferred resistance to carbenicillin, but not cefoxitin.

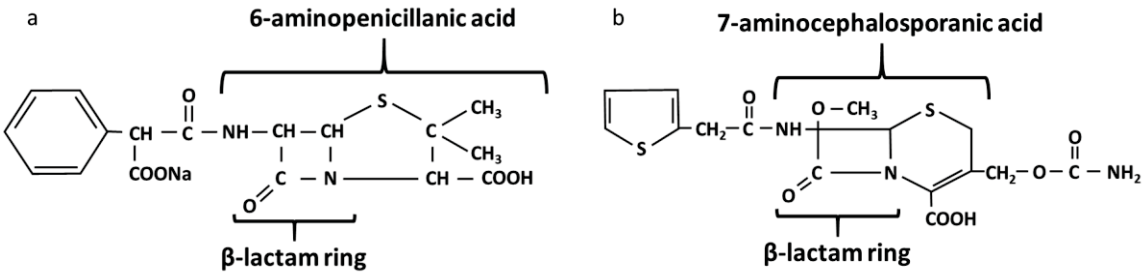

Figure 13. Chemical structures of cefoxitin and carbenicillin. The structural differences between (a) carbenicillin and (b) cefoxitin from Prescott's Microbiology (Willey, et al., 2017).

Structural differences between carbenicillin and cefoxitin may explain the ability of L2 to hydrolyze carbenicillin, but not cefoxitin. Figure 13 shows the differences in each structure. Carbenicillin, a penicillin, contains a 6-aminopenicillanic acid group, whereas and the cefoxitin, a cephalosporin, contains a 7-aminocephalosporanic acid group. A possible explanation for the difference in binding and hydrolyzing of these antibiotics by L2 could be caused by steric hinderance due to the structural difference between the antibiotics.

MIC results (Figure 11) showed, as expected, that L2 displays high resistance to ampicillin. The wild type generally showed higher resistance, though not statistically different, presumably due to additional resistance genes such as L1. Significant difference between *E. coli* strains with and without insert supported the hypothesis that L2 confers resistance to ampicillin. One trial of MICs was performed at 1500 µg/ml to determine the minimum amount of ampicillin required to inhibit wild-type and insert sample growth, however growth still occurred at this

concentration. Adding more trials of MICs will make the data more statistically accurate and decrease the size of the error bars. Further MIC testing should be performed with higher concentrations to determine concentration required to inhibit growth.

There may also be a third ampicillin resistance gene. While sequencing an arsenic resistance operon in *S. maltophilia* 02, a related reference sequence suggested that there may be a gene for a type C beta-lactamase. The existence of three penicillin resistance genes may explain why *S. maltophilia* 02 has an MIC that is higher than 1500 µg/ml for ampicillin.

Secondary structure could have caused problems with sequencing, especially at the 3' end of the *lysR* gene. Stem-loop structures with a T_m higher that 60°C, the extension temperature, make it difficult for DNA polymerase to replicate DNA in these regions.

Future Work

L1 primers should be designed to complete L1 sequencing. Once L1 sequence is completed, it will be inserted into a cloning vector and transformed into competent cells. The L1 insert will be tested for resistance on ampicillin plates and colonies will be used for MIC testing as was performed with L2. Following MICs with ampicillin, MICs will be performed using carbenicillin and cefoxitin as discussed in the objectives of this study. It can be hypothesized that L1 will also show resistance to ampicillin. In addition, it is expected that L1 will also confer resistance to cefoxitin since L2 was shown to be inhibited by the antibiotic. Future research will also aim to knock out both L1 and L2 to test antibiotic susceptibility to ampicillin. Primers will also be designed to sequence the third, class C beta-lactamase gene.

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Appendix

CGCCCTTCACCAGATGCGCCAGCAGCGGCCACATG
CCCCGCCGCAGCGCCTGCAGGTCCACTTCCAGGCCGGCGAACAGGAACAGCGAGGAAATG
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CGTTGCTGCCGGTCTTGTCGCCCACCCGCCAGCGCTTGCCGAGGCCTGcACGcAGGCAGG
CATCACCgGTTTCATTGTCGATCAGCCAGTCGgCCAACTGCTGCCGCGAGgCCGGtTGCA
GCACCTCGCCCAGCACCACGCGCTGCAGGGTCGCGGCCATCGCCGCCGGCGTGGTGGTAT
CGCGTgGATCGCCCTCGgCGAAACTGTTCAGTTCCGGTTCCAGGCGGTCACTGCGGCTCA
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AAGGGGAC

Figure 7. LysR and L2 DNA consensus sequence. Obtained DNA sequence using LysR_L2 primers.